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Tools for Resolving Functional Activity and Connectivity within Intact Neural Circuits

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Abstract

Mammalian neural circuits are sophisticated biological systems that choreograph behavioral processes vital for survival. While the inherent complexity of discrete neural circuits has proven difficult to decipher, many parallel methodological developments promise to help delineate the function and connectivity of molecularly defined neural circuits. Here, we review recent technological advances designed to precisely monitor and manipulate neural circuit activity. We propose a holistic, multifaceted approach for unraveling how behavioral states are manifested through the cooperative interactions between discrete neurocircuit elements.

Introduction

> “Can the brain understand the brain? Is it a giant computer, or some other kind of giant machine, or something more? The brain is a tissue. It is a complicated, intricately woven tissue, like nothing else we know of in the universe, but it is composed of cells, as any tissue is. They are, to be sure, highly specialized cells, but they function according to the laws that govern any other cells. Their electrical and chemical signals can be detected, recorded and interpreted and their chemicals can be identified; the connections that constitute the brain's woven feltwork can be mapped. In short, the brain can be studied, just as the kidney can.”
>
> — David H. Hubel, *Scientific American* 1979

This thought-provoking quote from the Nobel prizewinning neurophysiologist David Hubel (1926–2013) highlights how the architecture and function of the brain is exceptionally complex, but also suggests that this intricate biological system can be dissected with the right strategy and techniques. The need to map the connectivity and activity of precise neurocircuits is becoming ever stronger as the toll exacted by neurological and

neuropsychiatric disorders on society increases, and given the current goals set forth by large-scale research endeavors such as the NIH Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative. Recent developments in neurotechnology will likely assist in achieving these goals.

In this review, we shall discuss the new toolsets that are being developed to study neural circuit function, such as optogenetic techniques and *in vivo* measurement strategies, and explain how a combinatorial approach can be taken to systematically characterize the connectivity, function, and neurophysiological dynamics of precise neural circuits during or following discrete behavioral states. Collectively, assembling a genetically precise neural circuit wiring diagram and unraveling the choreography of neuronal network dynamics within a defined neurocircuit with advanced *in vivo* measurements and manipulations should provide critical insights into how neuronal networks orchestrate behavioral states.

The Neurophysiological Dynamics of Distinct Neurocircuits

The ability to identify single-unit activity originating from genetically defined neurons provides an avenue for elucidating how specific neuronal subpopulations are engaged by environmental stimuli [1–5]. Without these genetically guided electrophysiological approaches, the readout from extracellular recordings within brain tissue that originates from a vast array of diverse cell types, oftentimes with their own unique function, makes it virtually impossible to definitively characterize the activity patterns of select neuronal subpopulations. As extracellular recordings within a given brain region usually reveal a multitude of discrete firing profiles time-locked to behaviorally relevant stimuli, it is now critical to determine if these functionally distinct activity patterns arise from genetically distinct neuronal subpopulations. Identifying distinct activity patterns will likely be fundamental for illustrating how whole neurocircuit systems are equal to the sum of their individual parts (genetically and functionally distinct cell types).

In order to distinguish the firing profiles of genetically defined neuronal populations, a Cre recombinase-dependent viral vector encoding the light-activated cation channel, channelrhodopsin-2 (ChR2), can be introduced to genetically distinct neuronal populations in various Cre-driver transgenic mouse lines [6–9] (Figure 1A; Table 1). Additional recombinases, such as Flp or Dre, can also be used to generate cell-type specific expression of ChR2, and they can be combined with Cre-dependent targeting strategies to isolate genetically separate subpopulations within the same subject [10]. The number of available transgenic mouse lines is rapidly increasing, and they have become readily available from the Allen Brain Institute for Brain Science, GENSAT, Jackson Laboratory, and independent laboratories.

While genetically guided tools offer cell-type specificity, region-specific targeting of ChR2, via localized delivery of a ubiquitous viral vector (using human synapsin [11] or CAG [12,13] promoters), provides anatomical specificity (Table 1). Spatial targeting of ChR2 to a discrete brain region can reveal global information about how a neurosubstrate encodes certain behavioral states [14]. Integrating genetic- and region-specific targeting strategies is a powerful way of acquiring cell-type and spatial resolution; however, neighboring brain

regions can be similar in their cytoarchitecture, gene expression patterns, and connectivity [15], making it difficult to isolate the unique function of a region. Furthermore, the spread of viral particles is difficult to control, even when small-volume viral microinjections are employed, and can result in superfluous transduction of regions surrounding the target area. For these reasons, the initial experimental design should involve the investigation of surrounding regions that are prone to infection and subsequent contamination of data analysis. Improvements in viral delivery methods are needed for completely restricting circuit analysis to specific cell types in discrete brain regions that are situated in homogenous zones of tissue.

When extracellular recordings are performed, ChR2 can be a useful physiological tag or marker, as a brief pulse of blue light elicits a short-latency action potential in cells expressing ChR2 that is reliably detected across multiple light presentations (Figure 1B,C) [4,16,17]. Consequently, cells expressing ChR2 are distinguishable from ‘ChR2-negative’ neurons during *in vivo* extracellular recordings based on their electrical responses to light. Under certain circumstances, however, using phototagging methods to identify light-responsive neurons may generate false-negatives or false-positives. For example, some light-evoked responses may be mediated through both local and distal polysynaptic circuit modulation, as photoexcitable ChR2-expressing neurons may respond to light and elicit detectable spikes from neighboring synaptic partner neurons that do not express ChR2, thus giving a false-positive. When trying to identify neurons *in vivo*, there are certain criteria that can be used to minimize such problems. First, demonstrate a high-spike fidelity in response to high photostimulation frequencies [1,2]. Second, detected units possess short light-evoked latencies that have minimal variance (spike jitter) [1,2,5]. Third, light delivery at certain intensities should not alter the shape of the detected waveform, indicating that the responses to light are not caused from direct photoelectric artifacts [1,2].

While these criteria may uphold for particular cell types, other cells of interest may require adjustments or alternative criteria for properly identifying light responses. False-negative occurrences are influenced by factors underlying the detectability of light responses in recorded neurons. The detectability of light-responsive neurons depends on the level of ChR2 expression and on the volume of tissue that is efficiently illuminated by light. ChR2 expression strength is contingent upon the diffusion and spread of viral particles as well as the viral titer, while the volume of illuminated tissue relies on the intensity and scattering of the light in tissue. Thus, the absence of light-evoked responses does not completely rule out the possibility that a recorded neuron expresses the target gene.

The functional role of particular neurons depends on how they are integrated within a circuit. Thus, specific neuronal subpopulations that project to different postsynaptic target regions often display unique firing patterns compared to cells that project elsewhere. Therefore, phototagging of genetically defined neurons in a projection-specific fashion can further define and refine the computational role of participant cells within a complex circuit [2]. To accomplish this, ChR2 localized in axons and presynaptic terminals is activated via light delivery through implanted optical fibers. Congruently, multielectrode recording devices are implanted near the somas of these ChR2-targeted neurons (Figure 1D,E). Photostimulation of ChR2-expressing axonal fibers elicits an antidromic spike that

propagates from the axons back to the soma region where the implanted electrode is capable of detecting these antidromic-initiated spikes (Figure 1E,F). To confirm that detected light-evoked spikes originate from antidromic action potentials, orthodromic and antidromic photostimulations that occur close in time can be used to demonstrate that the photoinitiated spikes, traveling in opposite directions, collide with each other and occlude the detectable spike. Spike collision is a key indicator that the action potentials are conducting along the same axon and thus are not due to synaptic transmission within a polysynaptic circuit [2,18,19]. Because ChR2 is expressed along axons, in certain situations the photoinitiation site of the antidromic spike is not restricted to the terminals and may arise from photoactivated fibers of passage that traverse through the postsynaptic target region. This key factor should be kept in mind when antidromic photostimulation methods are used to identify projection-specific activity.

Current developments in high performance *in vivo* extracellular recording equipment along with the advances in light sources have significantly facilitated the application and versatility of electrophysiological phototagging methods. Compared to other light sources (lasers), light-emitting diodes (LEDs) offer stable light output, have exceptionally long lifetimes, and are cost efficient. Thus, independent research labs are implementing LED based systems with electrophysiological devices; for instance, the design of wireless micro-LED devices that are incorporated with electrophysiological sensors permit the ability to control and monitor the activity of circuit elements during complex behavioral tasks [20]. Furthermore, the construction of optrode devices that allow for simultaneous electrophysiological recording and optical stimulation [21], as well as the fabrication of an array of thin microwaveguides that delivers light in a three-dimensional pattern throughout the brain [22], have also extended the ability to precisely monitor and manipulate neural circuit activity.

While *in vivo* extracellular recordings can produce valuable neurophysiological information in a precise temporal fashion, there are many challenges faced with this technique that can drastically hinder the investigation of neurocircuit dynamics. The poor spatial resolution (∼100 mm) of multielectrode arrays [23] results in a low yield of detectable light-responsive units and thus limits the ability to record the neural activity from a large ensemble of neurons. For example, the small sample size obtained from extracellular recordings may not accurately represent the activity of the larger discrete neuronal population, as minor but important, subpopulations may exist within the larger genetically defined network, leading to a plethora of various firing patterns to go unnoticed. The long-term performance of multielectrode arrays for chronic *in vivo* recordings can often diminish over time because of obstruction from scar tissue (gliosis), protein fouling, and increased electrode impedance [24–26]. And the instability of the recording device in tissue prevents the unequivocal isolation of the same unitary signal on the same electrode across multiple recording sessions [27]. This limitation in particular may greatly reduce insight into neural circuit function achieved by single unit recordings, as the activity patterns of unequivocally the same neuron cannot be observed over chronic time scales (days to months), where neuronal networks may adapt in response to stimuli or environmental demands. *In vivo* imaging of neuronal activity provides a complementary strategy to electrophysiological phototagging methods by

defining the activity patterns of distinct participant neurons within a circuit over longer time scales.

Modifications that have been made to long-term *in vivo* imaging techniques [28], along with the advent of newer variants of genetically encoded calcium indicators [29,30], have partially alleviated some of the limiting factors of extracellular recordings by resolving the activity from large genetically-defined neuronal populations over extended timescales [31–34]. Given that calcium ions (Ca^{2+}) enter the cell in response to neuronal activation [35], and that engineered fluorescent proteins display changes in fluorescent intensity as they bind Ca^{2+} [36], action potentials and synaptic transmission are reliably measured by imaging changes in intracellular free Ca^{2+} [37]. Chen *et al.* [30] recently developed a family of ultrasensitive protein calcium sensors (GCaMP6.0) that allow for reliable detection of single action potentials in neuronal somata during *in vivo* two-photon imaging. Furthermore, two-photon imaging of GCaMP6-expressing neurons permits detection of synaptic calcium signaling within individual dendritic spines over several months (Table 2) [30].

Future additions to the color range of calcium indicator proteins [38], such as red fluorescent calcium indicators (RCaMP), will further improve the imaging depth within intact brain tissue, since near-infrared light, containing longer wavelengths, scatters less through biological tissue (Table 2) [39]. Additionally, chromatic variants allow for simultaneous multi-color activity imaging of multiple genetically distinct neuronal populations. As calcium transients can be detected in specific presynaptic terminals [40], these new engineered sets of chromatic variants may be utilized to simultaneously monitor the *in vivo* responses of a genetically defined postsynaptic neuron together with its presynaptic axon terminal input to reveal the precise mechanism by which information is transmitted and integrated between distinct cells within a circuit during a particular behavioral state (Figure 2A). Color-shifted indicators enable the integration of optogenetic neuronal activation and silencing manipulations with functional *in vivo* calcium imaging to control and monitor concurrently specific circuit interactions or connections [38]. This integration will enable a biofeedback mechanism for controlling neurocircuit activity by allowing the natural circuit dynamics to direct the optogenetic manipulation parameters during behavioral tasks — frequency, duration, and the precise time point of stimulation or inhibition — instead of relying on the artificial control that is typically employed by the experimenter. The information provided by these types of experiments could inform future human applications that involve a biofeedback system paired with deep-brain stimulation.

Two-photon imaging in head-fixed, awake behaving rodents permits the detection of Ca^{2+} associated activity in neural circuits [41,42] and makes possible long-term imaging with the application of non-invasive thinned-skull cranial window techniques [43]. The behavioral readouts from such experiments are limited, however, as the animals are unable to freely move during two-photon imaging (Figure 2B). Innovative virtual-reality systems that use linear or spherical treadmills and simulated environments in head-restrained mice [31,44], as well as voluntary head restraint methods that involve operant conditioning systems [45], help increase the complexity of behavioral tasks during two-photon imaging.

The optical-fiber based approaches [34,46,47] that are being developed are better suited for more complicated freely moving behavioral experiments that require *in vivo* imaging of activity in large neuronal populations situated in deep brain regions. Microendoscopes are composed of a relay lens with a gradient-index (GRIN) lens attached at the bottom. Because GRIN lenses have a flat optical surface to refract light through a refractive index gradient, they can efficiently focus and collect light through highly scattered tissue in deep brain regions [48] (Figure 2C). High-speed, miniature epi-fluorescence microscopes (weighing less than 2 g) equipped with a complementary metal oxide semiconductor (CMOS) image sensor and interfaced with a microendoscope are able to resolve simultaneous Ca^{2+} signals in approximately 1,000 neurons in the hippocampus per freely behaving mouse. Notably, these integrated microscopes can repeatedly image neuronal populations for weeks to months [34] (Figure 2D). Further technical developments, including innovative new microendoscopic methods and Ca^{2+} indicators, will likely prove to be pivotal for addressing how large ensembles of genetically defined neurons encode aspects of multifaceted behavioral tasks.

Cataloging the Connectivity and Function of Distinct Neural Circuits

Unraveling the intricate neural wiring patterns within molecularly defined circuits is essential for elucidating the anatomical specificity underlying particular behavioral states. While gross neuroanatomical tracing methods have been used since the dawn of modern neuroscience to provide critical insight into the axonal wiring between brain regions, these classical strategies typically cannot delineate neural circuit connectivity originating from ‘genetically’ distinct neuronal populations (that is, populations differing in their states of gene expression). Anterograde tracing viruses, such as Adeno-associated viruses (AAVs) [49] encoding a channelrhodopsin tagged with a fluorescent protein (ChR2-eYFP), are useful for mapping specific axonal projections and their selective destination points within distinct brain regions. AAV-mediated delivery of ChR2-eYFP to projection neurons in one brain region results in somata transduction [50,51] and subsequently leads to anterograde transfer of the membrane-bound fusion protein to the axon terminals in downstream regions [52–54], allowing anatomical visualization of the pattern and density of innervation (Figure 3A). However, these AAV-based anterograde tracing strategies cannot exclusively identify functional connections between multiple circuit nodes and so should only be used as an initial guide for visualizing possible neurocircuit interactions.

Transsynaptic viral tracers can be used to identify putative synaptic partner neurons between multiple brain regions. Wheat germ agglutinin (WGA) [55,56] is a transsynaptic tracer protein that is efficiently taken into neurons and transported anterogradely and retrogradely to axons and dendrites (Figure 3B). Thus, AAV-based constructs containing WGA transgene products label both first-order neurons (starter neurons) and second-order downstream neurons [57,58] (Table 3). While this neuroanatomical tracing tool can uncover multisynaptic neural pathways, both anterograde and retrograde traversing of synapses make it difficult to analyze the directionality and specificity of a particular circuit [57]. Other viral tracers, such as Herpes simplex viruses (HSV) [59–62] and Vesicular stomatitis viruses (VSV) [63] can be used to achieve greater anatomical specificity, as they can travel in a unidirectional manner (Table 3).

Axonal terminal targeting with retrograde transsynaptic viruses can be used to determine the specific connections between postsynaptic seed neurons and their upstream presynaptic input cells. The Pseudorabies (PRV) and Rabies viruses (RABV) are well characterized and serve as effective transneuronal tracers of multisynaptic circuit connections [64,65] (Table 3). Given that any particular brain region contains a multitude of cell types and connections with other structures, and that the fast rate of retrograde viral spread increases the number of synaptic phases, it becomes increasingly challenging to decipher the precise wiring from the initial viral infection site [66]. Thus, the ideal tracing scenario would involve targeting genetically defined postsynaptic starter neurons and their monosynaptic inputs with a transsynaptic retrograde tracer.

Wickersham *et al.* [67] made this possible by developing a modified monosynaptic rabies virus, EnvA-SADΔG-GFP, which restricts viral infection to a genetically distinct postsynaptic cell type and its monosynaptic input cell (Figure 3C). Under normal circumstances the modified rabies virus does not infect mammalian tissue, but infection will occur in the presence of the envelope glycoprotein of avian sarcoma/leukosis (EnvA) receptor, TVA. Thus, postsynaptic target neurons must express TVA for initial rabies infection, as well as the rabies glycoprotein (RG) for the formation of new viral particles and transsynaptic spread. Cre-dependent viral delivery of TVA and RG along with the infusion of the modified rabies virus sufficiently labels presynaptic neurons with GFP from a single postsynaptic seed neuron [68–70] (Table 3). This viral construct enables specific presynaptic neurons to be targeted with many new rabies virus variants to perturb and record activity from discrete circuit connections. Some of these variants encode ChR2 for photostimulating specific presynaptic neurons and GCaMP for imaging Ca^{2+} activity in discrete upstream regions [71].

The further development newer variants could expand the utility of transsynaptic tracing tools by implementing the viruses as functional modulators and/or neural activity monitors, thereby making these tools more than just an anatomical visual guide. Despite the utility of these viral tracing tools for circuit mapping, complementary approaches, such as ChR2-assisted slice electrophysiology experiments, should be used in combination to validate the presence of a functional synaptic connection between virally identified circuits. Another important factor to consider when applying these viral tracing techniques is the complexity and diversity of various synaptic connections that may display differences in transsynaptic viral spread. Accordingly, the absence of viral labeling does not exclusively rule out a specific circuit connection.

Mapping circuits with neuroanatomical viral tracers and molecular labeling with immunohistochemical techniques can generate large volumes of data, and information from an intact brain may be required to interpret that data set and allow the accurate and efficient reconstruction of the architecture of a specific neurocircuit. Traditionally, sectioning and reconstruction [72] of an intact system have been used for mapping connections between populations of neurons, but these methods are tremendously time consuming and do not preserve the structural integrity of an intact circuit. Moreover, because of the limitations of working with biological tissues, large volumes of tissue (~300–400 mm thick) drastically reduce imaging resolution even with two-photon applications [73], further compounding the

difficulty of rendering detailed three-dimensional information from an intact biological system. Therefore, reducing the opacity of brain samples (increasing tissue transparency) and minimizing the amount of scattered light through deep brain areas to increase the imaging depth of undivided biological systems are major priorities for improving the image acquisition of fluorescent labeled neurocircuits.

Recent optical clearing methods, for example using the urea containing Scale clearing agent, enable deep tissue imaging of fluorophores, but they require long incubation periods (months) and sacrifice the integrity of the sample via tissue swelling and expansion [74]. In contrast, a water-based optical clearing agent that utilizes different concentrations of fructose, See Deep Brain (SeeDB), reduces the incubation period to a few days and preserves the original morphology at the cellular resolution [75]. This method, however, appears to work best with smaller sample sizes and may not be suitable for completely clearing whole adult rodent brains.

A pioneering clearing method that shows promise for obtaining and maintaining fine biomolecular details in whole brains is ‘Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situhybridization-compatible Tissue-hydrogel’, conveniently known as CLARITY [76]. CLARITY operates through the removal of lipid membranes, while retaining biomolecules to preserve the structural integrity. First, the tissue is transformed into a nanoporous hydrogel-hybridized structure to support the sample and to prevent anatomical deformation. This hydrogel-support system allows for the removal of lipids that are responsible for the light-scattering properties of brain tissue, thereby making the tissue more transparent following electrophoretic-clearing procedures (Figure 4A). In addition, the electrophoretic-clearing technology permits multiple rounds of antibody staining and de-staining in whole intact tissue samples (Figure 4B). Passive diffusion (∼1 to 2 week incubation period) of the sodium dodecyl sulfate (SDS)-boric acid clearing solution without electrophoresis can also be applied to efficiently clear thick slices of tissue (500 mm to 1 mm). Future optical clearing applications may consider combining SeeDB and CLARITY methods to optimize tissue transparency.

The major caveat with these optical clearing strategies is the ability to acquire and analyze detailed biological information at the cellular resolution with a fast volumetric imaging system. Two-photon systems are ideal for imaging deep in tissue, but the limited focal plane of the microscope yields time-consuming tile scans for large volumetric samples (Figure 4C). Laser-scanning light-sheet microscopy [77] is suitable for achieving high-speed volumetric imaging and for obtaining high-resolution features at the cellular level [78] (Figure 4D). Ultimately, both microscope systems require the proper objectives that possess a long working distance and a refractive index that matches the clearing solution in order to efficiently image large volumetric cleared tissue samples.

Manipulating neurons to control behavioral responses has been a hallmark feature of neuroscience [79–82]. The brain, however, embodies a mosaic of diverse cell types that are exquisitely woven together; thus, manipulations involving non-specific electrical stimulation are not sufficient for ascribing brain function to behavior. The functional role of different cell types can be defined by their genetic complexion; therefore, genetically directed

manipulations are vital for understanding the causal link between specific circuit function and mammalian behavior. Advances in molecular genetics and genetically guided activity modulators have provided entry points to identifying precise neurocircuits that modulate the intensity and direction of certain behaviors. To selectively target genetically defined neuronal populations, as described previously, Cre recombinase in transgenic animals can be used to selectively turn on gene expression for Cre-dependent viral vectors encoding optogenetic or chemogenetic modulators [83] for activating (ChR2) and silencing neurons (archaerhodopsin (eArch3.0)) [9,84–90] (Figure 5A,B). Additions to the microbial opsin toolsets and the growing availability of Cre-driver lines are rapidly driving the ability to optically control activity in genetically distinct neuronal populations. In addition, new genetically encoded activators utilize radio frequencies to activate neurons by allowing calcium influx through the opening of temperature-sensitive ion channels [91], which may prove useful for translational and less-invasive applications. Although gene expression targets are viable tactics for isolating discrete processes within a circuit, functional heterogeneity within a genetically distinct network may mask the precise role of certain brain regions in regulating behavior.

Targeting subsets of neurons based on their activity is advantageous for isolating discrete functional processes within a circuit. Hence, particular behavioral processes involved with specific memories and sensory representations are regulated by sparse neurons that may share genetic similarity with other local neurons that are not involved with these behaviors. Therefore, activity-dependent expression of optogenetic manipulators would allow restricted reactivation of only the subset of neurons that had been activated by a previous behavioral experience [92]. To accomplish this, *Fos*, an immediate-early gene that is a marker for neuronal activity [93], is used as a promoter to drive ChR2 expression in neurons activated by discrete behavioral episodes [94]. These naturally activated neural ensembles can be subsequently reactivated artificially to simulate the previous experience [95,96]. Targeting functionally defined neuronal ensembles will advance our understanding of the causal relationship between environmentally induced neural firing patterns and complex cognitive processes, such as learning and memory. However, improving the poor temporal resolution of *Fos* expression by utilizing other markers of neuronal activation will likely be important for expanding upon the specificity of behavioral mimicry experiments. Ultimately, the functional significance of a given circuit should be dissected not only by its genetic complexion, but also by its activity and connectivity. Therefore, a combinatorial approach should syndicate all of these neuronal targeting strategies to properly decipher the precise neural codes of behavior.

Although optogenetic manipulations offer precise temporal control of neural circuit activity, the propensity of decreased opsin efficiency and phototoxicity significantly increases during chronic behavioral manipulations. Thus, behavioral experiments that demand sustained neuronal modulation may consider using 'designer receptors exclusively activated by designed drugs, or DREADDs [83,97]. DREADD methods utilize a pharmacologically inert compound, clozapine-*N*-oxide (CNO), that potently activates a family of muscarinic receptors on a longer timescale (up to several hours), allowing for selective modulation of signal-transduction pathways that is sustained through long durations [98]. In comparison to

the properties of optogenetic manipulators, longer-term chemogenetic approaches have the capacity to induce prolonged hyperpolarization and depolarization effects in genetically defined neuronal populations [6,99–101], making it a more viable option for reproducing the natural physiological characteristics of particular behavioral/disease states that are associated with persistent hyper- or hypo-activated neural circuits.

Concluding Remarks

The current renaissance in systems neuroscience has been fueled by innovative technologies that can synergistically be applied to dissect neural circuit function. These collaborative approaches will continue to transform our understanding of the function of genetically defined neural circuits. While the neurotechnology described here opens the door to experimentation that would have been considered in the realm of science fiction a decade ago, the possibility exists that unforeseen limitations with these approaches will arise as they are more widely adopted. Thus, continued refinement of these tactics and scientific vigilance are critical to ensure these methods do not produce erroneous discoveries. Collectively, these tools will help generate a holistic understanding of circuit-wide function that underlies behavioral processes.

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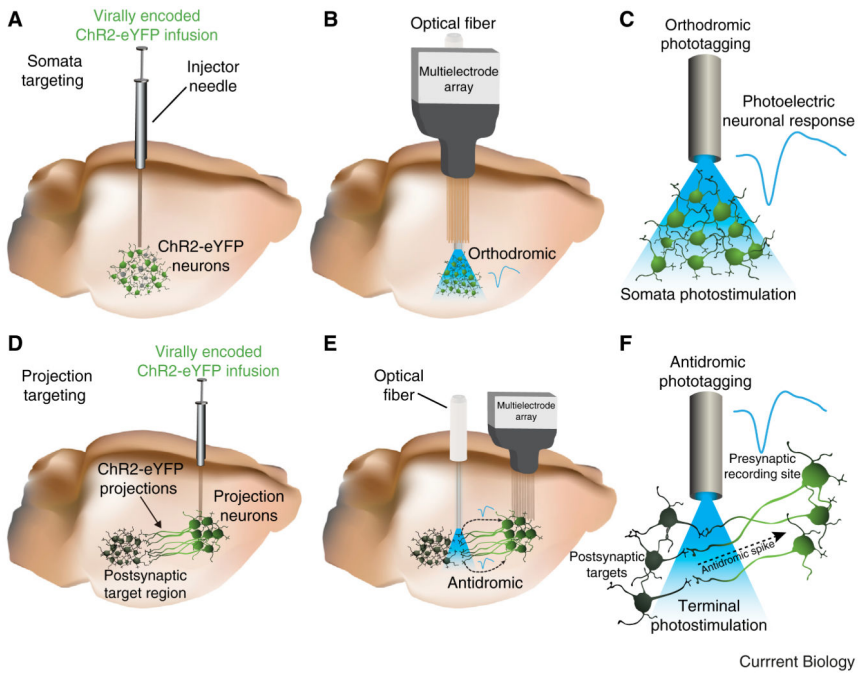

Figure 1.

Phototagging neuronal populations based on their genetic identity and projection targets during *in vivo* electrophysiological recordings.

(A) Microinfusion of virally encoded ChR2-eYFP into a particular brain region isolates genetically defined neuronal subpopulations for optogenetic identification. (B) Multielectrode array is coupled to an optical fiber and implanted within the viral-targeted brain region to detect orthodromic elicited spikes. (C) Somata photostimulation via blue light delivery from an optical fiber evokes detectable orthodromic spikes at the tip of each electrode wire. (D) Virally encoded ChR2-eYFP is introduced to the presynaptic brain region, allowing the fused protein to traffic down to the axonal terminals within the postsynaptic brain region. (E) Optical fiber is inserted directly above the postsynaptic region to photoactivate the ChR2-expressing presynaptic fibers, while the multielectrode array is implanted in the presynaptic region to record the antidromic elicited spikes originating from the terminals within the postsynaptic target region. (F) Terminal photostimulation elicits a back propagating action potential (antidromic spike) that is detected near the cell bodies of the presynaptic region where the multielectrode array is located.

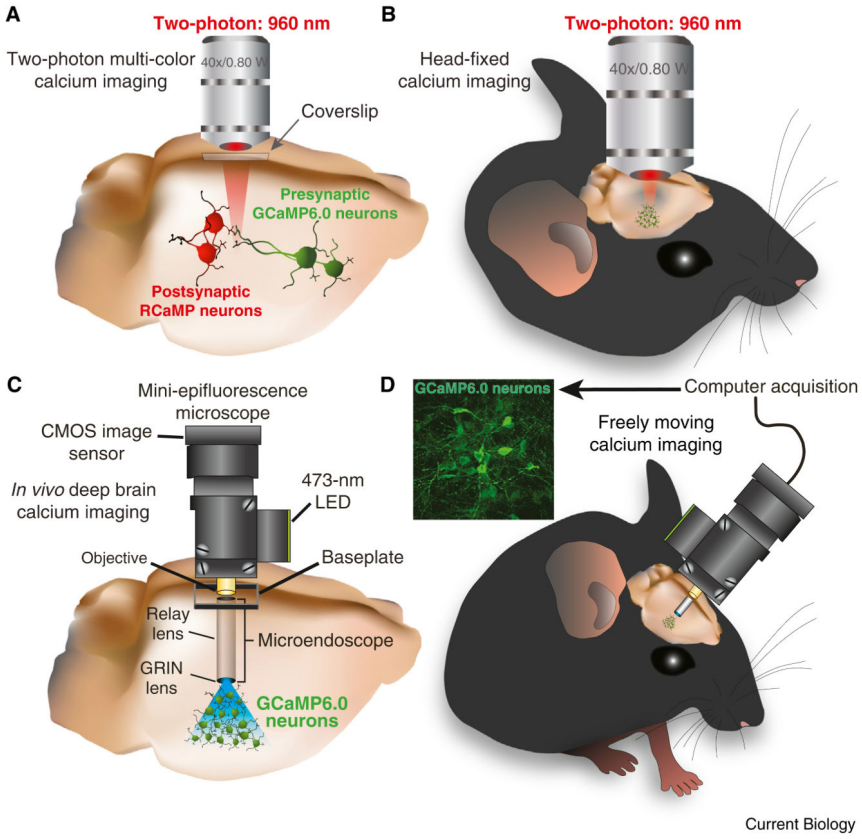

Figure 2.

In vivo calcium imaging using genetically encoded indicators reveals discrete neuronal network activity.

(A) Microinfusion of virally-encoded RCaMP (red) into the postsynaptic region allows two-photon imaging of somata calcium activity. In parallel, virally-encoded GCaMP (green) is targeted to the presynaptic input neurons, enabling simultaneous imaging of terminal calcium activity. (B) Schematic illustrating *in vivo* somata calcium imaging with a two-photon microscope in a head fixed mouse. (C) Configuration for calcium imaging in freely behaving mice using mini epi-fluorescent microscopes that are equipped with microendoscopes. (D) Illustration of microendoscopes that can detect calcium signals from GCaMP6.0-expressing cells during complex behavioral tasks.

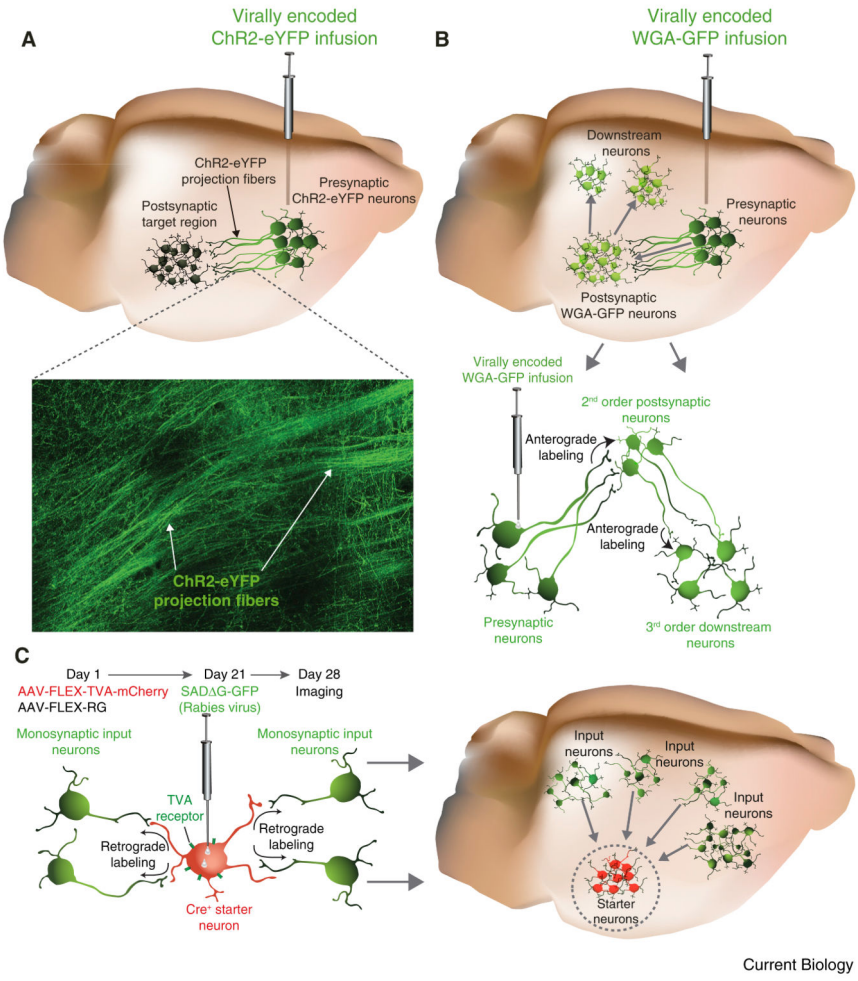

Figure 3.

Virally encoded neuronal tracing of genetically defined neuronal populations.

(A) Schematic outlining viral targeting of ChR2-eYFP (top) and a confocal image showing ChR2-eYFP labeling of axon projections (bottom). (B) Diagrams for anterograde multisynaptic viral tracing using WGA. (C) Timeline and schematic for modified rabies virus tracing. Cre-expressing neurons (red) are first infected with TVA and RG (left) allowing subsequent monosynaptic retrograde viral spread of rabies virus to presynaptic input neurons (green; right).

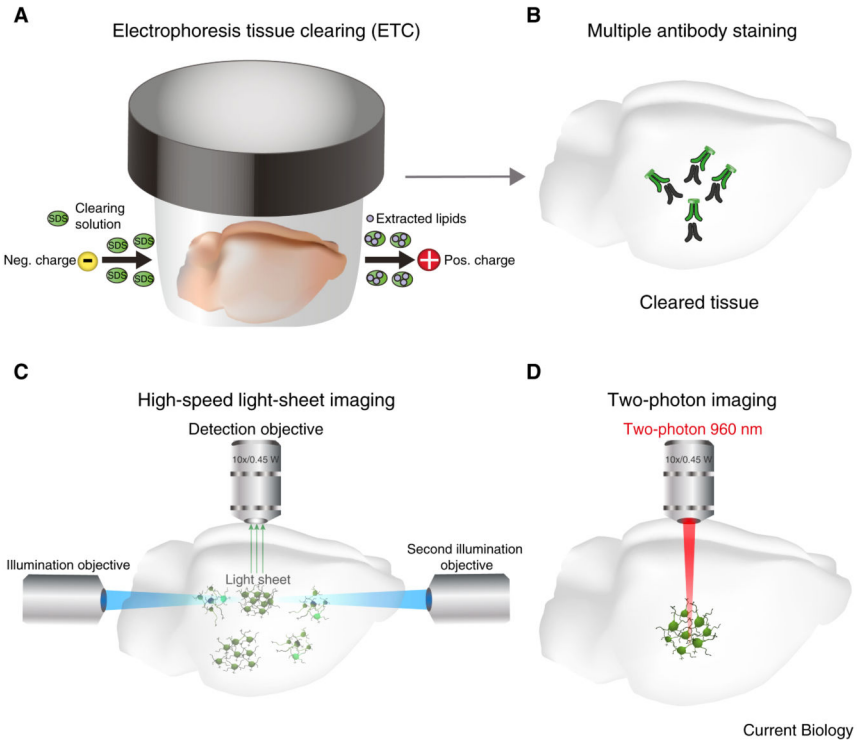

Figure 4.

Clearing tissue for imaging intact neural circuits.

(A) Diagram for electrophoresis clearing of hydrogel-hybridized tissue. (B) Multiple rounds of immunostaining in cleared whole brains. (C) Configuration for high-speed light sheet imaging of intact neural circuits in cleared tissue. (D) Two-photon imaging of neuronal populations in a cleared whole brain.

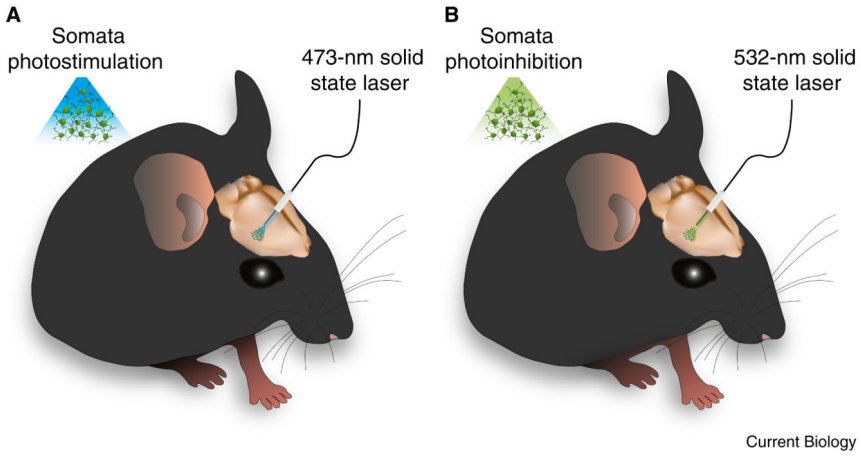

Figure 5.

In vivo optogenetic manipulations of genetically distinct neurons during behavioral measurements.

(A) Somata photostimulation of genetically defined neuronal populations during a behavioral task. (B) Somata photoinhibition of genetically distinct neuronal populations during a behavioral task.

Table 1

Commonly used viral constructs for optogenetic and chemogenetic experimentation.

Manipulators	Targeting method	Function	References
hSyn-ChR2,	Ubiquitous spatial targeting of a brain region	Elucidating regional and projection-specific function	[11]
CAG-ChR2			[12,13]
Ef1α-DIO-ChR2,	Cre-dependent: cell-type specific	Phototagging, anterograde tracing, and behavioral manipulations	[7,8,84–88]
FLEX-rev-ChR2			[6,9]
Ef1α-DIO- eArch3.0	Cre-dependent: cell-type specific	Photoinhibition for behavioral manipulations	[89,90]
Ef1α-DIO-hM3D$_q$	Cre-dependent: cell-type specific	DREADD receptor: long-term neuronal excitation	[98–100]
Ef1α-DIO-hM3D$_i$	Cre-dependent: cell-type specific	DREADD receptor: long-term neuronal inhibition	[6,98–100]

Table 2

Commonly used genetically encoded calcium indicators for reporting neural activity.

Calcium indicators	λ_{abs} (2-P), nm	Function	References
GCaMP6	940	Imaging somata, dendritic and axonal calcium activity	[30]
RCaMP	1070	Imaging somata calcium activity with ChR2 manipulations	[38]

Table 3

Genetically encoded viral tracers.

Tracers	Targeting method	Function	References
VSVΔG (LCMV-G), rVSV(RABV-G)	Ubiquitous spatial targeting of a brain region	Monosynaptic and polysynaptic anterograde and retrograde tracers	[63]
HSV-H129, HSV-1	Ubiquitous spatial targeting of a brain region	Transneuronal anterograde and retrograde tracers	[59–62]
PRV, RABV	Ubiquitous spatial targeting of a brain region	Transneuronal retrograde tracers	[64,65]
EnvA-SADΔG	Cre-dependent: cell-type specific	Monosynaptic retrograde tracers	[67–70]
WGA	Ubiquitous spatial targeting of a brain region	Polysynaptic anterograde and retrograde tracer	[55–58]