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Targeting neurons and photons for optogenetics

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Optogenetic approaches promise to revolutionize neuroscience by using light to manipulate neural activity in genetically or functionally defined neurons with millisecond precision. Harnessing the full potential of optogenetic tools, however, requires light to be targeted to the right neurons at the right time. Here we discuss some barriers and potential solutions to this problem. We review methods for targeting the expression of light-activatable molecules to specific cell types, under genetic, viral or activity-dependent control. Next we explore new ways to target light to individual neurons to allow their precise activation and inactivation. These techniques provide a precision in the temporal and spatial activation of neurons that was not achievable in previous experiments. In combination with simultaneous recording and imaging techniques, these strategies will allow us to mimic the natural activity patterns of neurons *in vivo*, enabling previously impossible 'dream experiments'.

The introduction of optogenetic tools—light-activated proteins that can activate or inactivate neural activity—is transforming the field of neuroscience. For the first time it is now possible to use light to both trigger and silence activity in genetically defined populations of neurons with millisecond precision. In principle, this enables fundamental experiments that probe the causal role of specific neurons in controlling circuit activity and behavior with unprecedented power and precision. Over the past decade a wide variety of different opsins have become available, and the 'optogenetic toolkit' is already part of the standard repertoire for investigating the functional properties of neurons at the molecular, cellular, circuit and behavioral levels[1–3]. While the adoption of optogenetics by thousands of laboratories worldwide has led to many new scientific insights, it has also exposed some of the weaknesses of current optogenetic approaches. These include a lack of specificity for the cell types being targeted, imprecise control of the number and spatial location of cells being manipulated, variability in the level of optogenetic modulation across a neuronal population, and the synchronous activation (or inactivation) of cells expressing optogenetic probes. In short, these are targeting problems: they reflect the inability to precisely deliver optogenetic probes, and the light that controls them, to the right neurons at the right time. In this Review, we discuss these problems, explore various strategies for solving them (**Fig. 1**) and give examples of dream experiments that will become possible with the application of these new approaches.

Targeting optogenetic probes to the 'right' neurons

The brain is composed of a large variety of morphologically and functionally different neurons that can be grouped into 'cell types' or 'cell classes' depending on the circuit. For example, *Caenorhabditis elegans* has 302 neurons, and the morphology of every neuron is known. Some of these neurons have their own defined function in the circuit, and therefore it is common sense to define the single neuron as the functional unit. In the mammalian retina, most neurons with a defined morphology and function exist in multiple copies occupying nodes of a spatial mosaic that covers the retina. Here the functional unit is often considered to be a mosaic of cells with the same properties, referred to as cell type. In this Review, we use "cell type" to refer to a population of neurons that cannot practically be divided into smaller units and "cell class" to refer to a population of neurons that is defined by some common property but which can be further divided into smaller populations.

A key advantage of optogenetics compared to electrical stimulation is that, in principle, the 'right' neurons—as opposed to a random set of neurons—can be manipulated. The 'right' neurons could be a cell type, such as a single retinal ganglion cell mosaic; it could be a cell class, such as parvalbumin-expressing neurons in a given brain area; it could also represent a functionally defined cell type, such as neurons in visual cortex responding to a particular stimulus orientation; and finally, it could mean subcellular localization; for example, the axon terminals in a given region. Targeting the right neurons is still a largely unsolved problem, especially in species, such as non-human primates, where genetic manipulations are often not feasible. Targeting optogenetic probes is not only important for research but also for the possible therapeutic use. In this review, we describe various approaches for targeting optogenetic probes, focusing on using viruses, alone or in combination with transgenics[4].

Viruses as 'Lego' machines for optogenetic targeting. Viruses are especially useful for optogenetic targeting because they are small (roughly 20–200 nanometers) compared to neurons, they can be injected at any time into any brain region, and they can lead to high expression of optogenetic tools. Viruses can be regarded as small machines containing modules with specific functions that can be modified. Many viruses incorporate only a few proteins that confer

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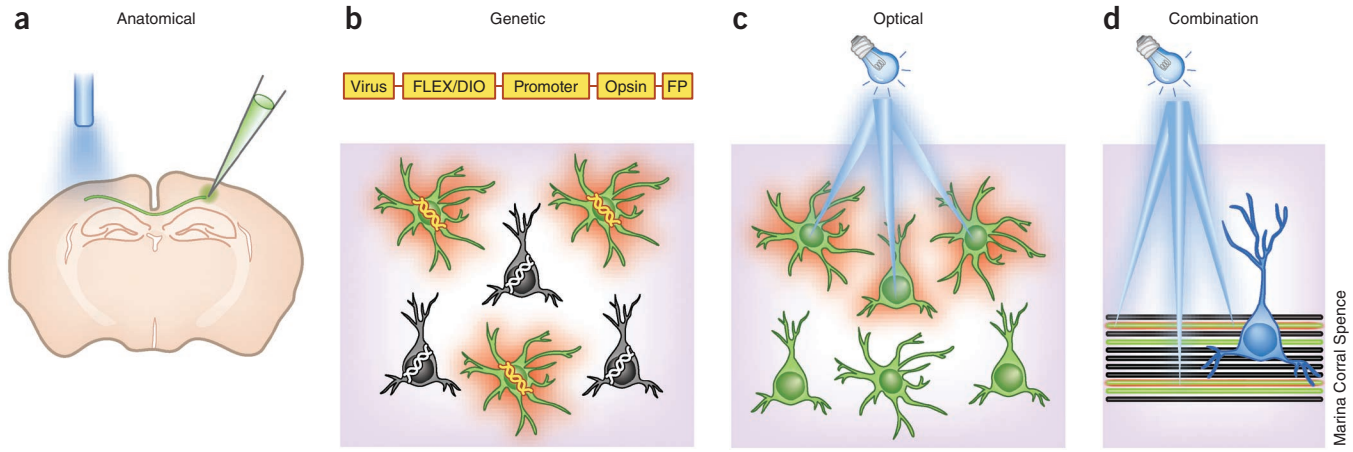

Figure 1 Intersectional strategies for targeting optogenetic manipulation. (**a**) Physical delivery of virus to a given anatomical location can exploit or uncover circuit connectivity patterns either by making use of axonal projections or by using viruses that are able to cross one or more synapses. (**b**) Cell types can be addressed if the cell type of interest has a known genetic identity. (**c**) Directing the illumination source to a given set of cells or even individual neurons and processes is useful when the targets of interest are separated in space relative to the spatial resolution of the technique used. (**d**) These three strategies can be combined, as shown in this example, in which axons of a particular cell class projecting to a subcellular domain of a neuron are photostimulated at different distances from the neuron.

essential properties. In a given viral family, these proteins exist in many variants, and this diversity can be further increased by synthetic approaches. For example, the virus used most frequently for targeting, the adeno-associated virus (AAV), has a coat protein that exists in 100 different variants in nature—and millions more can be made by DNA synthesis or mutagenesis[5]. A particular coat protein can confer a useful property, such as an affinity for a neuronal class or a preference to enter via axon terminals. By mutating that protein or providing a variant of that coat protein from a related virus the viral property can be modified, for example, changing the entry site from axons to soma or dendrites. Not only can variants of a given protein be exchanged within a viral family, but proteins can also be exchanged across highly different viruses. For example, the vesicular stomatitis virus G coat protein is often used in other viruses, such as lentiviruses, which enable efficient cellular entry[6]. Furthermore, combinations of different viruses can be used to enhance versatility. Rabies virus, for example, can be helped to cross one synapse with an engineered AAV or herpesvirus[7–9]. None of these viruses is used in its wild-type form; rather, they are assembled element-by-element by careful selection of the right components.

The thousands of viruses made by nature and the many variants made by researchers can therefore be thought of as a 'Legoland' for neuroscientists performing optogenetics experiments, or other experiments where precise gene targeting is needed. Once a new and useful property of a viral component is published, this component can be tested in any virus. Indeed, the way viruses are made is highly modular: the different properties are stored in different plasmids, and by mixing these plasmids and adding them to cells the virus is self-assembled. This modular nature of viruses facilitates innovation, providing new solutions to previously intractable problems.

Virus properties relevant for optogene targeting include the concentration at which it can be produced, whether it is an RNA or a DNA virus, whether it is replication competent or incompetent, whether it is lipid-coated or not, its physical size and its packaging capability. The concentration of the virus is an often-overlooked variable: it can vary over many log units (10^6–10^{13} ml^{-1}) and it can decrease substantially if the virus is handled improperly. Replication-competent viruses are toxic to varying degrees, but if long-term stimulation is required, replication incompetent viruses are needed. Our experience is that lipid-coated viruses, such as rabies, lentivesicular stomatitis and herpesviruses, do not penetrate well into tissues, and therefore infection occurs mostly along the needle track. The best penetration is achieved with small, non–lipid-coated viruses such as AAVs. The injection volume, injection speed and affinity of viruses for the surface of neighboring cells can influence access to cells further away from the injection site. Larger injection volumes deliver more viral particles but also can result in tissue damage. Slow injection speed may help to distribute viruses better; however, it is not clear whether the speed or the time before needle withdrawal is the more important variable. Early needle withdrawal could result in distributing the virus along the needle track before the particles have the chance to diffuse into the tissue. High virus affinity for non-target cells can substantially decrease target cell gene expression[10]. Finally, packaging capabilities vary widely among viruses, which represents a serious limitation for the more ambitious experiments with large genetic payloads.

Targeting viruses to different types or classes of neurons can be based on the genetic identity of these neurons (for example, expression of parvalbumin[4]), their specific circuit connectivity (for example, neurons presynaptic to a simple cell in visual cortex) or a combination of the two[3].

Virus targeting based on genetic identity. The morphology and function of different cell types is to a large extent defined by the pattern of genes they express. Past work has used the fact that some classes of neurons uniquely express particular signature genes—for instance, a large class of fast-spiking interneurons expresses parvalbumin—as a genetic handle that can be used to drive expression of various molecular tools exclusively in these cells. Some of these molecular tools, such as site-specific recombinases (for example, Cre or Flp) can be used to drive the expression of optogenetic probes from viruses infecting these cells[4]. Such conditional viruses can be made from DNA viruses, such as AAV[11] or herpesviruses[12]. Cell type–specific expression from RNA viruses such as rabies requires an additional component, such as a helper AAV[7]. Specificity of targeting may be increased using intersectional strategies[13]; for example, to express Cre and Flp in different but overlapping cell classes and make the virus expression conditional on both Cre and Flp.

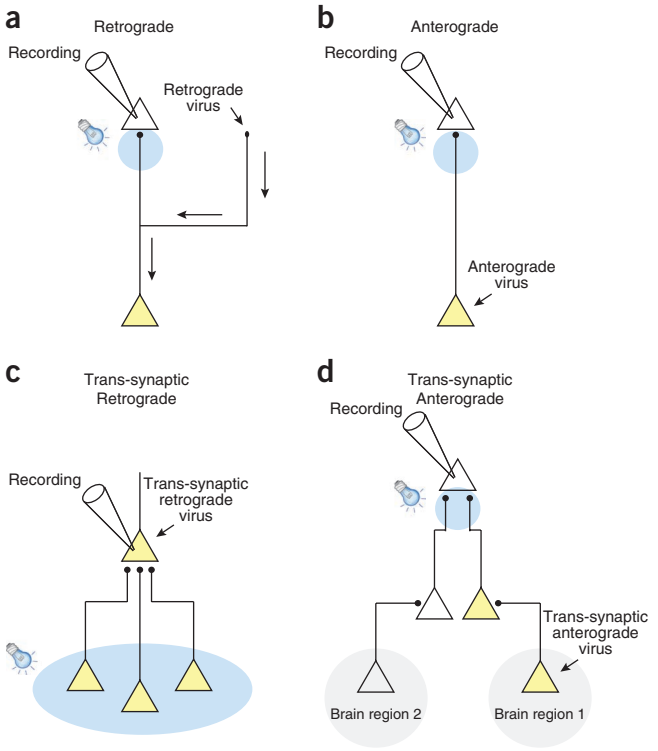

Figure 2 Viral targeting of optogenetic tools using knowledge of circuit connectivity. Schematic illustration of different strategies for targeting optogenetic tools to specific cell types based on their connectivity pattern. Neurons expressing an optogenetic tool are indicated in yellow, arrows next to cellular processes indicate the direction of viral spread, and the location of light stimulation is shown in blue. (**a**) Use of a retrograde virus with targeted virus injection to an axon projection region. (**b**) Use of an anterograde virus with targeted virus injection to the somatic region. (**c**) Use of a trans-synaptic retrograde virus starting from virus introduction (or infection) of a single postsynaptic cell, which leads to optogene expression in monosynaptically connected presynaptic partners. (**d**) Use of a trans-synaptic anterograde virus starting from virus injection in a given brain region to cause optogene expression in synaptically connected downstream neurons.

The main drawback of the conditional virus approach is that it requires expression of a site-specific recombinase, typically using a transgenic animal. The generation of a transgenic animal for a target neuronal type is both time consuming and unpredictable, and at present only feasible in a few model organisms, while viral expression requires an additional injection.

It would therefore be highly desirable to be able to target viruses directly to cell types of wild-type animals in a variety of species by using promoter elements. The most suitable virus for this purpose would be AAV because of the lack of observable toxicity and its long-term, often mouse-lifetime-long, expression[14]. Promoters are available for driving expression of AAVs in many cell types, but it is difficult to find one that restricts high transgene expression to one cell type. Screening AAVs for cell type–specific expression with random or guessed synthetic promoter elements would be highly valuable, both for basic research—as once a specific and strong promoter for a cell type is found it can be used in combination with any tool—and also for translational research and medicine, as specific and safe applications of optogenetic probes in humans may require cell type or class targeting[15,16].

Targeting based on circuit connectivity. In many cases, targeting based on genetic identity is not possible; however, some cell types can be thought of as having a 'connectivity signature' that defines them. This signature could be a specific long-range axonal projection[17], as well as specific local circuit connectivity to other neurons[18]. Where it exists, a connectivity signature combined with viruses specialized to either infect neurons at specific locations or to infect them via their synaptic connections (trans-synaptic infection) can be used for selectively targeting optogene expression (**Fig. 2**). Targeting based on connectivity can be performed in any species in which a particular virus is able to infect neurons.

Cells that project to a brain region can be targeted using injection of viruses that are able to infect neurons at axon terminals, such as some variants of herpesvirus, AAV, rabies, vesicular stomatitis, lenti- and adeno-associated viruses[19]. These viruses either naturally have the ability to enter axons or they are 'recoated' with a protein that allows them to do so. The soma of the target cell should be far away from the injection site to ensure that light used for optogenetic stimulation does not excite all the locally infected cells (**Fig. 2a**). A problem inherent to this approach is that the injection can cause damage exactly at the location where postsynaptic cells of interest reside. However, if the target cell also sends axon collaterals to another brain region, then this area could be used to initiate infection without damaging or infecting neurons in the intended postsynaptic zone. Rabies- and herpesvirus-based retrograde labeling methods, while suitable for short-term studies over days, are too toxic for studies in which long-term expression is needed. Among the viruses mentioned above, lentiviruses and AAVs are the least toxic; however, the efficiency of existing retrograde lentivirus and AAV variants is low, requiring identification of more efficient lentivirus and AAV coats for axonal entry.

An important use of optogenetics is the mapping of inputs, arriving from different brain areas, to different spatial positions on a given target neuron[20]. This can be achieved by viral delivery of the optogenetic probe to the cell bodies or dendrites of projection neurons. Once the probe is anterogradely transferred to the axon terminals of infected neurons, close to the target neuron, it can be focally stimulated by light (**Fig. 2b**). By systematically mapping regions around the target neuron, the spatial distribution of synaptic inputs from a given brain region can be reconstructed[20]. AAVs are excellent tools for anterograde delivery; however, existing AAVs are not exclusively anterograde, and further development of nontoxic, exclusively anterograde vectors is needed.

Optogenetic probes expressed using monosynaptic retrograde trans-synaptic viral tracers, such as rabies virus[21], could serve as important tools for proving putative connectivity between the virus-marked post- and presynaptic cells[22–24]. A particularly attractive strategy is single-cell electroporation of a postsynaptic neuron and the subsequent initiation of a retrograde virus from only the electroporated neuron[25]. Light stimulation of the tracer labeled cells and simultaneous electrical or optical recording from the electroporated cell could prove functional connectivity between these cells (**Fig. 2c**). A limitation of this approach is that the electroporated cell also expresses the optogenetic tool and therefore is directly stimulated with unfocused light. This can be solved by a combination of pharmacology—to compare stimulation before and after the application of synaptic blockers—and three-dimensionally patterned light stimulation as discussed below. A transneuronal approach for optogene expression that is likely to have little toxicity is the use of Cre recombinase fused to wheat germ agglutinin; this can be combined

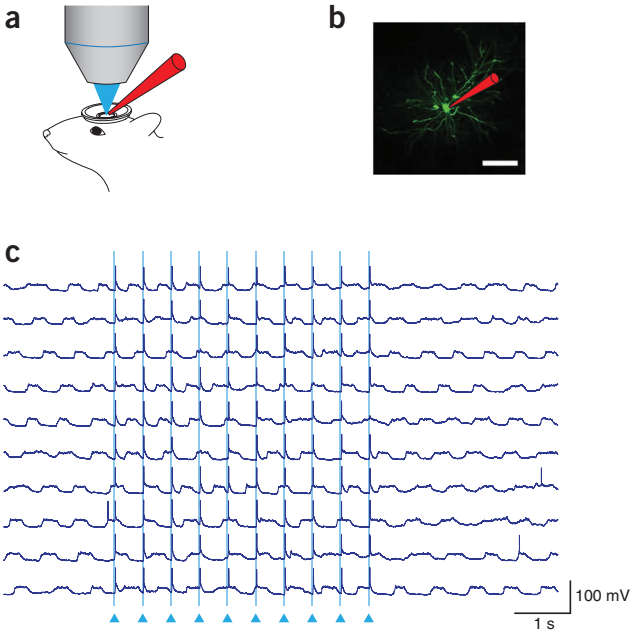

Figure 3 Targeting optogene expression using single-cell electroporation. (**a**) Schematic of the experimental setup for targeted single-cell electroporation *in vivo*. (**b**) Two-photon image of a small network of layer 2/3 parietal cortex neurons *in vivo* expressing channelrhodopsin-2 and enhanced green fluorescent protein (EGFP) 3 d after targeted electroporation of the respective plasmid DNA. Scale bar, 100 µm. (**c**) Targeted patch-clamp recording from a single layer 2/3 neuron (indicated with the red electrode in **b**) exhibiting spontaneous up and down states. Reliable and temporally precise spiking was triggered by illumination with brief pulses of blue light (5 ms; wavelength, 473 nm) to activate channelrhodopsin (ten consecutive traces are shown; 97% of pulses triggered a spike). Modified from ref. 31 with permission.

with conditional optogene-expressing viruses[26,27]. This approach is well suited for performing long-term studies and for studying optogenetically manipulated behavior.

Axonal projection–based mapping of synaptic inputs can be extended by using a monosynaptic anterograde trans-synaptic virus[23] two synapses away from the target neuron (**Fig. 2d**). This is useful when the brain area, one synapse away, contains different types of neurons that receive input from different brain regions. In many experiments, targeting is performed with the combined use of the knowledge of circuit connectivity and genetic identity[26]. Trans-synaptic tracing from Cre-expressing neurons[7,28], as well as the combination of axon projection–based retrograde labeling with labeling based on genetic identity, are powerful ways of increasing the specificity of targeting.

Despite the many available viral vectors and the possibility of combining different viruses to target the desired cell types, viral targeting is not yet robust and simple: replication-competent viruses are toxic to various degrees, growing different types of viruses in the lab requires specific safety conditions and expertise, and it often takes a long time until the targeting is optimized. These considerations highlight the need for the development of nontoxic versions of purely anterograde and retrograde as well as monosynaptic tracers. Furthermore, it would be highly desirable to create vector distribution centers for neurotropic viruses where all targeting vectors are available and where experts can produce viral kits for particular experiments and advise new users.

Long-term optogene expression. Major questions in neuroscience address the circuit basis of the formation, maintenance and elimination of synaptic connection over long time periods. Addressing these using optogenetic methods requires low toxicity and long-term stability of optogenetic probe expression. Expression with lower toxicity can be achieved using mouse genetics, AAVs, electroporation or a combination of these three. However, achieving stable expression is a key limitation. Expression via AAVs or *in utero* electroporated plasmids increases over several days or weeks, and it is unclear when equilibrium is achieved. This is a particular concern because high-level, long-term expression has been shown to cause abnormal axonal morphology[29]. Furthermore, AAVs form deposits in the target tissue after injection, which could lead to continued infection over time and a slow shift in optogene expression. This long-term increase in copy number is likely not a problem with electroporation, but for both delivery methods the number of optogene copies could vary considerably from cell to cell. The most stable method for long-term expression is the use of transgenic animals, where the changes across cells of the same type are uniform.

Single-cell targeting of optogene expression. An elegant way to precisely target optogenetic probes to individual neurons is via single-cell electroporation[30,31] (**Fig. 3**). This involves using two-photon microscopy to target a plasmid-filled patch pipette to individual neurons *in vivo*, followed by electroporation[30,31] to deliver the plasmid to the cell under visual control. Neurons can be targeted in this way on the basis of their somatodendritic morphology (using 'shadowimaging'[30]), their genetic identity (using GFP expression as a marker) or their functional properties (such as tuned responses to sensory stimuli) for subsequent optogenetic activation[31]. As up to a few dozen neurons, in any arbitrary spatial arrangement, can be electroporated using this approach, it therefore allows targeted optogene expression in a precisely defined ensemble of neurons, enabling tests of the relationships among neuron number, identity and spatial arrangement on circuit processing.

Activity-dependent expression of optogenes. It would be extremely useful to target expression of optogenetic probes to neurons not only based on genetic identity, but also based on activity patterns. This would open up many exciting experimental avenues, enabling functionally defined neuronal ensembles—rather than simply genetically defined populations—to be targeted for manipulation. For example, this approach would allow the reactivation of only the subset of neurons that had been active during a recent behavioral episode, such as during learning, allowing the minimal ensemble required for reactivating the behavior to be defined. At present, the options for implementing such a strategy remain limited. This is primarily due to the lack of known promoters that are unambiguously and specifically linked to spiking activity in neurons. Initial efforts in this direction have been made using a promoter for *Fos*, an immediate-early gene that has been shown to be switched on by neural activity[32], to drive ChR2 expression in neurons activated during a memory task[33]. However, the precise relationship between spiking activity and the resulting ChR2 expression in this system remains unclear. Moreover, as *Fos* expression, like that of other immediate early genes such as *Arc* and *Egr1* (*Zif268*), has a timescale of hours, it lacks the temporal precision to uniquely label ensembles active on a millisecond timescale during behavior, leading to problems with background and specificity (though these can be ameliorated to some extent using a combinatorial approach, such as with the tetracycline system for

gene regulation[32]). An alternative approach would be the use of light-sensitive promoter systems[34] to label cells with optogenetic tools. For example, cells that were activated during a specific behavior and were observed via two-photon calcium imaging could be forced to express optogenetic inhibitors, provided that the two-photon scanning required for the calcium imaging does not activate the promoter. In a subsequent experiment these cells could be optogenetically inhibited during the same behavior. Ultimately, it may be possible to find an appropriate promoter that is precisely temporally 'gatable' and yields a linear relationship between spiking and optogene expression, allowing well-calibrated reactivation (or inactivation) of functionally defined neural ensembles.

Targeted light delivery

Once the challenge of targeting optogenetic probes to the right neurons is overcome, the next challenge is to deliver light to those neurons. Ultimately, if light targeting is sufficiently precise and rapid to allow selective activation of individual cells, this automatically relaxes the constraints for genetic targeting (because all cells could then express the opsins and only the 'right' neurons be activated). However, we are still relatively far from this goal. Though an advanced treatment of the relevant optics is beyond the scope of this Review, we describe below some theoretical and practical considerations necessary for performing a successful optogenetics experiment. There are many options for delivering the required amount of light to a desired location, but careful consideration of the scattering nature of light in biological tissue requires control experiments to confirm that the relevant physics has been correctly taken into account. Getting enough light to the right place depends on specific experimental goals, but the key factors to consider are the wavelength, intensity and scattering of the light in the model system being used, in addition to the optical delivery system.

Selecting an animal model. Optogenetics approaches have been applied to animal models ranging from *C. elegans* and zebrafish to rodents and non-human primates. The optical access afforded by transparent animals is obviously advantageous for light-based approaches to activation, while mammals, whose nervous system tissue is much more difficult to access, are key species for modeling computations in the human brain. Most recent work using optogenetics has focused on rodent models, although recent work in non-human primates shows great promise. The first report of optogenetic excitation in non-human primates[35] was followed by the application of opsins with additional functionality such as inhibition and step-function capability[36]. The continued optimization of optogenetics approaches in non-human primates[37], which present their own experimental challenges, such as larger brains and the need for very long-term chronic installations, has recently yielded breakthroughs, with the appearance of the first reports of behavioral responses in non-human primates to excitatory[38,39] and inhibitory[40] optogenetics.

Selecting an opsin. The next step is to consider which opsin to use for optogenetic manipulation. This depends, of course, on the experiment and requires consideration of the polarity of manipulation (exciting, inhibiting or bidirectionally manipulating activity), the time course of the manipulation (involving millisecond control of spiking, or a more prolonged or subtle modulation) and the selected wavelength of light (for example, using longer wavelengths for deeper penetration or differential wavelengths if two opsins are being used simultaneously). As the rapidly growing range of opsins with different properties has recently been described extensively elsewhere[3,41], we will not treat this comprehensively.

The expression level of the chosen opsin is a key issue to consider, as viruses and promoters can often drive production of opsin molecules to extremely high levels. Overexpression can be useful in overcoming low conductances per molecule but high-level long-term expression can lead to toxicity[29]. In addition, driving all opsin-expressing membranes at once does not mimic physiological activity, and particular care should be taken when interpreting the results of such experiments, for example when all neurons of a particular cell type are driven synchronously. Finally, action potentials evoked optogenetically by illuminating the axon terminals can have different kinetics from spontaneous action potentials, resulting in differences in neurotransmitter release[42].

Selecting a light source. Practically, the first crucial piece of equipment is the light source. This can be a mercury or xenon bulb, a light-emitting diode (LED), a continuous-wave laser or an ultrafast pulsed laser (for two-photon excitation, see below). Mercury and xenon bulbs produce a wide spectrum of light that must be subsequently band-pass filtered for the desired wavelength. Bulbs produce the highest power output across the spectrum, but they need to be replaced often (200–2,000 h) and disposed of appropriately. LEDs last much longer (10,000–100,000 h), do not produce as much heat as bulbs, are generally inexpensive and can generate a specific wavelength or a wide spectrum of light. Both bulbs and LEDs emit light over a wide angular area, which can make coupling into a fiber or microscope inefficient. Nevertheless, both sources, if installed correctly, have sufficient power for reliable optogenetic activation. Laser light sources produce coherent light, which means the photons emitted are in phase with each other, a necessary property for generating holographic patterns (see SLM below) that also aids coupling efficiency into fibers. Lasers capable of yielding two-photon excitation emit 'ultrafast' pulses of light tens to hundreds of femtoseconds long.

Given the range of intensity of the various light sources, care should be taken to ensure the appropriate amount of light is delivered to the sample. Too few photons will result in insufficient activation of opsin molecules in the sample. Too much light can result in phototoxicity and photobleaching, or even activate neurons directly[43]. In addition, optogenetic tools exhibit desensitization to light over the course of seconds[41], a process noted in the initial characterization of channelrhodopsin-2 (ref. 44). Ultimately, only simultaneous electrophysiological recordings can confirm directly that enough photons impinge on the opsin molecules to drive sufficient current throughout the duration of an experiment. Such a calibration experiment is crucial, particularly when prolonged or repeated photostimulation is necessary.

Delivering light from source to sample. Transmitting the light from the source to the sample is the next practical consideration in designing an optogenetics experiment. This depends on whether the experiment is performed *in vitro* or *in vivo*. An *in vitro* experiment, for example electrophysiological recordings in acute slices during optogenetic manipulation, typically involves using a microscope onto which the light source can be coupled. The excitation light on many fluorescence microscopes can be simply repurposed for optogenetic stimulation once the appropriate wavelength and light intensity parameters are chosen. Alternatively, an LED or laser light source can be installed on the fluorescence excitation port of such a microscope. Light sources can also be mounted remotely to the microscope and the light delivered to a fluorescence port via a fiber or liquid light guide. An advantage of *in vitro* preparations is not only the stability

Table 1 Comparison of light targeting strategies

Targeted light strategy	Number of neurons addressed	Pros	Cons	Biological questions addressed	Representative references
1P full field	100–1,000	Many neurons activated simultaneously, high temporal resolution	Low spatial resolution using viral transfection	Circuit analysis of cell types	44,94
1P full field + sparse labeling	1–100	High spatial and temporal resolution; can identify cells individually	Only suitable for low numbers of neurons	Single- to many-neuron computation	31
1P fiber-optic	100–1,000	Can be used in freely moving animals	Low spatial resolution	Effect of cell types on behavior	95
1P directed beam	10–100	Spatial resolution ~50 μm	Cannot activate individual neurons	Mapping anatomical features of cell types and projections	20,96
1P DMD	100–1,000	Commercially available	Low spatial resolution	Effect of activation of cell types in spatial patterns	63,64,93,97
1P SLM	100–1,000	Holographic patterns enable photostimulation in three dimensions	Low spatial resolution	Effect of activation of cell types in spatial patterns	98,99
2P directed beam	1	Single cell spatial resolution	Only one neuron at a time	Mapping inputs from individual neurons	68,75,76,100
2P SLM	~50	High-resolution holographic patterns can activate multiple individual neurons	Low temporal resolution	Manipulation of neural coding at the individual neuron level	70,76
2P temporal focusing	1–10	High spatial and temporal resolution: can activate multiple individual neurons	Few neurons at a time given high laser power required for each neuron	Manipulation of neural coding at the individual neuron level	69,70
2P AOD	1–?	High spatial and temporal resolution: can activate multiple neurons sequentially over very short intervals	Untested	Manipulation of neural coding at the individual neuron level	None

1P, one-photon; 2P, two-photon.

of the sample, enabling higher resolution optical manipulations, but also the ability to leverage opportunities for optical access provided by the microscope. In addition to fluorescence ports, for example, light can be focused through the condenser or tube lens using one of the various targeting strategies (**Table 1**).

In vivo experiments can also be performed with a biological microscope if the animal is head-fixed, though the presence of the animal of course blocks optical access through many standard entry points (for example, from below via the condenser on an upright microscope). This situation is alleviated, however, in the case of transparent animals such as zebrafish. If freely moving behaviors in rodents or non-human primates are being investigated, fiber illumination can be used. This requires a stationary light source coupled to a flexible fiber that terminates in a mount on the animal's skull[45,46]. An alternative is an LED mounted directly on the animal, which can be controlled wirelessly[47]. Head-mounted miniature microscopes also offer the potential to deliver patterns of light stimulation for one[48] or two-photon excitation[49], but the usefulness of these devices has yet to be extended to optogenetics.

The impact of light scattering. The effect of light scattering must be considered when attempting to deliver light in biological tissue. Scattering is the process by which photons are deflected from their path of travel. A turbid medium, such as biological tissue, is highly scattering in an anisotropic manner owing to its dense and mixed composition. The mean scattering length, or distance a photon travels before being scattered, is on the order of 100 μm in biological tissue for visible light, and slightly higher for infrared light. This means that the distribution of light inside a specimen will not match the distribution of light observed when viewing the output of a light source or fiber outside a specimen. Simulations taking into account optogenetic as well as optical properties indicate that under certain experimental conditions action potentials may be initiated as far away as 1 mm from the fiber source[50]. The distribution of light in the sample is difficult to obtain, but can be estimated with Monte Carlo simulations or calibrated using simultaneous electrophysiological recordings.

The importance of simultaneous readout of activity. Given the difficulties associated with targeting of opsins to a particular cell type, strong and stable opsin expression, and adequate light delivery, the reliability of optogenetic manipulation in a given neuron cannot be guaranteed. It is therefore essential to have some form of readout of the activity of the neurons being manipulated. The best way to achieve this is by combining optogenetic manipulation with electrophysiological recording, which offers the highest fidelity measurement of neural activity[51]. A range of combinations are currently available, including simply inserting a tetrode together with a fiber; combination with patch-clamp recording[52]; up to the development of sophisticated optrodes[46,53,54]. These approaches suffer from the following problems. First, due to photoelectric effects, electrical artifacts arising from light stimulation are almost inevitable[45]. Second, electrophysiological approaches are limited to recording from only a few, and up to hundreds of neurons; and it is challenging to target particular cell types. As a consequence, assaying activity in a targeted way across the entire optogenetically manipulated population can be difficult.

These problems could, in principle, be circumvented by an all-optical approach, in which the use of calcium sensors in combination with optogenetic probes in the same cells is used to assay activity patterns from the same neurons that are being optogenetically manipulated. Such an approach faces difficult challenges, such as delivery of both sensors and activators to the same neurons with appropriate expression levels, clean wavelength discrimination, as well as stimulation and imaging at the necessary resolution *in vivo*. Using channelrhodopsin-2 simultaneously with voltage sensitive dye imaging has provided coarse anatomical mapping at the resolution of brain regions[55]. Simultaneous one-photon photostimulation and imaging has been performed in worms by making use of a digital micromirror device (DMD) to direct stimulation patterns while simultaneously performing calcium imaging of a genetically encoded calcium indicator at low intensity to avoid inadvertent photostimulation[56]. Simultaneous one-photon photostimulation and imaging has been performed with fiber optics *in vivo*, enabling manipulation and imaging on a finer scale of approximately hundreds of neurons[57]. Simultaneous one-photon

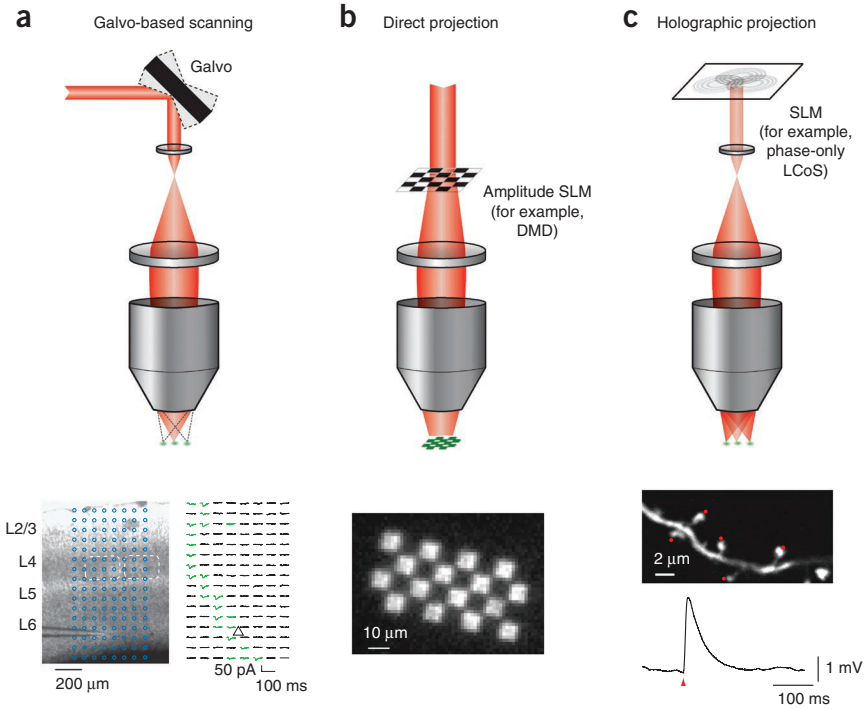

Figure 4 Patterned illumination strategies. (**a**) Top, pointing a single beam with galvanometer (galvo) mirrors is the most straightforward implementation of directing a focused beam of light onto different locations within a sample. Bottom, this approach is particularly useful for mapping studies[91] in which independent activation of small, localized subsets of labeled neurons or axons is desired for readout by downstream neurons. (**b**) Top, pointing multiple beams with a digital micromirror device[92]. Bottom, this enables more complex patterns of activation across large areas of tissue, which has proven useful in studies of retinal circuitry[63] and zebrafish behavior[93]. (**c**) Top, creating holographic patterns with a spatial light modulator combines the power of generating multiple individual beamlets with high efficiency in directing power into those beamlets. Bottom, this enables multi-site activation[70,76] when combined with two-photon excitation (see **Fig. 5**).

activation of sparsely labeled interneurons and two-photon calcium imaging could theoretically provide single-cell resolution for both activation and imaging. This has been done by disregarding any imaging data collected during the photostimulation, but single-cell resolution was probably not obtained due to likely photostimulation of axons from many neurons[58]. One-photon photostimulation of inputs to dendrites imaged with two-photon calcium imaging has enabled the dissection of subcellular circuitry, again using a blank imaging period during the photostimulation[59]. Expressing both an activator and a genetically encoded calcium indicator in one fusion construct enabled direct measurement of spectral crosstalk, highlighting the usefulness of a new red indicator (RCaMP) in combination with low-light-sensitive variants of channelrhodopsin-2 to activate and record in separate populations in *C. elegans*[60].

Patterned illumination. The simplest form of patterned illumination is to direct a diffraction-limited spot of light to the region of interest either by moving the sample[61] or by using a pair of galvanometer mirrors to direct the beam[62] (**Fig. 4a**). To generate more complex patterns, multiple beams of light can be independently directed using a spatial light modulator (SLM) to generate spatial patterns of light, for example by using a DMD[56] (**Fig. 4b**). This optically simple method is restricted by its low power efficiency, as a great deal of light is lost; however, this is often not an issue given the high intensity light sources available. DLP projectors incorporating DMDs can be installed to deliver illumination via a standard microscope condenser[63] and have even been programmed to track movement in *C. elegans*[64,65]. A third alternative for producing patterns of light is to use holographic projection, often achieved using liquid crystal on silicon SLMs (LCoS-SLM) to create holographic patterns under a microscope objective[66] (**Fig. 4c**). The holographic approach has the advantage that less light is wasted compared to a direct projection approach because the light is reshaped into a pattern. In any patterned illumination experiment, careful controls must be performed to ensure that scattering does not cause unacceptable distortion of the desired pattern at the intended location.

Two-photon excitation. The patterned illumination strategies discussed above rely on one-photon excitation, which excites any opsins in the cones of light above and below the focal plane. Two-photon excitation, on the other hand, provides high spatial resolution in both the axial and lateral dimensions by requiring two photons to be absorbed simultaneously, which only occurs within the extremely confined focal volume[67] (**Fig. 5a**). Combining two-photon microscopy with optogenetics is difficult due to this small illumination volume. While the number of opsin molecules must be expressed at levels low enough for the neurons to remain healthy, sufficient numbers of them must be activated to generate the desired current. Sufficiency depends on the current generated per opsin molecule (which depends on its two-photon absorption cross-section and its conductance); expression level (that is, number of opsin molecules per membrane area); temporal kinetics of the opsin (mainly the off time); and the particular spatiotemporal illumination strategy.

Optimization of these parameters can lead to successful action potential generation in neurons in acute slices and *in vivo*. In the initial work on two-photon excitation of channelrhodopsin[68], the most commonly used variant, ChR2(H134R), was shown to absorb two photons effectively. Scanning the somata of highly expressing cultured neurons in a spiral pattern for 32 ms can result in efficient spatiotemporal integration of photostimulated current leading to reliable action potential generation (**Fig. 5b**). Subsequent work showed action potential generation via two-photon excitation in acute brain slices using temporal focusing to create a disk-shaped illumination pattern. This enabled simultaneous stimulation of many opsin molecules in neuronal somata with very short stimulation times (≤5 ms)[69]. Combining temporal focusing with an SLM allowed structuring the two-photon illumination to match the shape of neuronal somata, further enabling the activation of more than one selected neuron simultaneously[70]. Such methods to shape the illumination to the soma require relatively high power on sample (70 to >100 mW) to obtain sufficient power density over the extended surface area, and calculations indicate that these 'parallel' excitation methods may require upward of 20 times as much power as 'serial' scanning methods[71]. This may be a particularly serious problem for *in vivo* applications, where light scattering is severe[72], although adaptive optics can be used to increase

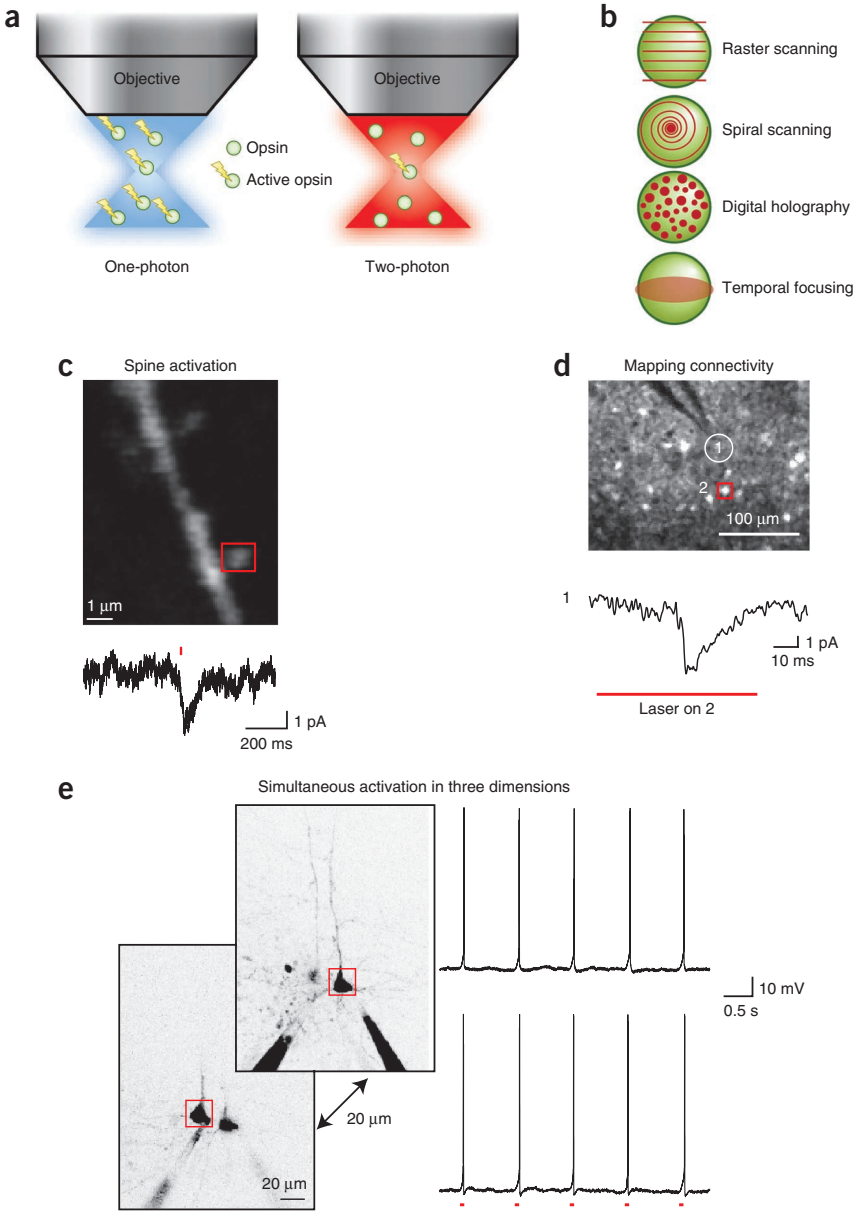

Figure 5 One-photon versus two-photon activation strategies: from spines to circuits. (**a**) In one-photon excitation (left), opsin molecules illuminated above and below the focal plane of interest are excited. In two-photon excitation (right), generally only opsin molecules in the focal plane are excited (but see ref. 68), leading to optical sectioning that allows activation to be restricted to the particular neurons of interest. (**b**) Spatiotemporal patterns for illuminating neurons with two-photon beams require different power budgets and yield different spatial and temporal resolutions (see **Table 1**). (**c**) Two-photon point stimulation of a dendritic spine on a neuron expressing C1V1 (top panel) generates current detectable at the soma (bottom trace). (**d**) Two-photon raster-scanning of neuron 2 (top panel, red box) during electrophysiological recording from neuron 1 (white circle, top panel and bottom trace) indicates that neuron 2 is monosynaptically connected to neuron 1. (**e**) Simultaneous action potential generation in two neurons in three dimensions using a spatial light modulator to generate separate laser beamlets over each neuron. Data in panels **c**–**e** adapted from ref. 76.

two-photon excitation deep in the tissue[73]. Recent work using temporal focusing (**Fig. 5b**) has shown particular robustness against scattering, enabling action potential production >200 µm deep in tissue[74]. If simultaneous stimulation of multiple neurons *in vivo* is the goal, improving the power per neuron ratio is required.

The recent introduction of C1V1, a red-shifted opsin with several variants, has addressed this issue directly. Expression of this opsin enables action potential generation in highly expressing neurons via conventional two-photon raster scanning, as performed during standard two-photon imaging[75] (**Fig. 5b**). One of the C1V1 variants, $C1V1_t$, has off kinetics approximately twice as slow as ChR2(H134R), easing the constraints on the integration of photostimulated current. With this variant, relatively short illumination times (10–15 ms) trigger robust action potential generation with only 20 mW of laser power on sample. The superior spatial resolution of two-photon microscopy allows zooming in to even finer levels of detail of neuronal function, at the level of subcellular compartments. Two-photon excitation pinpointed to dendrites and individual dendritic spines also generates reliable optogenetic excitation (**Fig. 5c**)[75,76]. This technique enables the mapping of monosynaptic connections from individual neurons to electrophysiologically recorded neurons (**Fig. 5d**)[76]. Alternatively, an SLM can be used to split the laser beam into individual beamlets, mediating the activation of multiple selected neurons in three dimensions (**Fig. 5e**)[76]. The reduced power budget implies that more selected neurons can be activated, albeit with less temporal precision.

Acousto-optical deflectors (AODs) allow dramatically increased speed in directing light versus conventional galvanometer-based systems[77–80]. Given the favorable two-photon absorption cross-section of channelrhodopsin-2, it seems the optimal excitation strategy would be to minimize scan time[68], though a lower limit to the effective scan time has been reported in raster-scanning applications[75,76]. Temporal focusing strategies[69,70] can alleviate this issue by illuminating the entire neuronal soma simultaneously, but if multiple neurons are to be stimulated, AODs should be particularly helpful given their ability to redirect a laser beam in less than a microsecond[77–80], compared to conventional galvanometer-based systems, which take ~100 µs. By enabling highly complex and rapid spatiotemporal activation patterns, AODs could also potentially be used to excite individual neurons or subcellular processes more efficiently than current strategies, particularly if new opsins with different kinetic properties become available.

Tradeoffs among light targeting strategies. The tradeoffs between the number of cells activated, to what level of activity, at a given resolution are important to consider when determining which targeted light strategy is appropriate for a given experiment (**Table 1**).

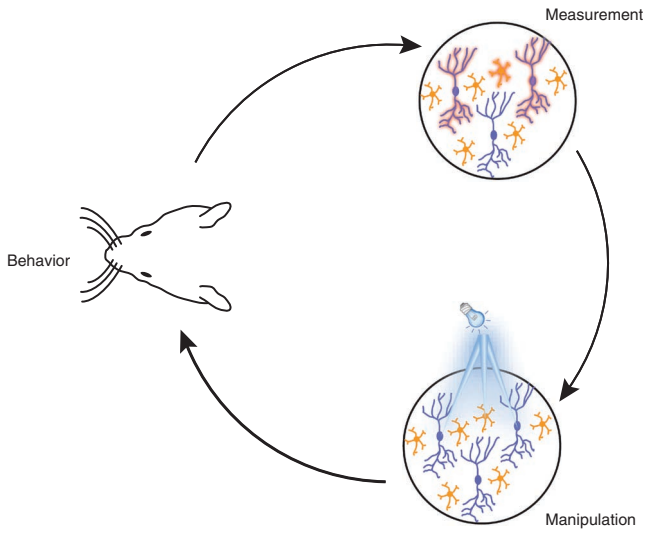

Figure 6 Using targeted optogenetics to enable 'dream experiments'. A schematic illustration of how 'targeted optogenetics' can be used to probe the neural code in a cortical circuit. The figure highlights the close interplay that is necessary between behavioral experiments, optical readout of patterns of activity and replay of the same patterns in the 'right' neurons using optogenetics. Targeted optogenetics allows the precision of temporal patterns and the precise membership of the neuronal ensemble to be tested directly to investigate their importance for the neural code driving the behavior.

Applications of successful optogenetic targeting

The genetic and optical targeting strategies described above are under active development worldwide and should yield substantial improvements over the next decade. Moreover, the various strategies can be used in concert, potentially dramatically enhancing the power of optogenetics. In order to spur further development and provide a yardstick for progress, it is useful to consider what 'dream experiments' may eventually become possible using these more sophisticated optogenetic strategies—which we call 'targeted optogenetics'—and what fundamental questions in neuroscience they can be used to answer. Here we provide a few selected examples of such experiments.

Probing neural identity. The question of what defines neuronal identity continues to remain a contentious issue in neuroscience[81]. While traditional definitions of identity on the basis of morphological features (dendritic and axonal shape, projection patterns) have recently been complemented by electrophysiological and genetic 'fingerprinting'[82,83], rigorously defining cell types continues to be a difficult challenge. The ability to target optogenetic probes to precisely defined genetically specified populations should allow the anatomical, genetic, and physiological definitions of identity to be combined in unprecedented ways. In particular, it may be possible to identify the precise functional role of a particular cell type during behavior—or to reveal further subdivisions in a defined population—by activation or inactivation of that cell class. For example, restricting expression of ChR2 in a particular cell class (identified on the basis of projection target or genetic identity) allows these neurons to be 'tagged' and identified by optogenetic activation during conventional electrophysiological or optrode recordings[84]. Such an approach has been used to identify and distinguish cortical interneurons from pyramidal cells[84–86], GABAergic and dopaminergic neurons in the ventral tegmental area[87], and striatal interneurons and projection neurons[88]. Experimental strategies such as these should allow greater security of cell type identification during *in vivo* experiments and ultimately may also lead to new, and richer, definitions of neuronal identity.

How many neurons are enough? Recent experiments have suggested that the activity of only few neurons[89]—or perhaps only a single neuron[90]—may be enough to change network activity sufficiently to influence behavior. The relationship between the number of active neurons and behavioral readout remains unknown and would put fundamental constraints on the design of neural circuits and their sensitivity to perturbations (such as noise). Being able to target expression of optogenetic probes to defined numbers of neurons, and/or being able to activate (or inactivate) precise numbers of neurons using a targeted optical approach, should allow this relationship to be determined for different cell types in different circuits during behavior. This would provide fundamental information about the sparseness of representations in neural circuits. It may also identify whether there are particular types of neurons (defined by their anatomical, genetic or functional identity; see above) or even single neurons that are unusually influential in regulating the activity of their local circuits and ultimately their behavior.

Cracking the neural code. The nature of the neural code has long been a fundamental problem in neuroscience. Given that behavior can engage thousands of neurons in intricate patterns on millisecond timescales, probing the nature of the code presents a formidable challenge to the optogenetic approach, which in its conventional incarnation only permits synchronous activation of neural populations. Cracking the neural code—in other words, determining which spatiotemporal patterns of activity in which genetically defined sets of neurons causally drive behavior—will require 'playing in' spatiotemporal patterns of activity into the circuit with the same temporal and spatial precision as the physiological patterns. Therefore, it will be necessary to combine optical readout of activity using activity indicators (for example, for voltage or calcium) followed by optogenetic manipulation of the same neurons, both with high temporal and spatial resolution, ideally in a volume encompassing the entire engaged circuit (**Fig. 6**). Such an experiment is not yet possible given the combined constraints of opsin properties and optical hardware, although the recent advances in targeted and patterned illumination described above suggest that this will soon be in reach. Moreover, because behavior engages activity differentially across different populations of neurons in the same circuit—at a minimum, excitatory and inhibitory neurons—it will be necessary to use a multi-color approach for selective manipulation of the different populations. Once these problems are overcome, however, it should be possible to test which neural codes—for example, involving different levels of temporal precision—in which neurons are required to drive specific behaviors. Similar experiments (in combination with the use of activity-dependent opsin expression) may be used to probe which activity patterns drive memory storage and retrieval. The interplay between experiment and theory is expected to be crucial for answering these questions, not only because theoretical approaches are extremely useful for refining design and interpretation of optogenetic experiments but also because theories can provide clear, experimentally testable predictions.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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