Integration of optogenetics with complementary methodologies in systems neuroscience

Christina K. Kim[1], Avishek Adhikari[2]* and Karl Deisseroth[2–4]*

Abstract | Modern optogenetics can be tuned to evoke activity that corresponds to naturally occurring local or global activity in timing, magnitude or individual-cell patterning. This outcome has been facilitated not only by the development of core features of optogenetics over the past 10 years (microbial-opsin variants, opsin-targeting strategies and light-targeting devices) but also by the recent integration of optogenetics with complementary technologies, spanning electrophysiology, activity imaging and anatomical methods for structural and molecular analysis. This integrated approach now supports optogenetic identification of the native, necessary and sufficient causal underpinnings of physiology and behaviour on acute or chronic timescales and across cellular, circuit-level or brain-wide spatial scales.

Fibre-optic patch cord
A flexible and lightweight optical fibre that is used to connect a light source (such as a laser diode or a light-emitting diode (LED)) to a fibre-optic cannula implanted on an animal, allowing light delivery to target cell populations in freely moving animals.

[1]Neurosciences Program, Stanford University, 318 Campus Drive, Stanford, California 94305, USA.
[2]Department of Bioengineering, Stanford University, 443 Via Ortega, Stanford, California 94305, USA.
[3]Howard Hughes Medical Institute, Stanford University, 318 Campus Drive, Stanford, California 94305, USA.
[4]Department of Psychiatry and Behavioral Sciences, Stanford University, 318 Campus Drive, Stanford, California 94305, USA.
**These authors contributed equally to this work.*

Correspondence to K.D.
deissero@stanford.edu

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Study of the neural underpinnings of behaviour is rapidly progressing, and many discoveries over the past decade have been enabled by technologies that address a fundamental principle: to determine how neural circuit activity controls behaviour, experimental interventions should be performed with genetic, anatomical and temporal precision. Owing to the development of optogenetics[1,2] (in which single genes encoding light-activated ion-conductance regulators or biochemical signalling proteins are introduced into targeted cells; FIG. 1; see Supplementary information S1), researchers can now control activity in defined neuronal populations and projections while examining the consequences on behaviour and physiology. Unlike pharmacological and lesion-based interventions, optogenetics (although readily applicable in bringing cellular specificity to slow or chronic timescales) also opens up causal investigation and specificity for the fast timescales of natural nervous system communication.

As with rigorous experiments in other fields of science in which causal hypotheses can be tested, optogenetic experiments include the triad of disrupting, providing and observing endogenous operation of specific hypothesized causal agents — in this case, activity patterns of neural circuit components during behaviour. Building on the fundamental process of hypothesis testing in other fields of biology — wherein, similarly, for example, a researcher studying a specific protein or gene might inhibit or knock out, activate or deliver, and measure corresponding endogenous activity — the flexibility of optogenetics is unique in further enabling causal tests over a broad range of spatial and temporal timescales to meet the unique challenges of neuroscience. Indeed, to take full advantage of the temporal capabilities of optogenetics, comparably fast behavioural and physiological readouts have been developed; modern optogenetics now includes naturalistic behavioural paradigms with fast quantitative readouts and even closed-loop feedback based on changes in the physiology or actions of the experimental system — enabling an unprecedented level of hypothesis-testing precision[2]. Such experiments require exchange of light with the nervous system; this can be achieved by implanting a small fibre-optic probe into the brain[3–5], which is typically interfaced with a lightweight fibre-optic patch cord coupled to a laser diode or to a light-emitting diode (LED) light source for input and to a fast camera or photomultiplier for readout. This robust hardware is compatible with diverse cognitive and motor tasks such as those measuring social, defensive, aggressive, navigational and indeed virtually all validated freely moving rodent behaviours[1]; recent developments in fully implantable light devices[6,7] may even further facilitate certain increasingly complex behavioural paradigms.

One example (among thousands of other published findings) was the finding that the ventrolateral portion of ventromedial hypothalamus (VMHvl) specifically modulates aggression[8]. The authors failed to elicit aggression with electric stimulation of the VMHvl, probably because this manipulation activated intermixed subnuclei and axons subserving other (for example, defensive) functions. The authors next optogenetically activated only VMHvl cells and found that this gain-of-function

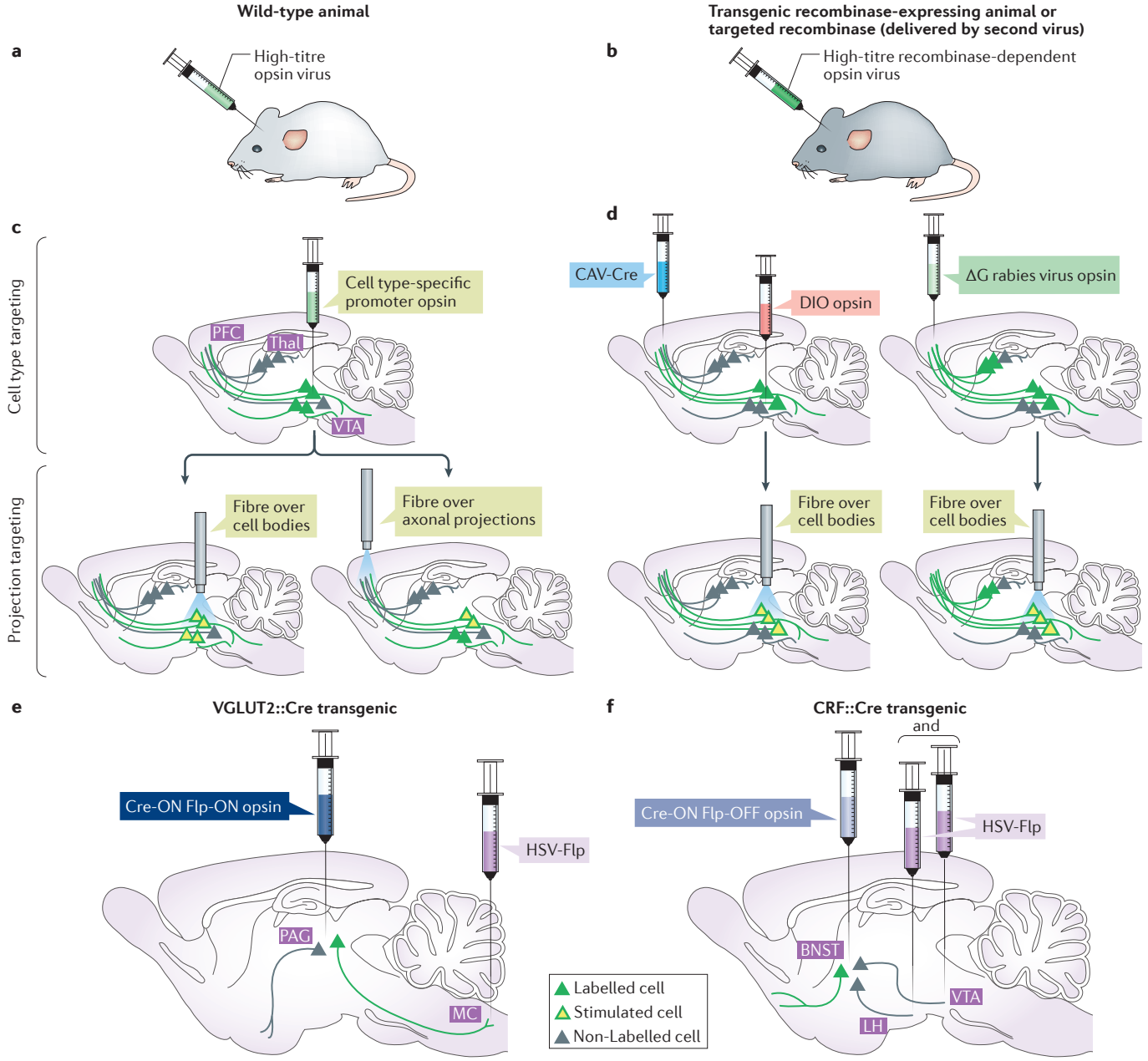


Figure 1 | **Approaches to opsin targeting with anatomical and cell type specificity. a**,**b** | The schematics demonstrate the method for expressing opsins in neurons. A DNA vector encoding an opsin is packaged into a high-titre virus (most often adeno-associated virus (AAV)), and this virus is injected into the brain region of interest, inducing opsin expression in target neurons. Cell type specificity of opsin expression can be achieved either by using a cell type-specific promoter virus in a wild-type animal (part **a**) or by using a recombinase-dependent (for example, Cre-dependent) virus in a transgenic recombinase-driver animal or with a secondary virus containing a targeted recombinase[13–15] (part **b**). **c** | Following opsin expression in the cell population of interest (green cells; top panel), a light-delivering optical fibre can be placed either over the cell bodies to target all projection neurons (bottom left panel) or in a known downstream region to target a specific projection (bottom right panel). **d** | A retrograde virus such as canine adenovirus (CAV)-Cre and a Cre-dependent doubly floxed inverted opsin (DIO) virus can be injected into the downstream and upstream region, respectively, to label only a specific projection with opsin. The fibre can then be placed over the cell bodies to manipulate that projection (left panel). Glycoprotein-deleted (ΔG) rabies virus can also be injected into a brain region to retrogradely label all presynaptic inputs. To activate a specific presynaptic input, the fibre can be placed over the input structure studied (right panel). **e** | The retrograde virus herpes simplex virus (HSV)-Flp can be used in conjunction with combinatorial INTRSECT Cre-dependent and Flp-dependent viruses in transgenic Cre animals (or with Cre viruses) to heighten projection-labelling specificity[15]. For example, HSV-Flp and a Cre-ON Flp-ON channelrhodopsin (ChR) virus can be used to label vesicular glutamate transporter 2 (VGLUT2)-expressing neurons in a Cre transgenic mouse that project from the periaquaductual grey (PAG) to the magnocellular nucleus (MC) of the medulla. **f** | This method can also be used to exclude a particular projection target. Corticotropin-releasing factor (CRF)-expressing neurons in the bed nucleus of the stria terminalis (BNST) that do not project to either the lateral hypothalamus (LH) or the ventral tegmental area (VTA) can be labelled in a Cre transgenic mouse using a Cre-ON Flp-OFF ChR virus in conjunction with HSV-Flp. PFC, prefrontal cortex; Thal, thalamus. Part **e** is adapted with permission from REF. 100, Macmillan Publishers Limited. Part **f** is adapted with permission from REF. 99, Macmillan Publishers Limited.

approach elicited a flurry of behavioural attacks on intruder mice, whereas stimulation of nearby surrounding regions did not — instead, it induced freezing, flight or no change. Countless such discoveries (often coupling optogenetic gain-of-function and loss-of-function interventions, measurements of endogenous activity and increasingly rigorous behavioural paradigms) led to results that would not have been achievable through other modes of neural intervention (such as electrical stimulation, pharmacology or lesion).

These earlier methods nevertheless remain powerful and relevant, especially as optogenetic experiments build on this strong foundation that underlies systems and behavioural neuroscience. Interestingly, when expressed nonspecifically in all neurons[9–11] to disrupt function, excitatory optogenetic tools are sometimes even used to mimic inhibitory interventions by achieving a 'lesion-like' influence with diverse global nonspecific effects[9–11]. Although this approach leverages neither optogenetic specificity nor optogenetic matching of naturally occurring local and brain-wide patterns, such intentionally nonspecific lesion-type efforts are still potentially interesting, as they could (if properly controlled) capitalize on the unique temporal flexibility of optogenetics over any acute or chronic timescale (from milliseconds to many days or more), to allow intervention-matched comparative testing of dynamical principles across timescales[12]. Current applications of optogenetics are instead much more commonly designed for ever-increasing specificity of direct targeting and modulation, with careful control experiments confirming that comparable different circuit modulations do not result in the effect of interest (such controls are standard practice in rigorous biology) and with precise matching (or tuning) of native local or global activity patterns.

As neural circuit components are highly diverse, targeting defined cell types or projections on the basis of multiple features was crucial for optogenetics and was enabled by the development of viruses with specially engineered properties, such as axonal transduction and/or retrograde propagation, cell type-specific promoter dependence and/or recombinase dependence (for example, Cre, Flp and Dre recombinases, which can be targeted to specific cell types using recombinase-driver rodent lines or recombinase-expressing viruses). For example, a two-virus strategy developed between 2010 and 2014 (REFS 13–15) entails delivering a targeted recombinase (for example, Cre-expressing) virus together with a strong Cre-dependent double-floxed inverted orientation (DIO) opsin virus to achieve both targeting specificity and high opsin expression levels even in wild-type animals[15]. Optogenetic control can be further refined by targeting neurons by virtue of their natural activity during behaviour; this is achieved either by recording activity and then manipulating the corresponding neurons with cellular resolution in the same animal or by preferentially labelling active neurons during behaviour via triggered opsin expression (using immediate-early gene (IEG) promoters) and then later reactivating those neurons with optogenetics[16–22]. Such activity-dependent opsin methods could also be useful for identifying circuit elements that are functionally downstream of specific previously activated populations, by driving or inhibiting those naturalistic populations selectively (all correctly targeted and behaviourally significant activity patterns — to regulate behaviour, just as with naturally occurring activity patterns — must exert long-range effects on other cells and regions across the brain beyond the initial directly targeted population). Cells labelled by virtue of wiring or activity can be later visualized, molecularly characterized and traced across the intact brain using tissue transparency methods, such as those based on hydrogel embedding[22–28] (see Supplementary information S1).

In summary, the past decade has witnessed remarkable developments in technologies that can be synergistic with optogenetics. Below we review these advances and highlight key demonstrations of successful integration with optogenetics. These techniques have individually yielded numerous discoveries, but recently some of the most informative lines of investigation have involved combining all of these supporting techniques together with the leverage of optogenetic control.

Natural and engineered opsin diversity

Some of the earliest steps towards genetically targeted optical control of neural activity and behaviour were reported in *Drosophila*; these elegant experiments were multicomponent in nature, requiring expression of multiple proteins[29] or optical uncaging of exogenous chemicals to activate engineered receptors[30]. By contrast, the single-component optogenetic method that was broadly adopted uses a fundamentally distinct class of molecule: microbial rhodopsin proteins[31] that act as unitary light-activated ion pumps or channels (for additional references to this microbial literature — which emerged in 1971 and has included heterologous expression to elicit light-activated ion flow as far back as 1994 — see Supplementary information S1). After the initial demonstration that microbial opsins could be used to control neuronal activity with light[1,32], microbial opsin-mediated optical control of neurons was soon reported in various different circuits and species[33–38], leading to further development for broad applicability in neuroscience[1] (see Supplementary information S1).

Excitatory or inhibitory effects can be elicited by expression of different subclasses of microbial genes encoding opsins; for example, many naturally occurring channelrhodopsins (ChRs) are nonspecific cation channels that depolarize (excite) neurons in response to blue light, whereas halorhodopsin-type Cl^- pumps and bacteriorhodopsin-type proton pumps (reviewed in REF. 39) induce hyperpolarization (inhibition) in response to yellow or green light, respectively, by pumping Cl^- ions into, or protons out of, the cell. Upon provision of eukaryotic cellular trafficking motifs that were discovered to be useful for safe and effective transport of all three classes of microbial opsins to vertebrate cell membranes[13,40–42], now all major classes of these light-activated channel and pump proteins are routinely used in neuroscience for temporally precise circuit manipulation[1]. As the special suitability of microbial

opsins for optogenetics became apparent, ion-selectivity variants were engineered to achieve new functionality — recently exemplified by Cl^--conducting ChRs. These Cl^- channels, initially engineered through crystal structure-guided mutation of ChR[43,44] and later found as naturally occurring variants[45–47], conduct multiple Cl^- ions per photon and thus can deliver more efficient and greater light-sensitive inhibition of neural activity[48] than Cl^- and H^+ pumps, which move one ion per photon.

Kinetic opsin variants were also discovered and developed for new domains of application. For example, faster-deactivating ChR variants (including ChETA[49], ChIEF[50], Chronos[51] and others[52]) have been developed and have been shown to reliably drive spiking up to 200 Hz (REF. 49). These high-speed variants have enabled studies wherein high-frequency modulation is required to mimic naturally occurring dynamics[53]. By contrast, step-function opsins[54] — including the excitatory stabilized step-function opsin (SSFO; a bistable excitatory ChR variant[42]), the step-waveform inhibitory ChR (SwiChR; a step-function form of Cl^--conducting inhibitory ChRs)[43] and others[55] — deactivate much more slowly (even over tens of minutes, instead of milliseconds); by inducing prolonged changes with brief light delivery, this class of opsin confers orders-of-magnitude greater light sensitivity to expressing cells and allows orders-of-magnitude reduced duration of light delivery, thus facilitating certain specialized chronic manipulations[56,57] (bright light exposure over hours can adversely affect cell health). The properties of these step-function tools also allow subtle modes of modulation. For example, the SwiChR variants[43,48] do not strongly hyperpolarize neurons but instead reversibly and stably open a Cl^- channel pore, as do native GABA type A receptor ($GABA_AR$) Cl^- channels. SwiChR and the second-generation version SwiChR++ by design have much slower kinetics than endogenous $GABA_AR$ channels, allowing long-timescale reversible inhibition. SwiChR is thus able to recruit (on long timescales) naturalistic inhibitory membrane properties such as shunting and, similar to native $GABA_ARs$, will display physiological sensitivity to natural influences of Cl^- balance, resting potential and input resistance[43,48]. Likewise, when SSFO is expressed, the targeted neurons do not strongly depolarize and do not experience directly light-driven action potentials (which would be useful in many but not all settings); instead, they display altered activity in a manner that is naturally timed or asynchronous in a population[42,57] depending on endogenous synaptic input activity.

Although controlling (providing or removing) neural activity with these tools can reveal whether activity in a neural circuit element is necessary or sufficient for behaviour, it is important to understand how the element and its connections across the brain are naturally used and respond to experimental or natural modulation; combining optogenetics with activity readouts such as IEG expression, functional MRI, Ca^{2+} imaging and electrophysiology is therefore important. Researchers have long integrated optogenetics with global-activity readouts[57–60] such as functional MRI, but limitations include slow temporal dynamics, lack of cellular specificity and incompatibility with freely moving behaviour. These limitations can be overcome by integrating optogenetics with genetically encoded Ca^{2+} indicator (GECI) fluorescence readouts; beyond the kinetic and selectivity opsin variants described above, spectral variants (such as the red-shifted excitatory opsins VChR1 (REF. 61), C1V1 (REF. 42), Chrimson[51], ReaChR[62] and bReaChES[63]) are compatible with blue-light-actuated GECIs[64–66] to simultaneously manipulate and record from neurons in the same animals. If the spectral properties of GECIs and opsins overlap substantially, the excitation light that is used to image activity causes unwanted cross-stimulation[67]; red-shifted opsins reduce this effect, enabling all-optical experiments that have taken great steps forward in practicality and application[68–72].

Cell type-specific opsin expression

The optogenetic toolbox can be applied with genetic specificity via viral vectors, recombinase-expressing driver animal lines and anatomical targeting strategies. The simplest way to target opsin expression to a specific cell type is to inject a virus expressing the opsin under the control of a cell type-specific promoter (FIG. 1a). Some commonly used promoter fragments that confer cell type preference and are applicable in lentivirus or adeno-associated virus (AAV) vectors are linked to the following markers[73]: calcium/calmodulin-dependent protein kinase type II subunit-α (CaMKIIα; biased towards excitatory cells in cortical regions[3,4]), hypocretin[74], oxytocin[75], D2 dopamine receptor[41], glial fibrillary acidic protein (GFAP; for astrocytes), myelin basic protein (MBP; for oligodendrocytes) and somatostatin[76]. This method has illuminated how hypocretin-producing lateral hypothalamic neurons regulate sleep–wake transitions[74] and how nucleus accumbens (NAc) D2 dopamine receptor-expressing cells modulate risk taking[41]. However, this strategy has not been used to target a wide range of cell types, because the sequences conferring specificity are usually too large to be packaged into viruses.

To address this issue, recombinase-dependent opsin-expressing viral vectors can be injected into transgenic animals or, along with targeted viruses[13–15] that drive recombinase expression, in cells of interest. For example, injecting an AAV designed for recombinase-dependent opsin expression from a strong but well-tolerated general promoter (for example, elongation factor 1 alpha (*ef1A*)), along with a second recombinase-expressing virus[13–15] or into a recombinase-driver transgenic mouse (FIG. 1b), will provide strong targeted opsin expression selectively in recombinase-expressing cells in the injected brain region, simultaneously intersecting genetic specificity with regional cell-body targeting (most AAVs do not robustly transduce axons). Hundreds of Cre-recombinase and many Flp-recombinase mouse lines are available, creating ample opportunities. Among the many results enabled by this approach, cortical parvalbumin-expressing interneurons were modulated to test social behaviour regulation[42], and septal cholinergic cells were modulated to explore regulation of hippocampal network activity[77]. 'Recombinase-off' viral vectors have also been developed to drive opsin expression in cells that do not

Kinetic opsin variants
Opsin variants that have been engineered to have slower or faster deactivation kinetics, such as the stabilized step-function opsin or 'ChETA' (E123T mutation-containing channelrhodopsin) variants, respectively.

Step-function opsins
Opsin proteins with very slow deactivation kinetics, which can thus remain activated for tens of minutes following brief light delivery and can also be switched off in a temporally precise manner with a different wavelength of light.

Red-shifted excitatory opsins
Opsin proteins such as VChR1 and C1V1 that have been discovered and/or engineered to be excited by light of longer wavelengths (that is, red-shifted), in contrast to blue light-activated channelrhodopsins, making them useful for integrating optogenetic excitation with Ca^{2+} imaging through blue-light-excited GCaMP sensors.

express the marker[78]; for example, such vectors have been used to investigate physiology of frontal-projecting non-cholinergic cells in the globus pallidus externa[79].

Projection targeting: light and opsins

Importantly, the strategies described above can be further enhanced to allow targeting of projections between two brain regions, by delivering light to opsin-expressing axon terminals via the fibre-optic interface ('projection targeting') (FIG. 1c). Optogenetic projection targeting enables versatile experimental leverage (for example, for selective inhibition of projections between brain regions[80–82] or for excitation of cells defined by projecting from one brain region to another[1,2,80]); however, several considerations must be kept in mind. For example, several weeks are necessary to obtain sufficient opsin expression in long-range axonal projections (although this issue has recently been ameliorated by the discovery that provision of a fused neuritin 3′ untranslated region can substantially accelerate the expression of heterologously expressed proteins in long-range projections[22]). In addition, if ionic milieu interactions (for example, Cl^- exchange, H^+ buffering and diverse H^+-induced currents) are different in axon terminals compared with somata, opsin effects may be different in the two locations, as would also be the case for native channels such as $GABA_A$Rs[48]; these issues suggest that the current best strategy for axonal inhibition is to use Cl^- pumps that are both insensitive to ion gradients and designed for robust axonal trafficking[13] rather than Cl^- channels[43] or H^+ pumps[83].

Additional solutions to these issues can be found in viral targeting of projections, in which opsin expression, rather than light, is targeted selectively to cells that project to a specific downstream population (FIG. 1d). One such strategy uses canine adenovirus (CAV)[84–88]; CAV driving Cre-recombinase can be injected into a given brain region (X) to transduce (along with local cell bodies) local axon terminals, resulting in expression of Cre in presynaptic cells. Injecting a recombinase-dependent opsin AAV in one of these corresponding presynaptic regions (Y) induces opsin expression exclusively in cells from the upstream region Y that project to the downstream region X. Thus, cells defined by projection can be recruited with light delivery at the soma instead of at the axon terminals. This 'retrograde' method can also be performed with engineered AAVs[89] or modified strains of herpes simplex virus[90,91] (albeit with some toxicity).

These virus-guided (in contrast to light-guided) methods can be stronger and faster in terms of enabling optogenetic control of cells defined by projections, because sufficient accumulation of opsin protein is only required in upstream cell bodies, not in axon terminals. However, true projection-specific control (for example, suppression of activity along a specific tract) cannot be readily achieved without also guiding light via the fibre-optic interface; moreover, most projection-targeting methods involving virus-guided opsin expression require two viruses, each of which will be only partially efficient, potentially resulting in a small number of labelled cells. Alternatively, one can inject a single retrograde vector encoding a recombinase (such as CAV-Cre) into a floxed-STOP (recombinase-dependent) opsin transgenic mouse line, resulting in opsin expression in all presynaptic cells and their fibres (not a single projection defined by origin and target). However, light delivery to a presynaptic population may then affect fibres of passage from multiple areas, potentially confounding the observed behavioural effects. Highly efficient rabies viruses have also been used to retrogradely target opsin expression[92,93], but rabies virus infection is toxic for neurons, and thus behavioural experiments must be performed within a few days of infection. The newly developed CVS-N2c$^{\Delta G}$ rabies virus strain is less toxic, potentially increasing the potential of rabies virus-based opsin targeting[94].

Two quantitative points are worthy of consideration regarding the physiology of optogenetic projection inhibition. First, useful information on the natural role of the projection is obtained even when potent but incomplete inhibition of evoked synaptic release is observed; inhibitory-projection targeting is especially important for certain specific experimental questions, as inhibition cannot propagate long distances back to the cell body and down axon collaterals. Second, although behavioural effects are reproducibly observed that are concordant with predicted effects of reducing projection influence in the target brain region (relative to other inputs), the effects of reducing this influence are not expected to be simply reflected in the form of altered mean native spike rates across all cells in the target region. Modern understanding of neural circuit operation in awake animals has progressed far beyond viewing circuit activity as simple summation from excitatory inputs, with local networks waiting passively for an input stimulus. Incoming inputs affect local computations through both excitation and inhibition, and mean spike rate is not expected to be altered in a simply predictable way following inhibition of a single input source among all other inputs to a brain region; instead, this intervention will reduce the influence of the projection in the ongoing computations performed in the local circuit without necessarily altering mean spike rates.

The above viral targeting strategies can be retrograde in nature (travelling from the axonal projection field of a cell to its soma), thus defining cells by virtue of output (creating output-defined elements[95]). Anterograde genetic targeting of cells defined by input is much less established, and a major limitation is the lack of robust trans-synaptic anterograde viruses. Two trans-synaptic anterograde viruses have been characterized: the H129 strain of the herpes simplex virus, which is polysynaptic[96,97], and a vesicular stomatitus virus variant, which can be monosynaptic[98] but is not extensively used because of its cytotoxicity. Although widely used monosynaptic anterograde-targeting strategies have not emerged, a method using multiple recombinases to target cell populations intersectionally on the basis of multiple genetic and/or anatomical features and using diverse Boolean logic-based operations has been developed[15] (FIG. 1e,f). This method (INTronic Recombinase Sites Enabling Combinatorial Targeting (INTRSECT)) was used to target only dopaminergic cells (but not other cell types) from the ventral tegmental area (VTA) that

Boolean logic
An algebraic framework in which the basic operations are "OR", "NOT" and "AND". These logical operators have been implemented for targeting cell types defined by the presence or absence of multiple features, such as through the use of multiple recombinases and INTRSECT viruses that allow expression of genes encoding opsins in neuronal populations that express Cre NOT Flp recombinase.

project to the NAc and only hippocampal interneurons that simultaneously express parvalbumin and somatostatin; later applications of INTRSECT have revealed broad utility[99,100] (FIG. 1e,f). Subsequent methods to target neurons defined by both input and output (defining IODES (input/output-defined elements); for example, with TRIO (tracing the relationship between input and output)[86–88]) have further extended capabilities of genetic and anatomical targeting. Together, these diverse genetic and optical advances have greatly expanded the power of optogenetics itself.

Electrophysiology and optogenetics

Patch-clamp electrophysiology is the unrivalled gold standard for high-speed single-cell monitoring of synaptic input and spiking output, but it is difficult to link the resulting data stream to defined cell types *in vivo* during behaviour. However, the integration of patch-clamp techniques with projection-targeted optogenetics has circumvented these limitations and allowed researchers to study functional connectivity of long-range projections with cell type specificity. One can ensure that only monosynaptic responses are optically elicited in acute brain slices (taken from the projection-target region after *in vivo* behavioural experiments and animal sacrifice) by pharmacologically blocking polysynaptic responses with tetrodotoxin while permitting ChR-driven monosynaptic transmitter release enabled by the addition of the stimulatory K^+ channel blocker 4-aminopyridine[101,102]; using this method, it has been shown that (contrary to prior predictions) excitatory projections from the dorsal, but not the ventral, medial prefrontal cortex (mPFC) synapse directly onto fear-suppressing GABAergic intercalated cells in the amygdala[82] and that dopaminergic cells in the dorsal raphe release glutamate on extended amygdala cells[103]. Integration of *in vitro* patch clamp with optogenetics has produced numerous additional insights into food consumption[104], anxiety[105], aggression[106], reward[92] and other behaviours.

Traditional *in vivo* electrophysiology alone is also difficult to link to specific cell types defined by genetics or connectivity, and therefore combining *in vivo* electrophysiological recordings with optogenetics has been an important and versatile technological integration, applied to numerous different circuits and behaviours (FIG. 2). For example, optogenetic stimulation of a specific input or cell type during multiunit extracellular recording allows the determination of circuit physiology effects (FIG. 2a,b); extending this approach to behavioural studies allows investigation of whether defined inputs from one region to another also contribute to encoding of a stimulus or behaviour. Using this approach, it was reported that activation of the basolateral amygdala (BLA) excites the NAc to drive reward seeking[107], that activation of GABAergic cells from the extended amygdala inhibits lateral hypothalamic neurons leading to increased food consumption[81] and that ventral hippocampal input is required by the mPFC to encode goal location[108] and aversive (open) versus safe (enclosed) spaces in the elevated-plus maze[109]. Similar principles can appear across circuits; the bed nucleus of the stria terminalis (BNST) uses BLA input to also encode closed spaces in the same maze[105]; moreover, optogenetic inhibition of BLA–BNST terminals powerfully reduced BNST representation of closed arms (as noted above, mean spike rate across all cells in the target (recorded) region can be modulated, but this measure is not necessarily expected to be altered by important modulation of a specific input during behaviour). These studies illustrate the power of integrating electrophysiology and optogenetics to create mechanistic explanations that causally link neural activity to functionally significant encoding of behaviour.

A less-commonly applied approach for combining optogenetics with *in vivo* extracellular physiology is phototagging[110–112]. This method[113] consists of expressing an excitatory opsin in the neuronal population of interest, recording and sorting the spike waveforms of units in that brain region and later attempting to assign individual electrically recorded cells to the genetically targeted population of interest by shortness of latency and low jitter (typically around 0.1 ms) of the corresponding spike waveform apparently evoked by light (FIG. 2c). Although this method will suffer from misidentification errors, in part because spike latency can be variably slow in directly excited cells or fast even in indirectly excited cells, for certain circuits that are well suited to this approach phototagging has been used to study spatial representation, food consumption, aversion and reward responsiveness, and other behaviours[91,112]. For example, phototagging has been performed with recombinase-driver mouse lines[111] to confirm that VTA dopaminergic cells encode reward prediction error and that VTA GABAergic cells are active between cue and reward delivery.

Closed-loop optogenetic interventions

Combining fast readouts (such as electrophysiology) with optogenetics creates the possibility of closed-loop optogenetic interventions, in which optical stimulation is guided by real-time readout of ongoing activity[2,114]. One study[115] recorded neuronal activity in the thalamus and cortex to detect seizure initiation in a cortical lesion-induced rodent model of epilepsy and showed that optogenetically silencing thalamocortical neurons after seizure onset sufficed to terminate ongoing epileptic activity (FIG. 2d); it would have been impossible to selectively silence thalamocortical neurons with an electrode. Similarly, on-demand activation of cerebellar parvalbumin-expressing Purkinje cells was reported to inhibit ongoing medial temporal lobe seizures[116]. It has also been reported that inhibiting CA1 pyramidal cells can modulate behavioural performance when applied during a specific portion of the task or during a specific phase of ongoing hippocampal theta oscillations: behavioural performance was enhanced either by optogenetic intervention in the encoding segment of the task during theta-wave peaks or by optical inhibition in the retrieval segment during theta-wave troughs[117]. These results illustrate the diversity and precision of discoveries that can be made at the interface of electrophysiology and optogenetics.

All-optical interrogation of circuits

Electrophysiological recording during behaviour from multiple genetically identified neurons is difficult, but advances in Ca^{2+} imaging using GECIs[64–66] now allow researchers to chronically record correlates of activity across hundreds of genetically defined neurons with either cellular or population-level resolution (see Supplementary information S1). Using cellular-resolution GECI fluorescence imaging, dynamics of local neuronal ensembles have been studied in head-fixed[63,69,118,119] or freely moving animals[120,121]; in general, this technique is limited to a small field of view to maintain cellular resolution and simultaneity, but this limitation is increasingly addressable with advanced wide-field optics. Alternatively, population-level fibre photometry[14,122,123] for Ca^{2+} lacks cellular resolution but

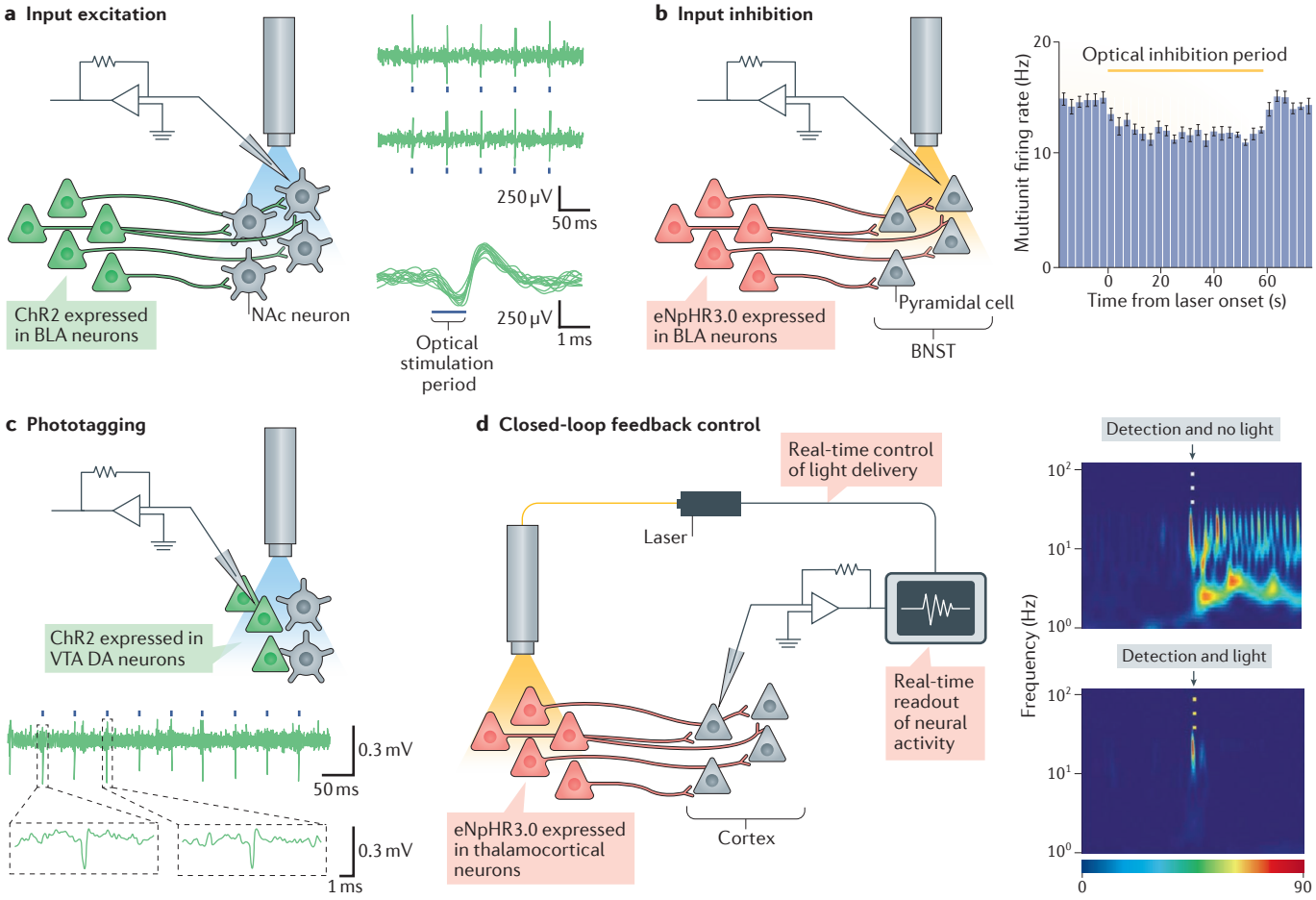


Figure 2 | **Integrating optogenetic control with *in vivo* electrophysiology. a** | The left panel shows optogenetic stimulation of axons from the basolateral amygdala (BLA) in which channelrhodopsin 2 (ChR2) has been expressed while simultaneously monitoring downstream activity in the nucleus accumbens (NAc). The right panel shows that optogenetic stimulation of BLA terminals in the NAc results in downstream electrical spiking in local NAc neurons. The top traces are example electrophysiological recordings exhibiting reproducible spiking in response to 1 ms optogenetic stimulation pulses (blue ticks). The bottom trace plots overlay trials of electrical spiking, aligned to the time of optogenetic stimulation (blue bar). **b** | Optogenetic modulation of bed nucleus of the stria terminalis (BNST) activity through optogenetic inhibition of afferent BLA axons is shown (left panel). Inhibition of BLA terminals in the BNST that express eNpHR3.0 results in net reduction in multiunit firing rate in the local BNST neurons (right panel; the yellow bar indicates the optical stimulation period); in other brain regions, highly efficacious *in vivo* input modulation may not cause such mean overall rate changes but instead modulates other features of regional computation and behavioural output. **c** | Phototagging can be used to identify and stimulate genetically specified ventral tegmental area (VTA) dopaminergic (DA) neurons expressing a ChR. ChR-expressing VTA DA cells, in this case, may be identified during *in vivo* recordings by responsiveness to pulses of blue light (blue ticks in voltage trace) and can be distinguished from non-expressing cells, which do not respond. Two examples of light-triggered spikes are shown in the bottom panel. **d** | Real-time closed-loop optogenetic inhibition of thalamocortical neurons is triggered when seizure activity is detected in the cortex by electroencephalography (EEG). Incipient seizures are detected in the cortex by EEG (in the figure, indicated by the black arrowhead above spectrogram), which in the absence of optical stimulation result in ongoing seizure activity, shown in pseudocolour (the red end of the spectrum indicates highest activity) by the rapid spiking and intense red spots in the upper panel. When seizure detection triggers yellow-light delivery to thalamocortical neurons, it results in an interruption of the seizure (bottom panel). Part **a** is reproduced with permission from REF. 107, Macmillan Publishers Limited. Part **b** is reproduced with permission from REF. 105, Macmillan Publishers Limited. Part **c** is reproduced with permission from REF. 111, Macmillan Publishers Limited. Part **d** is adapted with permission from REF. 115, Macmillan Publishers Limited.

is far simpler to implement while delivering genetically specified, behaviourally time-locked signals[14,41,72,87,123–125] and is easily scalable to multiple brain regions and axon tracts in the same animal[72].

Many studies have used GECI recording and optogenetics, as complementary tools, in separate subjects to study neural circuits deep in the brain of behaving mammals[14,41,63]. In these studies, naturally occurring neural dynamics during behaviour provide guidance for subsequent precisely timed optogenetic manipulation to mimic or block endogenous activity. For example, fibre photometry was used to identify differences in NAc D2 dopamine receptor-expressing neuron activity during the decision period before making risky or safe choices, and then optogenetic stimulation was applied only during this decision period to achieve single-trial control of risk-seeking behaviour[41] (FIG. 3a). Recently, simultaneous use of optogenetics and Ca^{2+} recording in the same animals has opened entirely new avenues for studying neural circuits, such as exciting or inhibiting axon terminals while imaging neural activity in downstream cell bodies[63], or manipulating one defined population of neurons while imaging a separate group of neurons; any of these populations could be defined for either control or imaging, or both, on the basis of genetics, anatomy or activity history during behaviour.

Perhaps the simplest approach for simultaneously controlling neural activity and monitoring Ca^{2+} transients in freely behaving mammals is wide-field one-photon activation (through a fibre) of a red-light-excited opsin-expressing population, alongside the use of the same fibre for blue-light-excited GCaMP fluorescence signals to monitor activity. Although this method does not allow control of individual neurons, it is compatible with freely moving animals, maintains genetic specificity, facilitates fast feedback and can be used on multiple brain regions in the same animal, as shown with frame-projected independent fibre photometry (FIP)[72]. Using FIP, it was possible to record from VTA dopaminergic neurons while stimulating the same neurons with a red-shifted excitatory opsin (bReaChES) for minimal cross-stimulation at the blue GCaMP excitation wavelength. Both perturbing and recording from the same VTA dopaminergic neurons in the same animal enabled titration of optogenetic light power to produce neural activity that matched endogenous VTA dopaminergic responses to reward, thus addressing a key goal of optogenetics: to match optogenetically induced activity to endogenous responses occurring during behaviour (FIG. 3b). Leveraging FIP for accessing multiple regions simultaneously, future developments can now include integration of optogenetics with multisite recording to study natural, causal cell type-specific brain-wide circuit dynamics.

Another method to integrate optogenetics and GECI recording is to use wide-field one-photon optogenetic activation in conjunction with cellular-resolution imaging in the same field; this approach involves a stimulation that is achieved through the same window, cannula or lens that is used to gain optical brain access[63]. Even more specifically, selective optogenetic stimulation of multiple individual neurons can be carried out while maintaining cellular-resolution Ca^{2+} imaging of the same and nearby individual neurons[67,69,70], enabled by the development of the first red-activated excitatory opsin (C1V1 (REF. 42)) robustly actuated by two-photon illumination[126,127]. Recently, targeted two-photon stimulation of C1V1 was used in combination with GCaMP two-photon imaging to achieve spectrally separated, all-optical readout and control[69,70]. In one study using head-fixed mice navigating a virtual reality environment, low-power optogenetic stimulation to a single hippocampal place cell was sufficient to alter firing dynamics and place-field activity of non-stimulated neurons[69] (FIG. 3c). Interestingly, these secondary-stimulation effects occurred in neurons that had place fields close to that of the stimulated cell, rather than in neurons that were anatomically in proximity. These results have substantial implications for models determining place-cell dynamics and could not have been obtained without the simultaneity of single-cell optogenetic stimulation and Ca^{2+} imaging. Newer methods using spatial light modulators[70,127] enable recruitment of dozens or more individual neurons at once, which may be important for studying causal effects of multiple individually defined cells on circuit dynamics or behaviour.

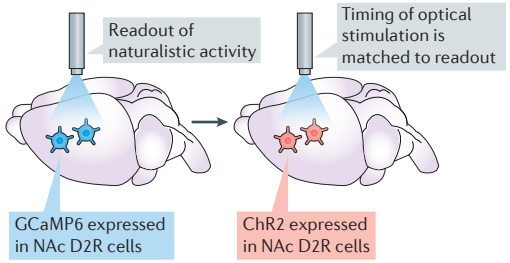
Other readout and control modalities

Although combining GECIs and opsins is a powerful approach to circuit analysis, the kinetics of GECIs are too slow to reliably resolve single action potentials *in vivo*. It may become possible to use genetically encoded voltage indicators (GEVIs) with fast kinetics instead[128–130]. However, GEVIs result in signal-to-noise ratios that are lower than those resulting from GECIs (membrane-bound, resulting in fewer sensor molecules per cell; and with fast kinetics, demanding high-speed imaging and collection of fewer photons per frame[131]). Researchers are actively engineering novel variants that may ultimately enable all-optical stimulation and interrogation of large scale networks and microcircuits alike with higher temporal resolution and full spectral separation between sensors and opsins[131].

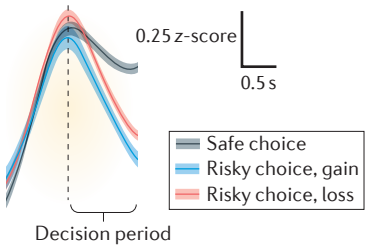
The broad action spectra of microbial opsins may pose fundamental limitations to the number of separable optical channels available for simultaneous control or observation. Adding non-optical axes of control, even if less precise, fast or targetable, could help to enhance increasingly complex forms of optogenetic experimentation. Designer receptors exclusively activated by designer drugs (DREADDs) provide a chemical option, in which G protein-coupled receptors (GPCRs) have been engineered with point mutations to be activated only in the presence of an exogenous ligand (commonly, clozapine-*N*-oxide). Such 'chemogenetic' methods allow activity modulation, although with much reduced spatial and temporal resolution compared with optogenetics[132]. Non-optical forms of energy delivery have also been explored. In magnetothermal work, magnetic fields cause neuronal depolarization via introduced nanoparticles that generate heat through hysteresis, which in turn activates overexpressed heat-sensitive transient receptor potential cation channels subfamily V member 1

a Matching timing

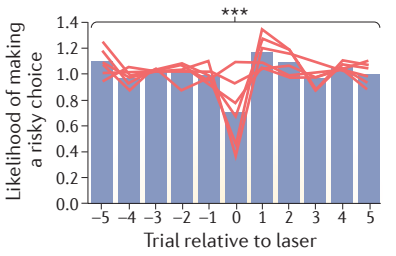
Sequentially record then stimulate neurons



'Decision period' readout of NAc D2R cells

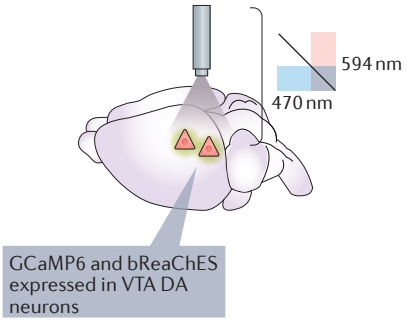


'Decision period' stimulation of NAc D2R cells

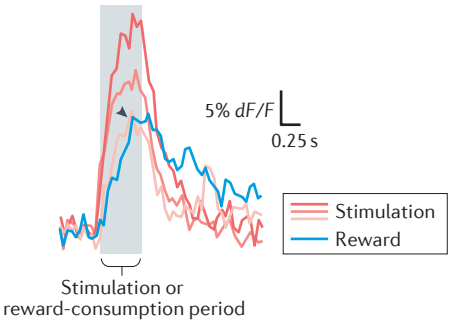


b Matching amplitude

Simultaneously image and stimulate neurons

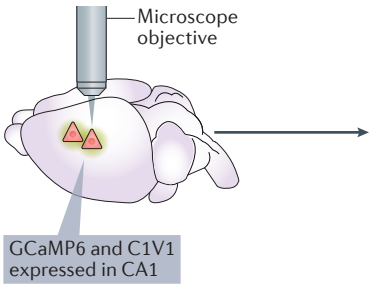


Tune the optogenetic stimulation response to match the naturalistic reward response

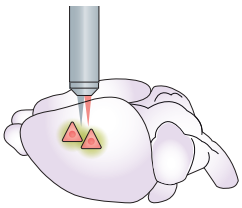


c Single-cell stimulation

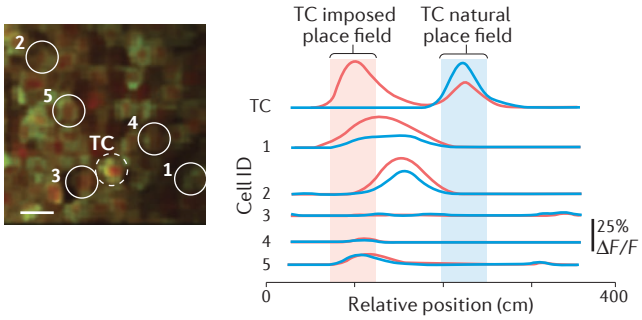
Image activity during behaviour



Stimulate cells during imaging

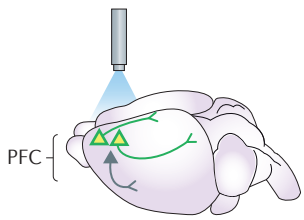


Stimulate TC while imaging neighbouring neurons

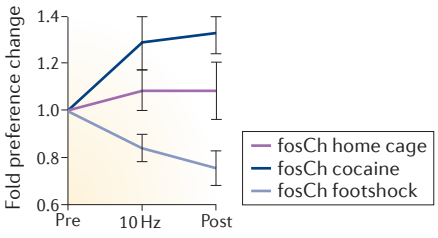


d Linking optogenetics with anatomy

Stimulation of neurons that had been previously active during behaviour



Real-time place-preference test



Use CLARITY to map projections and cell types of previously active neurons

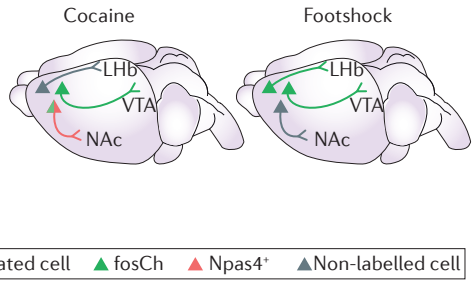


Figure 3 | **Integrating optogenetic control with optical methods: matching naturally occurring activity patterns and linking to brain-wide projection activity.** **a** | Matching timing: Ca^{2+} recording of nucleus accumbens (NAc) D2 dopamine receptor (D2R) neurons expressing the sensor GCaMP6 in one cohort of mice was used to guide optogenetic stimulation timing parameters of channelrhodopsin-expressing NAc D2R neurons in another cohort of mice (left panel). The activity of NAc D2R neurons was recorded during a risky decision-making task (middle panel). The graph shows that the activity during the decision-making period is lower on trials wherein the animal makes a risky versus safe decision, independent of whether the risky decision resulted in a gain or loss. Stimulation of NAc D2R neurons to mimic timing of activity during the task-decision period (using the method shown in panel **a**) produced an instantaneous, reversible and significant ($P < 0.001$) reduction in risk seeking (zero timepoint on the x axis); stimulation during other task epochs was much less effective. **b** | Matching amplitude: this paradigm is designed to simultaneously stimulate and record from ventral tegmental area (VTA) dopaminergic (DA) neurons to match evoked responses to naturally occurring responses (left panel). Right panel: three optogenetically evoked responses (shades of red) could be titrated to closely resemble VTA DA response amplitude to natural reward consumption (blue) in the same animal (in the figure, the black arrowhead indicates similar amplitudes of the evoked response and the natural reward response). **c** | The left panel shows simultaneous imaging and manipulation of local circuit dynamics in hippocampal CA1 on a cellular level. The example two-photon image shows individual hippocampal neurons expressing GCaMP and C1V1. Cells 1–5 and the target cell (TC) correspond to the traces shown on the right. Optical stimulation of the TC induces network-level changes in the place-cell firing of other neurons, causing some neurons to also fire within the imposed place field of the TC (cells 1 and 2), but not other cells (cells 3–5) (right panel). **d** | Left panel: integration of optogenetics and activity-dependent immediate-early gene labelling techniques in medial prefrontal cortex (mPFC) populations is shown. Middle panel: compared with the control response in the home cage (fosCh home cage), stimulation of mPFC neurons that had been previously activated by exposure to footshock was sufficient to drive place aversion, whereas stimulation of PFC neurons that had been previously activated by cocaine exposure drove place preference. Right panel: methodologies that enable clearing of brain lipid content after covalent hybridization of other biomolecules to a hydrogel have revealed distinct cell typology and projection patterning between cocaine-activated neurons and footshock-activated neurons. Cocaine-activated neurons (cells expressing the gene neuronal PAS domain-containing protein 4 (*Npas4*$^+$)) were revealed to have more dense projections to the NAc, whereas footshock-activated neurons project more densely to the lateral habenula (LHb). Part **a** is adapted with permission from REF. 41, Macmillan Publishers Limited. Part **b** is adapted with permission from REF. 72, Macmillan Publishers Limited. Part **c** is adapted with permission from REF. 69, Macmillan Publishers Limited. Part **d** is adapted with permission from REF. 22, Elsevier.

(TRPV1) channels[133]. These multicomponent systems are currently restricted by limited nanoparticle diffusion in tissue, limited compatibility with free behaviour and potential side effects of TRPV1 expression. Several groups have sought single-component magnetogenetics using non-channel iron-binding proteins, such as ferritin and MagR, to transduce magnetic fields, but possible magnetic or heating mechanisms remain unclear[134]; moreover, like chemogenetic or ultrasonic[135] approaches, the magnetic approaches would be much slower than optogenetics, and also could not be used to control neurons precisely defined by origin and target. Still, efforts like these following the optogenetic principle (genetic introduction of a transducer for external energy) should be pursued to identify new modalities for integrated control.

Activity-guided optogenetic stimulation

As described above, integration of fluorescence-based activity readout with optogenetics now allows experimentally imposed matching of timing and magnitude of activity in cells with particular activity properties during behaviour[41,72]. Although fluorescence readouts provide high temporal resolution and the option for cellular resolution, these methods do not allow whole-brain single-cell resolution readouts in mammals. Instead, whole-brain single-cell resolution readouts can be achieved by preferentially driving gene expression in previously active neurons via IEG promoter-based transgenic or viral strategies[16–22,136,137], and these strategies can be adapted for playing-in activity if the expressed gene is a microbial opsin.

In neurons, IEGs such as *FOS* or *ARC* (or engineered promoters such as E-SARE[138]) are transiently active beginning less than 1 hour after strong bouts of activity or powerful behavioural experiences and therefore are often tracked by investigators using molecular and histochemical approaches (often in combination with optogenetics) to determine which neurons or brain regions were activated by a particular behaviour or intervention (for example, REFS 14,105). Although IEG expression has been demonstrated to correlate with neural activity[139], expression can also be seen in non-neuronal cell types. Furthermore, this approach is susceptible to labelling 'background-activity' neurons that are active during the broad labelling window of IEGs (on the scale of many hours) but are not involved in the behaviour of interest. However, whereas IEG-driven optogenetics has limited temporal resolution for defining a behavioural experience, the speed of subsequent optogenetic control remains fast, and this approach confers the advantage of simultaneous access to many activity-defined neurons in a large tissue volume, unlike cellular-resolution light delivery. Just as genetically defined and projection-defined optogenetics revealed many previously unknown functions of brain areas and connections in behaviour[1], activity-guided optogenetics has the potential for large-volume spatially unbiased discovery of new pathways wherein only a subset of the neurons in a given brain region causally control a behaviour.

In a landmark demonstration of this approach, IEG-defined activity-guided ChR expression was used to preferentially label neurons in the dentate gyrus that were active during the encoding of a fear memory[16]. The authors designed and used a transgenic mouse strain in which ChR expression from a viral vector was elicited in *FOS*-expressing dentate neurons activated by fear conditioning. Optogenetic reactivation of these cells was sufficient to induce freezing in a novel, neutral context, showing that contextual memories may be recruited from the dentate gyrus and that stimulation of an ensemble of these neurons is sufficient to reactivate fear behaviour. Importantly, it has been shown that nonspecific stimulation of dentate gyrus granule cells instead disrupts fear-memory encoding and retrieval in mice[140], highlighting the value of activity-dependent optogenetics. Related methods have been used not only to reactivate prior fear memory but also to implant a false fear memory in neutral[17] or rewarding[18] contexts and to improve fear-memory retrieval during chemically induced amnesia[20], among other studies[19,21,141].

Tissue–hydrogel composites

New techniques allow time-locked labelling of previously activated neurons without use of transgenic mice. Recently, whole-brain optical-access technology via hydrogel embedding[142] has been combined with activity-dependent opsin labelling in mice[137] to discriminate cell types in the mPFC recruited by behavioural experiences of different valence[22]. A novel viral vector driving ChR expression under a *FOS* promoter (linked to a protein-destabilizing sequence for greater temporal precision) enabled temporally precise, activity-dependent labelling of mPFC neurons during either cocaine or footshock exposure and revealed that reactivation of mPFC neurons that had earlier been recruited by footshock induced place aversion, whereas reactivation of neurons recruited earlier by cocaine induced instead place preference. Surprisingly, experimental integration with a hydrogel-based method (in this case, CLARITY[24,95]) revealed that these two mPFC cell populations not only had different causal effects on behaviour but were in fact different cell types defined by distinct molecular and brain-wide wiring features[22] (FIG. 3d). Brain clearing and/or labelling techniques with diverse capabilities continue to be developed that could be partnered with optogenetic techniques (reviewed previously[95]; see also Supplementary information S1).

Current limitations and future directions

As always, limitations must be considered while designing experiments and interpreting data (reviewed previously[1,2,71,95]); for example, opsins should be carefully chosen for each application, owing to trade-offs among desirable features (for example, opsins with higher light sensitivity also tend to have slower off-kinetics[143]). Some limitations specifically affect projection-targeting experiments, such as the time that is required to achieve high levels of opsin expression in long-range axon terminals, or the potential for antidromic spiking that is caused by axon stimulation or nonspecific stimulation of fibres of passage. Integrative activity-tracking solutions can help to circumvent some of these potential caveats. For example, electrophysiology[82] and IEG expression measures[80] can be used to test whether optogenetic stimulation of axon terminals modulates action potentials in an upstream brain region, and CLARITY-based imaging can be used to demonstrate absence of potentially confounding fibres of passage[82]. Other controls include verifying that induced effects are abolished by positioning the fibre-optic instead over the potentially confounding fibres of passage themselves[82,105] or repeating the experiments while pharmacologically inhibiting different downstream structures to test for blockade of light-induced effects[80,144]. Another issue that affects projection-targeted experiments is that the most commonly used inhibitory opsins (Cl^- or H^+ pumps) are less efficient than channels; H^+ pumps have an additional issue, as activating these in axons increases spontaneous neurotransmitter release[83]. Potential solutions include projection targeting by genetics rather than by optics for these cases, so that cell bodies, rather than axons, may be controlled as described above, and future efforts in opsin engineering may continue to advance integrative efforts along these fronts.

Opsin engineering may also be key to solving a remaining challenge for integrating optogenetics with Ca^{2+} imaging, as all 'red-shifted' opsins can still be partially activated by the blue light that is used to image GCaMP[2]. Although it is possible to circumvent this cross-stimulation issue by titrating down the intensity of GCaMP imaging light[69,70,72], this strategy will limit signal-to-noise ratio of Ca^{2+} transients, and there may also be subthreshold changes in activity that are not detected. Although the use of red Ca^{2+} indicators with blue-light-activated ChRs could address this issue[68,145–147], in general, the red indicators have reduced signal-to-noise ratio and slower kinetics compared with GCaMPs, and some variants exhibit non-Ca^{2+}-related fluorescence changes in response to blue light (photoswitching)[68,147]. Future modifications of existing red Ca^{2+} indicators may allow spectrally independent optical readout and control of neural activity, but currently, issues with photoswitching and photobleaching complicate implementation of this combination *in vivo*.

The rapidity of targeting-strategy development suggests that the specificity of optogenetic manipulations will continue to grow; emerging opportunities include increasingly diverse AAVs generated by directed evolution[89,148] and glycoprotein-deleted rabies viruses generated by pseudotyping[94]. A second potential future avenue to pursue higher specificity in opsin targeting is to combine different recombinases (for example, Cre, Flp and VCre[15]) using anatomical and/or genetic criteria to restrict opsin expression to specific cell types and projections. The addition of a third recombinase[15] would enable targeting based on intersection of three criteria, and higher targeting specificity can reveal effects not otherwise observable. Methods to express opsins with genetic specificity have led to discoveries about how specific cell types affect behaviour, but future work will need to involve integrated control of multiple cell types, regions and projections. Development of red-shifted opsins and multiregional light-targeting strategies have partially addressed this issue[42,63], but spectral overlap among opsins persists, and further discovery and engineering are required for multiple-population control; the development of chimeric ChRs pieced together from known variants[149] alongside the ongoing identification of naturally occurring opsins, as well as the structure-guided discovery and utilization of opsin mechanistic principles, may continue to provide new insights and tools.

Beyond precise excitation and inhibition, different kinds of modulation can be achieved. Step-function excitatory and inhibitory tools can alter excitability without directly driving spiking or powerfully hyperpolarizing the membrane, respectively[42,43,48], and optogenetic neuromodulation can also be achieved by driving neuromodulatory neurons (for example, dopaminergic[14], noradrenergic[150], cholinergic[77] and peptidergic[151] projections). Finally, several strategies have been devised to create neuromodulatory optogenetics in the form of chimaeras of GPCRs and non-microbial rhodopsins, which

enable light-activated biochemical signalling[152] and have been developed into a generalizable 'optoXR' strategy for fast optical control of neuromodulation and behaviour in freely moving animals[153] (the X in optoXR stands for the GPCR of interest; these approaches led to the development of tools such as opto-α1AR, opto-β2AR, opto-D1R, and so on, which can be targeted to only those cells naturally expressing the original GPCR using recombinase-dependent AAVs[14]). For example, the creation and testing of opto-α1AR (which is obtained by replacing the intracellular loops of bovine rhodopsin with those of the mammalian α1 adrenergic receptor) revealed that optical activation of α1 adrenergic receptor pathways in NAc neurons is appetitive by evoked place preference[153], whereas optical activation of β2 adrenergic receptor pathways elicits anxiety-related behaviours[154]. OptoXRs, generated by different groups around the world, also include opto-A2AR (adenosine 2A receptor), opto-μOR (μ-opioid receptor) and opto-mGluR6 (metabotropic glutamate receptor 6; which is obtained by using light-activated domains of the vertebrate melanopsin receptor instead of rhodopsin, resulting in light sensitivity sufficient to partially rescue vision in a mouse model of photoreceptor degeneration[155]). Rapidly expanding generation and use of optoXRs has opened the door to studying other neurotransmitter systems that are linked to GPCRs, including neuropeptide Y receptors and their ability to regulate pain, obesity and circadian regulation[156].

Real-time closed-loop feedback has been performed using optogenetics and electrophysiology[115] but has yet to be perfected in all-optical settings with Ca^{2+} imaging[2]. By virtue of relative simplicity, fibre photometry provides a ready platform for such real-time feedback control of neural activity, and genetically encoded Ca^{2+} signals (although slower than electrophysiological signals) enable closed-loop experiments with optogenetics linked to activity in specific cell types or projections. Given the expanded anatomical and genetic toolbox that optogenetics provides, additional possibilities include modulating the activity of one population of neurons on the basis of the Ca^{2+} readout of a separate population, either in the same or in a different brain region[2]. The ability to modulate one neural population on the basis of the activity of another population could lead to potential translational insights into disease states such as autism, in which the relative balance of opposing and genetically distinct neural circuit elements is thought to be crucial for maintaining typical behavioural function[42]. Another avenue would be to use FIP optogenetics to naturalistically stimulate a cell population in one region and to use FIP Ca^{2+} recording in multiple downstream axon terminal fields to readout activity patterns among different projection neurons. FIP optogenetics could also be used to impose or degrade brain-wide 'networks' such as the default mode network or saliency networks or to test causal relevance of brain-wide correlations during behaviour. These and other related approaches may be useful for studying multiple different circuit outputs, as well as for mapping (and testing the causal significance of) brain-wide responses to different behaviours.

Finally, recent developments in brain-clearing techniques[22–28] will enable complementary anatomical discoveries to be made[22,82,87] (as fibre tracts can now be preserved throughout entire intact brains, and immunostaining[22–27] and *in situ* hybridization[28] in this setting can reveal molecular phenotypes of multiple populations of neurons). One pathway to integrate brain clearing with optogenetics and activity imaging would be to first record the activity of a population of neurons during behaviour, selectively manipulate those neurons according to naturally occurring timing and/or magnitude of signals[41,72] using cellular-resolution closed-loop optogenetics contingent on activity to evoke changes in behaviour, and then use the tissue–hydrogel method to identify the neurons that were previously imaged and/or optogenetically manipulated and map their brain-wide projection patterns and molecular identities using labelling[22] registered back to cellular-resolution activity. Such integrated use of developments in optics, molecular biology, anatomy and behaviour is now feasible, enabling examination of neural circuitry from multiple different perspectives that contribute unique and synergistic information about how cells, connections and circuits modulate local and global network activity in the encoding and causation of behaviour.

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Integration of optogenetics with complementary methodologies in systems neuroscience

Christina K. Kim, Avishek Adhikari, and Karl Deisseroth

Supplementary Note. Additional background, technical detail and references

This note provides background, technical information, and reference material covering optogenetics, activity imaging, and tissue-hydrogel methods, supplementing the brief descriptions of these integrative techniques in the main text.

Optogenetics

Optogenetics is built upon a broad foundation spanning many decades and intersecting disciplines, involving research in the fields of microbial biology, biochemistry, optics, virology, and neuroscience. Francis Crick articulated the need in neuroscience for selective control of cell types in 1979[1], and then suggested light might be a useful modality in 1999[2], but without a concept for achieving this goal (which he termed "far-fetched")[2]. In fact, means for targeted optical control of neural activity date back decades before this suggestion, beginning with the use of laser light to modulate individual targeted neurons[3-6]. These early efforts did not employ genetics as a selectivity strategy, but rather spatial and anatomical targeting strategies for individual cells were demonstrated; this type of intervention was successfully leveraged to evoke and record light-elicited post-synaptic potentials in the mollusk *Aplysia*[4].

Partially overlapping with these lines of investigation were early efforts at developing multi-component genetic strategies for targeting; Zemelman et al. developed a three-component method to optically control depolarization and spiking in cultured hippocampal neurons by co-expressing several proteins from the *Drosophila* visual system[7]; Banghart et al. reported a two-component method involving introduction of a small organic photoswitch chemical and an ion channel designed to bind the photoswitch and thus respond to light[8] which they later applied to zebrafish[9], and Lima et al. described a related two-component method based on optical uncaging of an exogenous receptor ligand which they were able to apply to fruit flies[10]. The elegance of all these landmark efforts did not, however, lead to broad adoption in other laboratories, due largely to the multicomponent nature of the genetic approaches and limitations in light targeting, which together posed challenges for versatility, speed, reliability, and targeting.

A completely unrelated field of biology would ultimately provide the long-sought single-component genetic solution. In 1971, Oesterhelt and Stoeckenius[11] reported the first evidence for the existence of microbial rhodopsins, retinal-binding membrane proteins that can directly give rise to transmembrane currents in the presence of light; these diverse seven-transmembrane proteins, produced by microbial organisms such as archaebacteria and algae and each encoded by a single gene, were eventually found to include light-activated proton and chloride pumps, as well as light-activated cation and anion channels[12]. As previously reviewed[12], in 1994 it was reported that heterologous expression of microbial opsins (of the bacteriorhodopsin pump-subtype) in eukaryotic cells could give rise to light-activated transmembrane ion currents[13]. Similarly, in 2002 heterologous expression was observed for the channel-subtype (channelrhodopsins naturally found in algae) in oocytes[14] (and later in HEK cells[15]); a long

history of research on the basic biology on motile microalgae had earlier identified the corresponding photocurrents in the native algae[16] that were later hypothesized and shown to be subserved by rhodopsins[17,18].

All of these classes of microbial opsin were later discovered to serve as powerful and targetable tools in neural systems, thereby enabling the optogenetic approach, between 2005 and 2015. The initial paper describing neuronal control with a microbial opsin[12,19] was followed by several other reports over the ensuing year[20-25], revealing that many groups had been simultaneously racing to achieve this goal as previously reviewed[12,26]. Yet although feasibility for optical control of spiking was published in several systems from 2005-2006, including cultured neurons, acute slices[20,27] of mammalian CNS, and *in vivo* preparations of worms[21], flies[25], and mammalian retina[22], there remained considerable (and justified) skepticism that this approach would turn out to be generalizable for broad discovery in biology. Indeed, for several years after the experiment that led to the initial paper, use of microbial-opsin optogenetics was restricted to optically accessible invertebrates or to exposed and superficial brain structures in mammals[28]; among other challenges it was unclear how to deliver light *in vivo* in behaving rodents, or to deliver opsin-based light sensitivity to targeted cells or projections in an effective, safe, and most importantly versatile way.

The subsequent development of systems to deliver light to deep brain regions or projections using fiberoptics[29] was crucial, along with promoter- and recombinase-dependent virus-based expression systems that (together with the fiberoptic neural interface) allowed versatile optogenetic targeting for real-time modulation of mammalian behavior. By 2007, optogenetic control of hypocretin neurons deep in the lateral hypothalamus of behaving mice had been achieved, with particular patterns of spiking in targeted neurons found to suffice for sleep-wake behavioral-state transitions[30]. At this point, optogenetics had attained the fundamental form that most systems neuroscientists use today, employing targeted expression of microbial opsins with cell-type specificity using high-titer viral targeting vectors and delivery of light through implanted fiberoptics. And by 2009, generalizable targeting strategies had been developed and shown to be suitable for mammalian behavioral control, involving anatomical targeting of projection-defined cell types[31] and/or recombinase-dependent targeting of genetically-defined cell types[32,33].

Both necessity and sufficiency testing is possible with different subtypes of excitatory and inhibitory microbial opsins, which can be selected based on specifics of experimental configuration (reviewed in refs. 34,35); this combination of capabilities becomes particularly powerful when integrated with readouts of naturally-occurring activity of targeted neurons using immediate-early genes, electrophysiology, or genetically-encoded Ca^{2+} imaging (discussed next) so that provided activity patterns can be tuned to match (or differ from in controlled fashion) the timing and magnitude of naturally-occurring activity patterns in the very same cells and projections.

Ca^{2+} imaging

Synthetic Ca^{2+} indicator dyes[36] were developed to show fluorescence modulation of either excitation or emission spectra dependent on Ca^{2+} binding to the dye, which in turn is increased by membrane-depolarization-driven Ca^{2+} flux associated with neuronal activity. After

electroporating (or administering via acetoxy-methylester (AM) formulations) these synthetic dyes into neurons, neuroscientists were thereupon able to monitor intracellular Ca^{2+} concentrations within individual neurons. An interesting property of these synthetic dyes is that some display an isosbestic wavelength, at which (for example) the dye does not exhibit Ca^{2+}-dependent emission changes. Thus by imaging the synthetic dye at two different wavelengths, one at which the indicator fluoresces in a Ca^{2+}-dependent manner and another at the isosbestic wavelength (or a wavelength showing opposite-direction shifts in response to Ca^{2+}), a ratiometric calibration across the two measurements can be performed that accounts for external factors such as differences in dye concentration and imaging pathlength across experiments in order to estimate intracellular Ca^{2+} concentration $[Ca^{2+}]$. The following equations from ref. [36] demonstrate this idea:

$$F_1 = S_{f1}c_f + S_{b1}c_b \quad (1)$$

$$F_2 = S_{f2}c_f + S_{b2}c_b \quad (2)$$

$$c_b = c_f[Ca^{2+}]/K_d \quad (3)$$

where F represents the fluorescence intensity measured at the isosbestic wavelength (1) or Ca^{2+}-dependent wavelength (2), respectively, S_f and S_b represent the proportionality constant for free and Ca^{2+}-bound dye, respectively, c_f and c_b represent the concentrations of free and Ca^{2+}-bound dye, respectively, $[Ca^{2+}]$ represents that concentration of intracellular Ca^{2+}, and K_d represents the effective dissociation constant. By taking the ratio $R = F_1/F_2$ and substituting in equation (3), one can obtain the following calibration equation to calculate $[Ca^{2+}]$:

$$[Ca^{2+}] = K_d \left(\frac{R - (S_{f1}/S_{f2})}{(S_{b1}/S_{b2}) - R} \right) \left(\frac{S_{f2}}{S_{b2}} \right) \quad (4)$$

These early synthetic dyes were helpful for probing Ca^{2+} signaling but could only be transiently delivered in small volumes of tissue and in a non-cell-type-specific manner. The later development of genetically-encoded Ca^{2+} indicators (GECIs), such as the GCaMPs[37-41] vastly improved the utility of Ca^{2+} imaging to study correlates of neural activity *in vivo*. The GCaMP-family sensors have a useful Ca^{2+}-sensitive excitation wavelength band of ~470-490 nm, at which emitted fluorescence in the 500-530 nm band increases in the presence of intracellular Ca^{2+}. These GECIs have been used in numerous *in vivo* preparations and animal models, including cellular-resolution imaging techniques such as one-photon microendoscopy[42] and two-photon laser scanning microscopy[43,44], as well as population-recording techniques such as fiber photometry[45,46].

However, unlike the early experiments performed *in vitro* using ratiometric synthetic indicators such as Fura-2[36], generally *in vivo* Ca^{2+} imaging does not allow for a direct estimate of intracellular Ca^{2+} concentration using the ratio calibration equations – particularly during freely-moving Ca^{2+} imaging. This is because this ratio operation only holds when 1) there is low

background autofluorescence, and 2) there is stability within each individual ratio measurement[36] – two assumptions that cannot be met during freely-moving one-photon Ca^{2+} imaging where there is high autofluorescence from the tissue and optical components themselves, and the potential for instability in the background autofluorescence due to bleaching. Thus equations (1) and (2) listed above now have an additional autofluorescence proportionality constant, S_a:

$$F = S_f c_f + S_b c_b + S_a \quad (5)$$

Because this additional S_a constant is dependent on the autofluorescent properties of the brain tissue itself and each individual patch cord and ferrule, it would be difficult to empirically measure S_a for each animal in order to perform a calibration across experiments. Thus the ratiometric method is not typically used to report $[Ca^{2+}]$ from *in vivo* Ca^{2+} indicator fluorescence measurements, and instead researchers report the relative change in fluorescence over time relative to the baseline (dF/F). However, recently, researchers have taken advantage of the excitation isosbestic point of GCaMP6 (410 nm) to identify non-Ca^{2+}-related fluorescence fluctuations during freely-moving fiber photometry recordings that can be linearly scaled to and then subtracted from the overall fluorescence emission measured at the Ca^{2+}-dependent wavelength[45-47]. As the baseline emission of the Ca^{2+}-dependent and Ca^{2+}-independent signals may differ (due to differences in illumination power or excitation efficiency at the two wavelengths), the Ca^{2+}-independent signal is linearly scaled to match the Ca^{2+}-dependent signal prior to subtraction. Subtractive correction is empirically found to be superior to divisive normalization in dealing more robustly with fiber autofluorescence bleaching and background shifts (MS in preparation). This approach takes into account the fact that there is often a high and variable amount of background autofluorescence that linearly summates with the actual Ca^{2+}-dependent GCaMP6 signal during one-photon imaging, and that this background signal can be captured when illuminating the tissue and optical components with the isosbestic GCaMP6 wavelength. Work in progress will detail quantitative considerations for removing non-Ca^{2+}-related fluorescence changes from 1-photon fiber photometry imaging using the GECI isosbestic wavelengths (not shown).

Brain tissue clearing techniques

A crucial feature of the GECI approach is its stably-expressed and cell-filling fluorophore; these properties open up a broad new set of possibilities for data integration. In principle, it would be immensely powerful if brains from the very same animals in which activity had been observed and controlled with cellular resolution, could then be studied anatomically to resolve local and global wiring of the corresponding cells (and perhaps even to collect high-content molecular phenotyping of those same cells by multiplexed antibody staining and *in situ* hybridization). However, until very recently, technological capability had not existed for this sort of data integration. The opacity of adult mammalian brains (due chiefly to light scattering at lipid-water interfaces) had long prevented direct observation of these features in the intact state, while serial thin sectioning of multiple large cohorts of entire adult brains with axonal resolution had remained not only impractical but also incompatible with preservation and interrogation of the desired anatomical and molecular features.

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Despite pioneering work on chemical treatments of brain tissue for improved transparency (from the labs of Hans-Ulrich Dodt and Atsushi Miyawaki; the 3DISCO and Sca*l*e methods, respectively[48,49]), requisite optical and molecular accessibility for the adult mammalian brain had not been achieved. Artificial gels and polymers of various kinds had long been part of histology (to facilitate handling and sectioning); in 2013 a distinct approach was taken, in which a hydrogel was built from within, and covalently linked to, adult mammalian brain tissue for the explicit purpose of chemically anchoring native biomolecules into a gel-tissue hybrid-- using a cocktail of gel monomers, chemical linkers to span from gel to native biomolecules, and triggerable gel crosslinkers for polymerization—all as a first step toward high-resolution optical access and macromolecular-probe permeability. The history and process leading to this concept and discovery have been recently reviewed[50].

One form of this (an initial acrylamide-based version of the hydrogel) was termed CLARITY, and has been applied to diverse basic science and clinical applications[50]. This unusual process allowed for vigorous and near-complete removal of membrane lipids (the major source of light-scattering-based opacity; most lipids are unable to bind to the chemically engineered hydrogel which had been designed to target reactive amines and thus retain proteins and nucleic acids[51]. Since 2013, many variants of the original hydrogel-in-tissue concept have been described. A simple form of lipid removal ("passive CLARITY") was described in 2014[52,53] which eliminated an electrophoretic step of lipid removal that some labs had found difficult. A passive CLARITY strategy suited for whole organisms called PACT was described that same year[54], as well as new hydrogel formulations called A4P0[54] and SWITCH[55]. A great variety of possible tissue-gel compositions are possible, and this paradigm has spurred innovation from labs around the world[56-60] (reviewed in ref. 35), alongside the complementary development of high-speed automated microscopy/analysis hardware and software[53,61-64].

Beyond the tissue-hydrogel concept, distinct brain transparency methods have since been reported based on various combinations of refractive index matching by solvent immersion and lipid solubilization[63-68]. In particular, the iDISCO method has shown substantial promise for intact adult mammalian brain studies, showing particular utility in terms of procedural simplicity[64], although thus far lacking capability for multiround/multiplexed or nucleic acid labeling. In a recent integrative paper, Ye et al.[62] provided automated cell and projection-tract quantification software for hydrogel-embedded volumes, and achieved an integration of CLARITY and optogenetics to quantify distinct brainwide wiring patterns of cell in prefrontal cortex involved in responding to rewarding or aversive experiences.

Resource links

http://web.stanford.edu/group/dlab/media/papers/sciAm2016.pdf

http://clarityresourcecenter.org/pdfs/Table_S2_Transparency_Methods.pdf

http://web.stanford.edu/group/dlab/media/papers/deisserothNatNeurosciCommentary2015.pdf

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