Optogenetics
Maria G. Paez Segala & Loren L. Looger

Abstract
We review the state-of-the-art in optogenetics – the use of light to control processes in cells, tissues, and intact (often behaving) organisms. We focus on the available genetically encoded reagents, the principles behind their construction and usage, and potential complications in deploying them and correctly interpreting data from such experiments. We cover microbial rhodopsins, animal opsins and other light-gated G protein-coupled receptors, the opto-XR designed GPCR reagents, nucleotidyl cyclases and phosphodiesterases, dimerizers and allosterically regulated enzymes and other effectors, engineered light-gated ion channels, and several other niche reagents. We offer concrete steps to improve the quality of optogenetics experiments, including many critical controls. We also offer a few ideas of future reagents.

Key words
Optogenetics, channelrhodopsin, opsin, effector, LOV domain, ion channel, cryptochrome, dimerizer, photoactivation, rhodopsin

Introduction
To truly understand biological systems, one must possess the ability to selectively manipulate their parts and observe the outcome. (For purposes of this review, we refer mostly to targets of neuroscience; however, the principles covered here largely extend to myriad samples from microbes to plants to the intestine, *etc*.).

Drugs are the most commonly employed way of introducing such perturbations, but they act on endogenous proteins that frequently exist in multiple cell types, complicating interpretation of experiments. Whatever the applied stimulus, it is best to introduce optimized exogenous systems into the systems under study – enabling manipulations to be targeted to specific cells and pathways. (It is also possible to target manipulations through other means, such as drugs that acquire exquisite cell type-specificity through targeting *via* antibodies and/or cell surface receptor ligands, but as far as we are aware, existing systems fall short in terms of necessary specificity.) Many types of perturbations are useful in living systems and can be divided into rough categories such as: depolarize or hyperpolarize cells, induce or repress activity of a specific pathway, induce or inhibit expression of a particular gene, activate or repress a specific protein, degrade a specific protein, *etc*. User-supplied triggers for such manipulations to occur include: addition of a small molecule ("chemogenetics" – ideally inert on endogenous proteins) (1), sound waves ("sonogenetics") (2), alteration of temperature ("thermogenetics" – almost exclusively used for small invertebrates) (3), and light ("optogenetics"). (There are reports of using magnetic fields ("magnetogenetics") (4), but there is no evidence that such effects are reproducible or even physically possible (5), (6). Of these, the most commonly used, for multiple reasons, is light.

Many factors make light an ideal user-controlled stimulus for manipulation of samples. Light is quickly delivered, and most light-sensitive proteins and other molecules respond quickly to light stimuli, making many optogenetic systems relatively rapid in comparison to, for instance, drug-modulated systems. Light is also quite easy to deliver in localized patterns, allowing for

targeted stimulation. Multiple wavelengths can be delivered separately to distinct (or overlapping) regions, potentially allowing combinatorial control of diverse components. Finally, light can be delivered to shallow brain regions (and peripheral sites) relatively noninvasively, and to deeper brain regions with some effort.

However, there are also a number of shortcomings of using light for control. Robust and uniform penetration of light into the sample is the most significant concern. For systems requiring modulation of many cells, particularly at depth, use of systems controlled by small molecule drugs would generally be recommended instead of optogenetic approaches. When light is delivered through use of fibers, lenses, or other optical devices, such interventions can produce significant cellular death, scar formation, and biofouling. The foreign-body response of tissue to objects triggers substantial molecular alterations, the implications of which are incompletely defined, but can involve reactive astrogliosis, oxidative stress, and perturbed vascularization. Head-mounted light-delivery devices can be heavy and/or restrictive, and thus perturb behavior, particularly for small animals (*e.g.*, mouse behavior is much more disrupted than rat behavior). More generally, all light causes tissue heating, which can have dramatic effects on cell health, physiology, and behavior. This is most concerning for tiny animals such as flies. Light itself also damages tissue, most obviously through photochemistry (*e.g.*, oxidation and radicalization) and photobleaching of critical endogenous molecules. Furthermore, of course, light is ubiquitous, meaning that the sample is never completely unstimulated, despite precautions. Light passes through the eyes into the brain with surprising ease, and even through the skull with modest efficacy(7) – which can disrupt animal behavior (as can the converse: stimulating light in the brain perceived as a visual stimulus through the back of the eyes.) Light-responsive proteins exist in all samples, particularly in the eyes but to some extent in all tissues – notably, deep-brain photoreceptors (8).

The use of optogenetic tools has accelerated research on many fronts in disparate fields. Additional, perhaps most, limitations on the utility of optogenetics must, however, be placed squarely on the shortcomings of the current suite of tools (and potential inherent limits in their performance.) The vast majority of optogenetic effectors are gated by blue light, which has significant penetration issues and can be phototoxic under high intensity; redder wavelengths would in general be preferred. Furthermore, multiplexing requires tools making use of other parts of the visible spectrum (and redder wavelengths.) A related issue is that most chromophores for optogenetic reagents have very broad action spectra (~250 nm bandwidth for retinal; ~200 nm bandwidth for flavin), complicating both multiplexing and their use alongside many optical imaging reagents – narrower action spectra would be preferred for effectors in most situations. More generally, the current classes of optogenetic effectors are quite few: mostly limited to 1) channels and pumps (most with poor ion selectivity), 2) dimerizers, and 3) a handful of enzymes. The number of optogenetic tools that perform a very specific function in cells is small. Although progress has undeniably been made, much additional research and engineering will be required to dramatically expand the optogenetic toolkit.

Rather than providing a survey of research findings, this review covers general considerations of optogenetics experiments, and then focuses largely on molecular tools: the existing suite, their features and limitations, and goals for the creation and validation of additional reagents.

General considerations

As stated above, considerations differ substantially based on model organism, preparation, and experimental goals. Transparent organisms such as zebrafish allow straightforward light delivery – although for all samples, it is generally not known to where the stimulating light penetrated and to where it did not, potentially confounding experiments if one assumes that light reached all cells to the same extent. (Idealized optogenetics experiments would include a mechanism by which to indicate which cells experienced the perturbation – and perhaps to what extent. As an intermediate measure, photoconvertible fluorescent proteins could be co-expressed and used as a "dosimeter" of activating light delivery. This could be immediately tested with reagents that already include a fluorescent protein component, for instance to boost expression and membrane targeting. Alternatively, a tethered or co-expressed fluorophore could enable tracking of the delivered light – such a dosimeter would best be ratiometric to partially control for expression level differences.) In the absence of such a mechanism to label cells receiving the stimulus, it is generally assumed that all cells expressing the effector (perhaps imaged indirectly through use of the aforementioned fluorescent protein) received the stimulating light, and that all expressing cells underwent stimulation. In fact, of course, different cells will receive different amounts of light, and cell-to-cell variability in protein complement, ionic composition, state, *etc*. could dramatically alter function of the exogenous reagents. The extent to which such caveats come into play in optogenetics experiments is essentially unknown. For instance, rheobase, the minimal current required to elicit an action potential, is not a fixed parameter and varies between individual neurons of the same class. Therefore, even if the optogenetic tool was equivalently expressed and identically activated by light, it would not be expected to produce the same effect on all the neurons, implying that binary interpretations of "off" and "on" for populations of neurons in optogenetic experiments may be simplistic.

In contrast to transparent organisms, mice (**Figure 1a**) typically require the creation of a cranial window or implantation of a light guide such as an optical fiber. As mentioned above, some light does indeed pass through the skull and eyes, but optogenetic effectors typically require intense illumination, necessitating measures to increase light access. Adult flies (**Figure 1b**) have a dark, waxy cuticle that severely hampers light penetration. Before the development of red-shifted effectors, optogenetic experiments in adult flies were incredibly difficult; with red-shifted opsins (see below) like ReaChR and Chrimson (for activation) and yellow/orange opsins like GtACR (for inhibition), cuticle removal is now rarely required. Light delivery to larval flies (**Figure 1c**) is easier than to adults, but they exhibit numerous light-dependent responses, complicating experiments.

For a given preparation, there are numerous mechanisms for light generation and delivery. Obviously, light can be shone broadly on samples, turning on (or off) effectors widely – this is mostly done in essentially transparent organisms like fish and worms, and in preparations such as cell culture. More commonly, light is delivered locally (to a specific brain region), through an optical fiber (**Figure 1d**) or another such light guide in larger animals and focused illumination in smaller ones. Light emitted from the source may be delivered to the sample with whole field illumination (**Figure 1e**), reaching many expressing cells in the area; for some applications, delivery of patterned light (for instance, through a spatial light modulator (9), with holographic illumination (10), or with temporal focusing (11)) can be used to activate predefined cells (**Figure 1f**) – *e.g.*, cells that have been identified as being of interest through functional imaging. The

greatest spatial selectivity in optogenetic stimulation is afforded through the use of infrared 2-photon stimulating light (**Figure 1g**) (12). (Of course, the downside of this approach is the need to extensively scan the point over cells, given the low conductance and high desensitization of opsins and other optogenetic channels.) In addition to targeting the light stimulus to specific cell populations and individual cells, great progress has been made in subcellular optogenetics (**Figure 1h**) (13), *e.g.*, activation of ion transport across the mitochondrial membrane or localization of a synaptic protein to the active zone. In general, this requires reagent targeting through genetic fusion to specific targets, light patterning on desired areas, and optogenetic effectors engineered for small-volume activation (see the section on dimerizers below.)

We also note that Cre-dependent lines (mostly in mouse, but also used in rat and zebrafish) inducible by the drug tamoxifen (*i.e.*, $CreER^{T2}$ and related lines (14)) should be treated with great caution for neuroscience experiments. In addition to reagent-quality concerns such as high background and low levels of drug induction, tamoxifen of course signals through estrogen and other steroid receptors and thus dramatically alters behaviors (*e.g.*, affecting locomotor activity, social interaction, and anxiety after single-dose administration (15)). Furthermore, it has recently been found that tamoxifen inhibits sodium channel currents (16), rendering neuroscience results involving tamoxifen very fraught from a biophysical perspective, as well. Certainly, experiments should be done as long after tamoxifen administration as is possible. A much preferable solution would be the generation and validation of recombinases inducible by molecules with no physiogical effects; further, the effector should be insensitive to endogenous molecules ($CreER^{T2}$ retains binding to estrogen and other steroids and thus has background activation (17)). Cre is also associated with genomic toxicity through cryptic *loxP* site recombination (18).

Optogenetic reagents

The first optogenetic experiments were conducted with small molecules (*e.g.*, neurotransmitters) synthesized to bear "photocages" that are released by the incident light. Such reagents are critical and for many applications offer the only real way to probe a pathway's function. For instance, it is inconceivable to reproduce the function of photo-uncaging of nicotine (19) – a molecule with activity against a number of distinct receptors and targets – in a protein-based system. (Such systems, however, would be critical for determining the activity of nicotine on those specific proteins – which is impossible with uncaging of the cognate drug.) For this review, we limit ourselves to genetically encoded (*i.e.*, protein-based, although all known photoreceptors make use of small molecule cofactors as the actual light sensors) systems that are exogenously supplied to samples (sometimes along with the accompanying small molecule cofactor) to introduce light responsivity.

Microbial opsins

The underlying driving forces of many cellular processes are the standing gradients of many cations and anions, primarily Na^+, K^+, Cl^-, H^+, Ca^{2+}, and others to a lesser extent (**Figure 2a**). In animals and other eukaryotes, ions are primarily conducted through high-specificity ion channels, pumps, and transporters. It is clear that ancestors of these proteins arose first in bacteria and other microbes. Indeed, there are numerous extant ion channels and pumps in these organisms today, including the specific class of opsins (**Figure 2b**). Several decades ago, it was discovered that such proteins underlay one of the fundamental physiological processes in microbes (and indeed most living organisms): that of the diurnal cycle. In many microbes, this process is directly encoded

through light-gated ion influx and efflux (20). Once genes encoding such proteins were identified and cloned, it was determined that they displayed homology to eukaryotic photoreceptor proteins such as rhodopsin, the photopigment of retinal rods (21). Rhodopsins and other eukaryotic photoreceptors are G protein-coupled receptors (GPCRs; see next section) signaling through intracellular second-messenger pathways. Instead, many microbial homologues directly flux ions through a conducting pore and hence were dubbed "channelrhodopsins." Structural characterization confirmed the high degree of similarity between the microbial channels and the eukaryotic photoreceptors. Intriguingly, in addition to the relatively well characterized microbial opsins, channelrhodopsin homologues have been recently discovered in several viruses (22).

Alteration of ion gradients can drive many physiological processes, and so it is unsurprising that microbial opsins have found wide usage in numerous biological realms. In neuroscience, their use to elicit or inhibit action potentials is easily the most common application of optogenetics. The first depolarizing opsin identified was channelrhodopsin-2 (ChR2) (23). (Channelrhodopsin-1 was identified a year earlier – it conducts H^+ at quite low levels and did not find wide uptake in the field.) Pioneering experiments demonstrated that these opsins could be used to generate ionic currents across the membranes of various cell types and to depolarize neurons (23-27).

Over the past 15 years, numerous ChR2 homologues have been identified across many clades of microbial life (28). Structure-guided mutagenesis was first performed using homology models with mammalian rhodopsin and subsequently from high-resolution crystal structure determination of the microbial opsins themselves (29). Through genomic screening and mutagenesis, various mutants display a fair bit of variation in multiple channel properties: ion specificity, conductance, action spectrum, and kinetics of conductance onset and offset (including variants with very long offset, dubbed "step-function opsins" (30) – although these proteins suffer from dramatic desensitization (31)).

The precise choice of opsin will depend critically on the preparation and the question at hand. Generally, best-in-class microbial opsins currently include: activators Chrimson (32), Chronos (32), ReaChR (33), ChIEF (34), ChETA (*e.g.*, CrChR2-E123T–T159C) (35), and ChRger (36); inhibitors (the Cl^- pump *Natronomonas pharaonis* halorhodopsin 3 (eNpHR3) (37)), the H^+ pumps Arch-T (38) and Jaws (39); the Cl^--passing ChR2 mutant GtACR-1/2 (40); and Ca^{2+}-permeable ChR2 mutants (*e.g.*, CrChR2-L132C-T159S) (41). For a detailed discussion of the strengths and weaknesses of specific variants, see for instance (42). It should be noted that the "Ca^{2+} permeability" opsins (broadly referred to as "CatChR") in fact have only modest improvement (~1.5-3x) of Ca^{2+} selectivity – meaning that they remain fairly non-specific cation channels (with vast preference for H^+). Where redder activation light is required, the activators Chrimson and ReaChR, and the silencer GtACR, are preferred. Of course, this list will constantly evolve as new tools are developed and further characterization on existing tools is performed.

It must be noted that wild-type microbial opsins typically traffic quite poorly in eukaryotic cells, producing large intracellular aggregates with very little protein reaching the plasma membrane. For this reason, most modern opsins include multiple Golgi and endoplasmic reticulum export sequences, and almost always carry a fused fluorescent protein for improved expression level and membrane display (such fused fluorescent proteins can be made dark so as not to consume an imaging/activation optical channel while still improving folding and display).

However, such enhanced opsins still routinely show incomplete membrane trafficking – the activity of such intracellular pools of opsin is essentially unknown; ion passage across the endoplasmic reticulum and Golgi could further perturb cellular activity.

A recent innovation in opsin engineering is the discovery that it is possible to invert their natural topology through the addition of an N-terminal transmembrane helix (43). In addition to being of profound basic science interest, such topology-inverted opsins can provide functionality not seen in naturally derived proteins. The inverted-ChR2 mutant dubbed "FLInChR" (43) exhibits strongly hyperpolarizing currents and functions in some cells where other silencers fall short. It is, unsurprisingly, not as simple as "invert an activator to create a silencer," or any other potential rule of thumb. The ion conductance of FLInChR remains unclear, despite fairly exhaustive sampling of buffer compositions. It is almost certain that topological inversion alters numerous properties, including membrane targeting, ion specificity, conductance, desensitization, voltage-dependence, *etc*. It is imperative that new tools generated in such a fashion are thoroughly characterized in order for them to be used optimally.

Opsins can be targeted through protein fusions to diverse subcellular locales, such as the soma (44, 45), dendrites (46), axon initial segment (47), mitochondria (48), *etc*. An innovative method to tag active synapses (49) has been the use of targeted mRNA trafficking (for instance, through axon- or dendrite-targeting mRNA elements or use of the MS2-MCP system of protein-RNA hairpin binders (50)) followed by local transcription and/or translation through activity-dependent enhancers and ribosome-binding elements, respectively, delivered in construct untranslated regions (UTRs).

As a final note, many applications either require or benefit from the addition of exogenous retinal cofactor. For flies, this can be delivered through their food, and in fish and worms it can be placed into the medium. In addition to potentially boosting existing applications, this opens the door for the development and delivery of non-natural retinals with altered properties once bound by their apo-proteins. This has been demonstrated for worms with the synthesis and delivery of dimethylaminoretinal, naphthyl-retinal, and thiophene-retinal, which altered the spectral, kinetic, and conductance properties of ChR2, Arch, and Mac (51). Recently, bond-inserted retinals resulted in further red-shifting of ReaChR and Chrimson (52), although their *in vivo* utility has not been studied yet, as far as we are aware.

G protein-coupled receptors

G protein-coupled receptors (GPCRs) were among the first genetically encoded optogenetic effectors used and have of late been enjoying renewed interest. GPCRs signal through a heterotrimeric G protein complex (α, β, γ subunits – with α being the primary determinant of transduced pathway – **Figure 3a**; also important are arrestins and accessory Regulator of G protein Signaling (RGS) proteins, not shown). In 1988, expression of bovine rhodopsin (a hyperpolarizing transducin (G_t)-coupled receptor) in *Xenopus* oocytes, followed by incubation with 11-*cis*-retinal, facilitated light-driven inward currents (53), indicating that endogenous G proteins and related machinery are sufficiently conserved between cow and frog, and adequate expression of the genes occurs in oocytes, to essentially recreate the light-sensitive pathway of the vertebrate rod photoreceptor cell *ex situ*. However, there are two caveats for systems employing vertebrate opsins for optogenetics: vertebrate rhodopsins hyperpolarize upon light stimulation, whereas invertebrate

homologues depolarize – and critically, after a single cycle vertebrate rhodopsins expel *cis-trans*-isomerized retinal, which must be then enzymatically regenerated to 11-*cis*-retinal (in the retinal pigment epithelium *in vivo*) before it can be bound again to rhodopsin. Thus, vertebrate rhodopsins are quite prone to run-down; it is theoretically possible to supply the complement of enzymes required to regenerate 11-*cis*-retinal in the cells of interest, but this would require the delivery of more transgenes (*i.e.*, retinal photoisomerase), potential concerns about stoichiometry of expression, and likely limits on rhodopsin duty cycle despite efforts to address this.

Invertebrate opsins, by contrast, are by and large bistable molecules that photochemically regenerate light-sensitive retinal from the inactivated form without retinal dissociation or enzymatic activity. Thus, invertebrate opsins constitute a more robust set of optogenetic effectors than their vertebrate homologues (they are not without their downsides, however.) In 2002, expression of 10 *Drosophila melanogaster* photoreceptor light-dependent signal cascade in cultured mammalian neurons produced robust currents (54). Following the addition of 11-*cis*-retinal, shining white light on the cells produced large, slow (lasting ~30 seconds) depolarizations. Further experiments showed that a minimal set of three proteins (together called "chARGe": the blue light-sensitive rhodopsin NinaE, arrestin-2, and $G_{\alpha q}$) sufficed to reproduce the depolarization. (The evolutionary distance between flies and mammals is the reason that these experiments require onboarding the appropriate arrestin and G protein, whereas the lower cow-to-frog distance allowed endogenous machinery to suffice. It is likely that protein engineering of NinaE and related opsins could remold their intracellular loops to interact with endogenous arrestins and G proteins, thus creating a 1-component system.)

The eyespot opsin from box jellyfish ("JellyOp") (55) shows improved photobleaching relative to other opsins and rhodopsins. JellyOp has been harnessed as a $G_{\alpha s}$-signaling tool (as with opto-β_2AR; see below) to promote cAMP generation in neurons (56). A hybrid opsin, coupling the blue light-sensing components of blue opsin with the intracellular loops of JellyOp, produced "Crblue" (57) – which drives G_{α}s signaling with light blue enough to allow simultaneous imaging of fluorescent proteins such as GFP and YFP; without opsin blue-shifting, imaging of the fluorescent protein would have activated the effector. (It is not immediately obvious why a jellyfish opsin works well in mammalian neurons without arrestin and G protein complementation.) Recently, less well characterized photoreceptors such as parapinopsin, peropsin, encephalopsin (Opn3), melanopsin (Opn4), neuropsin (Opn5), long wavelength-sensitive bistable opsin, pteropsin, teleost multiple tissue (TMT) opsin, and others have been explored as optogenetic scaffolds (58) (59) (60).

Opto-XRs

In addition to the naturally evolved light-driven GPCRs discussed in the previous section, there is a class of wholly engineered proteins that function as light-evoked GPCRs. These reagents are created by forming chimeric sequences between an opsin (the photoresponsive machinery) and a GPCR (typically the three intracellular loops and the C-terminus, all of which are implicated in signaling through G proteins and arrestins (**Figure 3b**)). This family, termed the "opto-XRs," now contains a number of members: adrenergic receptors β_2AR and α_1AR (61) (the first opto-XRs created, in 2005 – and regenerated with very similar sequence in 2009 in (62); glutamate receptor mGluR6 (63); adenosine 2A receptor (64); serotonin receptor 5-HT1A (65)); serotonin receptor 5-HT2C (66); dopamine D1 receptor (67); μ-opioid receptor (68); and GPR37, an orphan GPCR

(69). A variety of opsins have been used in constructing these chimeras. Importantly, the opto-XRs lose the dimerization of the cognate GPCRs (which is mediated through the helices that have been replaced by opsin sequence) and thus may not necessarily maintain the localization and function of the cognate receptor (70). Due to this and overexpression/trafficking concerns as well as potentially perturbed G protein/arrestin signaling, opto-XR stimulation cannot be taken to reflect the effects of cognate receptor activation without additional experiments. Regardless, opto-XRs offer ways to manipulate signaling pathways not currently accessible through other reagents.

For both the naturally evolved light-driven GPCRs and the synthetic GPCRs, it should be noted that – as with all GPCRs – they can be driven into more G protein-mediated signaling or more arrestin-mediated signaling (known as GPCR "bias" (71); most commonly discussed with regard to agonist molecules, but increasingly understood in a more holistic form (72)). Thus, cell type and cell state differences could profoundly affect function of these reagents; in fact, cell type-specific chemogenetic effectors (GPCR designer receptors exclusively activated by designer drugs, DREADDs) have been created based on the differential expression of G proteins across cell types (73). Thus, it cannot be assumed that GPCR-based reagents act similarly across cells and organisms due to complement of G proteins, arrestins and other accessory proteins, phosphorylation state, *etc*.

Enzymes

Several naturally evolved soluble enzyme systems are light-dependent. Most notable among these are two major classes of nucleotide-transforming enzymes: the soluble nucleotidyl cyclases (74), and nucleotide phosphodiesterases (75). Together these constitute major inputs to photoavoidance/phototaxis and other light input-driven phenotypes of plants, fungi, and many prokaryotes – by controlling the production and degradation of the universal intracellular signaling molecules cAMP and cGMP (**Figure 3c**). Well characterized photoactivated adenylyl cyclases include those from *Euglena gracilis* (euPAC (76) and PACα (77), *Beggiatoa* sp. PS (bPAC) (78), and *Oscillatoria acuminata* (OaPAC) (79) – all driven by BLUF photoreceptors (see below) coupled to catalytic domains. bPAC was further engineered to have GTP – instead of ATP – specificity through structure-guided mutagenesis, producing a soluble photoactivatable guanylyl cyclase, named BlgC (80). The most commonly used photoactivated phosphodiesterase is LAPD (light-activatable cAMP/cGMP-phosphodiesterase), rationally designed as a chimera of a red light-driven phytochrome and an allosterically coupled human phosphodiesterase 2A (81).

In addition to the classical soluble enzymes, a recently discovered subclass of microbial opsins is the "two-component cyclase opsins" (82), which apparently carry an additional transmembrane helix relative to the standard seven for GPCRs (thus being classified as Ib opsins), and which seem to function as light-inhibited, ATP-dependent guanylyl cyclases rather than GPCRs. It appears that these "2c-Cyclop" opsins signal through light-induced inhibition of a histidine kinase domain, which in turn phosphorylates a response regulator domain, which then activates a guanylyl cyclase. Given this three-step signaling mechanism, it is unsurprising that the kinetics of the 2c-Cyclop proteins are quite slow, developing and declining over many minutes (82).

Dimerizers

Particularly powerful components of the optogenetics toolkit are reagents with the ability to bring proteins together, typically to induce a functional transformation requiring their mutual presence. This is accomplished through photodimerizers, of which quite a number have been demonstrated (**Figure 4a**). Most of these are built upon a framework of photoreceptors from plants, fungi, or cyanobacteria – where their *in vivo* role is to modulate signal transduction cascades in a sunlight-dependent manner. There are many photoreceptors in the Light/Oxygen/Voltage-sensing (LOV domain) superfamily (83, 84), originally discovered as the signaling mechanism behind plant phototropism (LOV domains have since been discovered in bacterial, archaeal, algal, fungal and plant species). Numerous other general photoreceptor classes have been discovered as well: cryptochromes (85), phytochromes (86), ultraviolet-B receptor UVR8-like proteins (87), and sensors of Blue-Light Using FAD (BLUF) proteins (88), among others. Other light-dependent binding events have been engineered, such as the oligomerization of the fluorescent protein Dronpa (89, 90). LOV domains couple to a wide array of signaling pathways – histidine kinases, response regulators, phosphodiesterases, nucleotide cyclases, sulfate transporter/anti-sigma factor antagonists, helix-turn-helix transcriptional regulators, serine/threonine kinases, and about 30 other known effector categories (91). The other photoreceptor classes in general couple to fewer possible signaling readouts, but the diversity is still quite impressive.

For the purposes of this review, however, we focus on the light-dependent conformational change in the photoreceptor domains themselves, which can be coupled to downstream allosteric regulation of single-domain effectors (next section) or binding/unbinding of multi-component tools for purposes of bringing together/apart effectors (this section).

A number of factors come together to determine the overall utility of an optogenetics dimerizer tool. For all dimerizers, utilization of endogenous cofactors is of course desirable. Similarly, small size is preferred, so as to minimize perturbation of the fused proteins. Lastly, optogenetic dimerizers should have low levels of association in the dark and very high association in the light (the opposite tools, optogenetic "de-dimerizers" are also useful.) For applications that require bringing two disparate components together, a heterodimerizer must of course be used, whereas a homodimerizer can suffice if the application requires dimerizing a single effector domain. For heterodimers, it is important that the two separate components do not themselves homodimerize, as this degrades heterodimerization efficiency and can disrupt localization.

For homodimerizers, commonly used reagents include Dronpa (which potentially tetramerizes); LOV domain homodimers including Vivid (92), EL222 (93), and relatives; and Cryptochrome-2 (CRY2) (94). UVR8 dissociates upon UV light illumination, thus constituting an optogenetic "de-dimerizer." For heterodimerizers, the most commonly used are CRY2 and its binding partner CIB(N) – although CRY2 homodimerization partially defeats its utility as a heterodimerizer; the phytochrome PhyB and its interacting partners PIF3/PIF6; the bacterial phytochrome BphP1 and its interacting partners PpsR2 (95) and Q-PAS1 (96); UVR8 and its partner COP1 – again, its homodimerization complicates its use; and the LOV domain heterodimers FKF1/GIGANTEA (97), Magnets (98) and enhanced Magnets (eMags) (99), iLIDs (100), and TULIPs (101).

Of these tools, PhyB is quite large and of unknown structure (also true of its interacting partners). CRY2 is of known structure (at least of a homologous cryptochrome) but is fairly large

– CIB is of unknown structure and appears to be an intrinsically disordered protein. The UV light required for UVR8 penetrates poorly and causes photodamage. PhyB also requires addition of the exogenous chromophore phytochromobilin; however, advantages of the PhyB system include bidirectional control with red and far-red light, allowing greater penetration. The dissociation kinetics of CRY2/CIB are quite slow, and fusion of CRY2 to membrane proteins can lead to organelle loss and even cell death (102). The dimerization efficiency of iLIDs is fairly low, and original Magnets requires use as a tandem trimer to achieve good dimerization efficiency (103) – Magnets also needs preincubation of expressing cells at a lower temperature (28 °C) due to low thermodynamic stability (103). eMags shows greater dimerization efficiency than original Magnets, even when used as a monomer; it also folds well at 37 °C and shows faster association and dissociation kinetics than original Magnets (99). Finally, to achieve optimal spatiotemporal control of activation, it is important that both components of a heterodimerizer system be photoreceptors and that association requires that light activate both components. This is not the case for CRY2/CIB, iLIDs, PhyB/PIF, and TULIPs, and accordingly, these systems have higher levels of background activation than LOV domain dimers, and furthermore, following illumination, activated effector molecules spread much more broadly throughout cells, delocalizing the intended effect (103). Taken together, the use of enhanced Magnets (99) is recommended for most optogenetic heterodimerizer applications, and Vivid for homodimerizer ones.

Notable reagents derived from dimerizer components include light-activated Cre recombinase (104), CRISPR-Cas9 (105), T7 RNA polymerase (106), a zinc finger transcription factor (107), control of organelle trafficking (108), manipulation of lipid transport through the trans-Golgi network (99), and botulinum toxin activity (109).

As a final note, we mention that photocleavable proteins can be (and usually are) used in place of de-dimerizers such as UVR8. Many fluorescent proteins undergo backbone cleavage during either chromophore formation or photoconversion of the chromophore to a second form. However, in most cases the two resulting polypeptides remain tightly associated with one another and thus, although technically cleaved, do not functionally act that way. Protein engineering on the photoconvertible fluorescent mMaple to promote dissociation following photoconversion yielded the more photocleavable protein PhoCl (110). PhoCl and related proteins can be incorporated into optogenetic reagents to yield light-dependent release of a component, *e.g.*, a moiety that once alone translocates within the cell – perhaps to the nucleus to enhance transcription. In a similar vein, selection of LOV-binding proteins from a library of *Staphylococcal* protein A mutants produced reagents (Zdk, LOVTRAP) that tether *Avena sativa* LOV2 (AsLOV2-Jα), but only in the dark (111). Upon optogenetic activation of LOV, the Zdk is released, along with a fused effector domain that had previously been tethered to the site of LOV expression.

Allosterically regulated proteins

In addition to using photoreceptors as dimerization moieties to drive association/dissociation of effector components, the conformational changes within an individual photoreceptor (most notably the dissociation of the Jα helix of the AsLOV2 domain from the β-strand bulk) (112) (**Figure 4b**) can allosterically alter function of a fused protein. A number of enzymes and other effectors have been successfully caged with AsLOV2 and related photoreceptors: the GTPases Rac1 and Cdc42 (113), dihydrofolate reductase (albeit with weak activity) (114), histidine kinases

(115), the molecular motors myosin and kinesin (116, 117), tetracycline repressor (118), calmodulin (PACR, to release Ca^{2+}) (119), membrane-tethered toxins (lumitoxins – note that light releases toxin from target) (120), the singlet oxygen generator miniSOG (not allosteric regulation – the flavin cofactor directly produces this) (121) – as well as the miniSOG-derived reagent for control of synaptic vesicle release (chromophore-assisted light inactivation; CALI) (122), nanobodies (123), receptor tyrosine kinases (124), tryptophan repressor (125), and the light- and Ca^{2+}-dependent transcriptional activators FLARE (126) and Cal-Light (127). Many proteins have been shown to be allosterically regulatable by the LOV2-Jα association, and many more will surely be designed in the future.

Other ion channels & pumps

Although the naturally evolved (but heavily improved in the lab) microbial opsins are the predominant class of light-gated ion channels and pumps, there are a number of optogenetic reagents to effect ion conductance that have been engineered by combining a transmembrane channel with a light-gated moiety. Here we review the most notable such reagents.

The first light-gated ion channel created through protein engineering was SPARK (synthetic photoisomerizable azobenzene-regulated K^+) (128). The high-conductance, high-specificity voltage-gated potassium channel Shaker was made light-gated through systematic introduction of cysteine mutations to the region surrounding the outer pore opening, followed by incubation with MAL-AZO-QA, a synthetic small molecule combining maleimide (for cysteine reactivity), azobenzene (a bulky photoswitch undergoing *cis-trans* isomerization around an internal double bond upon illumination), and a quaternary amine based on tetraethylammonium, an inhibitor of Shaker that occludes the pore. The MAL-AZO-QA is thus an "inhibitor on a stick," which in one configuration (in this instance *trans*) allows the QA group to bind and inhibit the channel, and in the other (in this instance *cis*) sterically removes the QA group from proximity to the pore, thus disinhibiting channel function. Through structure-guided design and extensive screening, it is usually possible in these situations to devise both attachment sites where the *trans* configuration is active and those where the *cis* configuration is. The voltage sensitivity of Shaker remains essentially unchanged despite the presence of the bulky MAL-AZO-QA moiety, and SPARK current is tunable from 0 to 100 pA *via* illumination light wavelength and intensity. However, numerous shortcomings of SPARK have hindered wide uptake of the reagent. The MAL-AZO-QA cofactor is synthetically difficult and not commercially available. Further, it is large and hydrophobic and does not penetrate well into tissue. The 360 nm absorbance peak of the *trans* configuration requires UV light, which has poor penetration and high phototoxicity. (The 440-540 nm peak of the *cis* configuration is more tolerable – it should also be noted that such reagents are independently tunable by these two wavelengths, enabling bidirectional control, which is lacking or at least poorly understood for most optogenetic reagents – excepting PhyB/PIF, discussed above.)

A similar design strategy on a kainate receptor (GluK2) produced the optogenetic Na^+ channel LiGluR (129) – this time with glutamate "on a stick" rather than an antagonist (**Figure 5a**). LiGluR has been demonstrated *in vivo* in a number of preparations (129, 130). Disadvantages of LiGluR are similar to those of SPARK, in terms of small molecule availability and delivery, and light penetration. It should also be noted that over-expression of endogenous receptors (or proteins similar to them) could interfere with the function of said receptors, for instance through

hetero-oligomerization. On the other hand, there are a number of key advantages that tools like LiGluR possess over the microbial opsins. Firstly, the ion selectivity for Na^+ is quite high, given that GluK2, like other iGluR proteins, has numerous protein features favoring preferred ion passage, and features blocking non-preferred ion passage (*i.e.*, the specificity filter). Secondly, the conductance and passed current are many times greater than those of microbial opsins.

Similar reagents have been created for GABA receptors "LiGABARs" (131) and acetylcholine receptors (132). (It should also be noted that arguably the first optogenetics experiment ever conducted was in 1978, when Henry Lester and colleagues created photoisomerizable nAChR agonists and showed that they indeed modulated nAChR activity upon light illumination. However, since such experiments involve no targeting of the drug, we consider these insufficiently genetically encoded to cover in this chapter. The same extends to modulators of GIRK, TRPV1, TRPA1, P2X, and ASIC channels – see (133) for a good review.)

We note that such "transmitter on a stick" approaches translate to other protein scaffolds, as well. Light-agonized and -antagonized versions of the metabotropic glutamate receptors mGluR2, mGluR3, and mGluR6 were constructed through similar methods (134).

A fully genetically encoded, light-gated potassium channel, BLINK1 (135), was created by incorporation of LOV2-Jα (see above) into a viral potassium channel, Kcv – the final design features the LOV2-Jα at the N-terminus of Kcv, with an additional myristoylation motif before the LOV domain, to create tighter allosteric linkage between LOV light activation and pore opening. An improved reagent, BLINK2 (136), exhibits better surface expression through incorporation of several targeting motifs (**Figure 5b**). The kinetics of the BLINK channels are incredibly slow: complete turn-on takes several minutes, and conductance remains elevated for tens of minutes following light cessation, which is good for some applications and fatal for others. Moreover, the K^+-to-Na^+ selectivity of Kcv is only ~9 – around three orders of magnitude lower than that of many eukaryotic ion channels (it is not clear whether the selectivity of the BLINK channels has been determined.) But BLINK2 permits *in vivo* utility in several preparations (136) (although other groups have reported nearly undetectable currents (137).) Similar techniques could in principle be extended to other ion channels, including those with greater ion selectivity and/or faster kinetics.

Other fully genetically encoded ion channels include the 2-component systems involving the light-gated enzymes discussed above, coupled with (typically directly fused to, although co-expression can suffice (78)) an ion channel gated by the enzymatic product. For instance, co-expression of bPAC (see above) and the olfactory cyclic nucleotide-gated (CNG) channel CNG-A2 in *Xenopus* oocytes produced sufficient cAMP from bPAC to gate the channel and produce reasonable currents (78) (**Figure 5c**). The kinetics of both the bPAC and the resulting CNG-A2 currents are fairly slow, however. Neurons express fairly high resting levels of phosphodiesterases, more closely linking CNG activity to PAC activity by rapidly degrading leftover cAMP. Even still, bPAC co-expressed with the channel CNG-A2 in cultured hippocampal neurons turned on in ~5-10 seconds and stayed on for ~100-200 seconds. Another design, using bPAC and the bovine olfactory cyclic nucleotide gated channel (OLF) to produce more Ca^{2+}-selective currents (137) had even slower kinetics: turn-on in ~1 minute, and turn-off in ~10 minutes. A more K^+-selective channel was made by the same group through fusion with the channel SthK from *Spirochaeta*

thermophila; kinetics were still quite slow but ~10 times faster than the bPAC-OLF channel. Many CNG channels exist with diverse ion selectivity, although they are not as selective as classical voltage-gated ion channels. Moreover, CNG channels can be modulated by both cAMP and cGMP, in opposing or concordant directions, potentially complicating regulation of currents.

Considerations for optogenetics experiments

Some applications of optogenetic effectors are quite clear in their interpretation – in general, these are experiments where the manipulations are used to generate hypotheses that are verified with other means, or where the manipulation merely generates a signal to identify cells of interest for probing by other means. Notable examples include "optogenetic tagging" of cell types to facilitate *in vivo* electrophysiological recordings (138) and tracing of long-range axon projections *via* a light-dependent signature (139), among many others.

However, caution is warranted when using optogenetics in behavioral and mechanistic experiments, particularly those done on the brain, for many reasons. As with any foreign (or endogenous, for that matter) genes, their overexpression can produce unexpected phenotypes. Overexpression of opsins, particularly long-term and/or through development, has been shown to produce defects in axon morphology, projection patterns, and neural activity patterns (140), even in the absence of light stimulation. Although the precise mechanisms by which this occurs are unknown, it is apparent that sufficient levels of Ca^{2+} are fluxed (even in the dark) through ChR2 (and likely other opsins) to perturb Ca^{2+}-dependent axon guidance factors (141) and morphological synaptic plasticity (142). Long-term (~4 week) expression of ChR2 produced enlarged axon trunks and gave rise to aberrant projections from L2/3 neurons to L4, which receives no such input in normal animals (140). It is also noteworthy that over-expression of ChR2 in cells dramatically increases (in the range of 40%) membrane capacitance (143), thus fundamentally altering the electrophysiological properties of the cells under study – again, even without explicit stimulation. It is unsurprising that microbial opsins have activation-independent effects, both because of the membrane loading with charge and because the pores are typically quite leaky (144). Thus, the first potential concern is that effector expression – even without activation – could perturb, potentially dramatically, the system under study. Further experiments are required to determine whether such effects are truly light-independent, *e.g.,* mediated through protein-protein interactions with endogenous signaling pathways, or if activity driven by ambient light is sufficient to produce the effects seen. If the latter, then animals could be kept in strict darkness, but that would, of course, have its own unintended, dramatic effects on development and behavior. Similarly, overexpressed GPCR-based effectors will no doubt disrupt signaling even absent explicit activation.

It is also the case that light, rather than the effector molecules, can damage and perturb tissue – even at low intensities. Probably the most salient concern is local heating (145), (146), which affects all cellular processes, with several noteworthy pathways being sensitive to even slight deviations above resting temperature. Illumination with commonly used optogenetic activation light intensities (~3-30 mW) resulted in local heating of 0.2–2 °C in mouse striatum (147). This level of heating was sufficient, even in the absence of opsin expression, to dramatically depress neural firing rates in a cell type-dependent fashion (in this case, medium spiny neurons, MSNs, were most inhibited), with hyperpolarizing currents appearing to arise from thermal activation of inwardly rectifying potassium (K_{ir}) channels. This light-driven, opsin-independent,

inhibition of MSNs was sufficiently strong to produce a clear behavioral phenotype: upon illumination of the dorsal striatum with 532 nm (green) light, mice exhibited rotational behavior biased in the direction ipsilateral to the side of the brain receiving illumination. This perturbation was evident at illumination intensities of 7 mW and became more pronounced at 15 mW. Heating effects can be even more profound on smaller animals with much less tissue to serve as a heat sink. Light, particularly high-intensity, can also have other adverse effects on cells, including effector-independent depolarization (148), optoporation (formation of holes in the membrane) (149), activation of the endoplasmic reticulum stress-response pathway and autophagy (150), and alteration of gene expression patterns, particularly immediate early genes (151). Light has also been shown to cause vasodilation in the brain, which would affect functional hyperemia and perhaps the interpretation of some experiments where optogenetics have been used in fMRI evaluations (152). In general, it is advised to use the lowest intensity possible of activation light, with longer wavelengths also being preferred.

Of concern to interpreting neuroscience experiments in particular is the nature of action potential generation and propagation. Because of the asymmetric expression of ion channels through neurons, cellular output from a stimulation regime depends critically on subcellular distribution excitation. Activation of the soma and dendrites simultaneously produces different output waveforms from cells than activation of soma alone: namely, this synchronized depolarization leads to increased spike rate, more bursting events, and greater backpropagation of spikes through the dendritic arbor, even to distal tufts (153). Even "axon-specific" optogenetic activation of axonal fibers (139) produces backpropagating action potentials that can travel back and depolarize the soma and dendrites. Thus, observed phenotypes can result from something other than the output of stimulated neurons or terminals (154).

Another issue is habituation of expressing cells to elicited ion currents. Cells may change their complement of expressed proteins in response to repeated perturbations from equilibrium. Phenotypes seen after long-term stimulation might, at least somewhat, reflect these cellular changes. This can be especially evident following long firing of H^+ channels and pumps, which disrupt local pH environments and can lead to (sometimes rapid) loss of responsivity of the effector, through poorly understood cellular mechanisms. Furthermore, effectors may drive cells into non-physiological states, such as activators spiking cells faster than they do *in vivo* (155) – persistent activation can even lead to apoptosis and cell death (156). Similarly, inhibitors can drop cells to potentials below that normally seen, leading to rebound excitation upon block release (157).

Another cause for concern, particularly for the microbial opsins, is that the effect that the optogenetic reagent is having on expressed cells may be very different from expectations. The ionic specificity of opsins is quite poor, with ChR2 passing guanidinium$^+$, methylammonium$^+$, dimethylammonium$^+$, Li^+, Na^+, Ca^{2+}, and K^+ within only an order of magnitude of each other – and a million-fold less than the passage of H^+ (23). Obviously, the effects of these cations on cells are profoundly different, with Na^+ and K^+ directly opposing one another in membrane polarization effects, with Ca^{2+} exerting profound signaling effects (as discussed above), and with dramatic pH changes possible following opsin activity. Obviously, the effect of a given channel or pump depends critically on the electrochemical gradients of the ions involved, which can vary widely between cell types, developmental stage (*e.g.,* the Cl^- reversal potential changes sign in the

vertebrate embryo), and activity state of neurons and other cell types, and the circuits to which they give rise. It was recently shown, for instance, that ChR2 stimulation led to profound increases (up to 5 mM) in extracellular [K^+] in living mice (158). As such, outward K^+ flux likely substantially contributes to depolarization, in contrast to the canonical model of ChR2 function, *i.e.,* inward Na^+ current. This observation is consequential for several reasons. Primarily, high extracellular [K^+] exerts its depolarizing effects on all nearby cells, rendering ChR2 activity essentially non-cell-autonomous; indeed, the study showed that ChR2 activity in striatal astrocytes promoted increased excitability and c-Fos expression in nearby medial spiny neurons, despite no explicit physiological coupling between the astrocytes and MSNs. Secondly, this contribution of extracellular [K^+] increases to cellular depolarization will depend on the precise complement of expressed potassium channels and their current state of openness, among other things. This implies that controls are needed in multiple experimental settings. Non-cell-autonomous effects are seen with other reagents, including ArchT, where activation in astrocytes led to dramatic acidification of the extracellular space (~0.2 pH units), sufficient to gate acid-sensing ion channels on neurons (159). These non-cell-autonomous effects of overexpressed channels and pumps complicates mechanistic interpretation of results – the salient perturbation may occur on a cell type not even considered.

Another example of counterintuitive effects of opsins is the observation that Arch (ostensibly passing H^+) activation in astrocytes of living mice instead produced large [Ca^{2+}] increases in astrocyte processes (160), despite no previously documented conductance of Ca^{2+} by Arch. The authors speculate that the H^+ conductance of Arch modulates the activity of glutamate transporters, with the subsequent signaling through glutamate receptors producing intracellular [Ca^{2+}] rises – although it is clear that sufficiently many processes are affected that it is impossible to definitively propose a straightforward mechanism. Thus, results from cell culture experiments may not be consistent with those *in vivo* – although of course it is not immediately clear which results to trust more. As mentioned above, the observed phenotype may result from a different pathway or even cell type altogether. Regardless, experimenters should mind that *in vivo* performance of effectors can differ from published results.

Critically, although deployed as light-dependent proteins, opsins are unsurprisingly affected by other cellular stimuli as well. The kinetics, particularly of recovery and desensitization, are quite dependent on temperature (161), indicating that they will not necessarily behave similarly in different organisms. Of greater concern, ChR2 and other rhodopsins are also quite voltage-sensitive, specifically that depolarization results in significant slowing of activation and deactivation (161, 162). Thus, during trains of elicited spikes, subsequent depolarizations are smaller than initial ones, sometimes much so. Furthermore, resting membrane potential heterogeneity (up to 30-40 mV in pyramidal cells) will engender different reagent properties across cells – even of the same fundamental cell type. Many of the other depolarizing/hyperpolarizing tools discussed here are likely voltage-dependent as well, but this has been less systematically characterized.

More generally, the exact effects of perturbations on a system depend critically on the precise properties of the tools used. For instance, three channelrhodopsin variants (ChR2 itself, Chronos, and Chrimson), despite ostensibly all functioning as light-gated elicitors of spiking activity, manifested wildly different effects on wide-scale cortical activity when stimulated in mice

(163). At the most basic level, Chrimson often failed to reliably elicit spikes, leading to a broad distribution of spike rates in expressing neurons. Despite reliably driving action potentials early, Chronos often dropped spikes during sustained firing, likely due to its more rapid inactivation in neurons. At the circuit level, where neurons recursively interact with one another (and with themselves), things became more complicated. ChR2 evoked robust cortical gamma-wave (γ) oscillatory activity, Chronos did not, and Chrimson not only did not evoke γ actively but also suppressed endogenous power in all oscillatory rhythms up to 150 Hz, essentially negating the critical contributions of underlying circuit oscillations to brain activity. In retrospect, this is perhaps unsurprising, given the wide diversity of neuronal types, most obviously excitatory and inhibitory, and extending to precise electrophysiological properties. This wide variation in spike rate, projection pattern, expressed ion channels and receptors, *etc*. between expressing cells can of course magnify small differences in single-neuron properties into large differences at the circuit level. Other examples of recurrent networks and tight coupling confounding optogenetic manipulations can be found in (157), (164), (165), (166), (167), (168), and more. These observations suggest that we need realistic biophysical and computational models (driven by careful biophysical measurements) of neurons and of opsins (and other reagents) to meaningfully interpret how they affect single neurons and ultimately circuits.

In conclusion, the diversity of optogenetic reagents, both naturally evolved and explicitly engineered, is breathtaking. Many previously unimaginable experiments are now routine. However, there are also a number of caveats associated with cellular perturbations, which by and large have not been grappled with sufficiently.

Recommendations

For these reasons and more, caution must be used when interpreting the results of optogenetics experiments. Here we recommend several experimental modifications that can increase the confidence in potential findings from studies involving the manipulation of cellular activity through optogenetic effectors:

- Toolmakers should build “internal control” versions of optogenetic effectors that are minimally mutated, ideally at a single site, in order to neutralize the effector activity but hopefully remain identical in expression level, targeting, *etc.* – akin to how non-responsive sensor variants help rule out artifacts from sources other than the target analyte or state. If these control versions alter the phenotype, it is likely due to reasons other than the ostensible effector function.
- Perform experiments using multiple similar tools (*e.g.*, ChR2, Chronos, Chrimson, as above; or both a chloride channel such as halorhodopsin and a proton channel such as Arch) – are the findings the same across effectors?
- Perform parallel experiments with no explicit light delivery, to help elucidate non-specific activation.
- Similarly, activate a population in one set of experiments and silence it in another. Of course, many receptors, cells, and populations exhibit “U-shaped” responses in which both sets might produce similar results. Regardless, such experiments would aid interpretation.
- If experiments suggest multi-neuron circuit-based mechanisms in a particular response, then probe the intervening neurons to further test the predictions. For example, if excitation of an upstream neuron produces a particular response, then explore if silencing the intermediate neuron(s) can block the effect.

- Complementary experiments using diverse reagents: *e.g.*, if activation of a downstream neuron is thought to signal through specific receptors in a second population, then test direct activation of that signaling pathway, through the appropriate opto-XR, caged neurotransmitter, *etc*.
- Ensure that the spiking rates elicited by optogenetic activation are consistent with behaviorally elicited rates. If the optogenetically induced rates exceed those measured in behavior, this may be non-physiologically relevant and inform about things cells "can" do, rather than what they actually do.
- Ideally, perform experiments using a different modality, for instance with a chemogenetic effector such as a DREADD (169).
- Overexpression of effectors creates multiple concerns: alteration of cellular and membrane properties, hetero-oligomerization with endogenous proteins, and disrupted targeting of proteins, thus producing non-physiological actions. When using an effector meant to recreate the activity of an endogenous protein, this should be done in a knock-in organism, where the photoswitchable effector replaces the endogenous protein. Of course, careful histology and physiology is required to show that the modified protein is expressed at similar levels and in similar places as the wild-type, and that its activity is similar (just photoswitchable.) This would allow experiments showing that signaling through a certain protein, in a certain place, at a certain time, definitively produced a given phenotype. When expressing effectors in another manner, expression level should be kept as low as possible through promoter choice, IRES sequences, degrons, *etc*.
- Perform immunolabeling to reveal effector expression patterns, particularly as regards subcellular distribution.
- Stain for activity from stimulation with *in situ* hybridization against c-Fos or another marker. Is staining consistent with the population meant to be stimulated? Importantly, are there signs that unintended populations experienced dramatic stimulation?
- Express the effector in populations that should not affect the phenotype under observation. Unexpected contributions from other regions could suggest that the effect is mediated through mechanisms other than that proposed.
- Consider and try to rule out the possibility that the effector is actually functioning through a different, perhaps unexamined, cell type and/or pathway. The non-cell-autonomous effects of overexpressed ion channels and pumps allow them to act on nearby cells. The effects of extracellular H^+ and K^+ appear particularly important.
- In general, inhibitory tools are considered to be more precise mediators of perturbation than activators. For example, silencing a group of neurons could reasonably be expected to block circuit signaling through those cells but not necessarily have far-reaching network effects. Injecting neuronal activity into a circuit, on the other hand (and particularly if the injected activity is non-physiological), will have knock-on effects on downstream (and even upstream) circuit components.

Future goals

A critical set of reagents that is lacking is tools to rapidly and specifically degrade target proteins – thus testing their effects in a circuit without the dramatic effects of compensation (as in knockouts). Many of the pieces are available, including light-regulated nanobodies (although the number of high-quality nanobodies – or any affinity reagent – remains low), E3 ubiquitin ligases, and potentially variants of botulinum and tetanus toxins with redesigned target specificity. Such reagents would be transformative for systems biology. There are also very few tools for

optogenetic modulation of specific synapses – reagents to activate or inhibit targeted synapses will be extremely useful for neuroscience.

A dream set of reagents in neuroscience is those that would allow the tagging of active neurons and synapses during a behavior, potentially facilitating a replay of the behavior by properly stimulating the active ensemble. However, the word "properly" is doing a lot of work in this sentence: if all neurons and/or synapses are identically tagged regardless of their temporal position in the physiologically evoked sequence (in addition to the firing strength and other concerns), then there is no reason to believe that simultaneously activating all of said neurons and synapses would produce anything other than behavioral noise. That is to say, neurons and synapses acutely "care about" their spatiotemporal activity patterns, and optogenetic tools that do not recapitulate this aspect run the risk of producing non-physiological results. However, tools allowing correct temporal replay of tagged neurons/synapses seem woefully difficult to create, and neuroscientists might need to content themselves with much smaller pieces of the puzzle.

Many more red light-gated tools are required, both for maximizing penetration and minimizing damage and allowing multiplexing. Better still, effectors gated by near-infrared (NIR) or fully infrared light would be transformative. However, given the lower energy of such photons and thus the more limited range of available photochemistry, such reagents may be difficult or even impossible to create. (Some nanoparticles allow upconversion of NIR light into visible wavelengths for effector activation (170), but they have their own limitations and being non-genetically encoded, are beyond the purview of this piece anyway.) For neuroscience applications, it would be extremely useful if many of the existing reagents could be made to work much faster. Effectors whose perturbations manifest over minutes allow no precision in manipulating specific cells or synapses. For instance, the advantages of a very slow optogenetic silencer over constitutive silencers such as inward-rectifying potassium channels (K_{ir}) are largely having fewer developmental effects and lower cellular habituation. Reagents with sub-second (ideally millisecond) level kinetics are required to drop out given cells and synapses to monitor their circuit contributions.

There are many tools for light-regulated transcription and recombination, but our overall feeling (and from discussions with other researchers) is that none is sufficiently robust to reliably produce believable results. In general, many of the tools covered here are at the proof-of-principle level and are insufficiently optimized and/or validated to robustly deploy.

In fact, many of the tool classes covered in this review would benefit from systematic improvement through protein engineering – we have tried to note their specific shortcomings. Despite hundreds (maybe thousands) of opsin variants having been characterized in neurons (and other excitable cells) – and many fewer yet a considerable number *in vivo* – it remains unclear which opsins are the best for each specific application. A larger concern, as we have discussed in detail above, is that the wide-ranging effects of opsins and other effectors on the systems expressing them has not received adequate attention (but see (157), (171), (172), (173), (174), (175), (176), (177), (178)), and there are reasons to question the interpretational validity of many optogenetics experiments without a thorough battery of controls and careful consideration of alternative explanations.

Taken together, optogenetics represents a ridiculously powerful suite of reagents and methods, and it will only improve with time – particularly as manipulations become more and more precise with new tools and techniques. In the meantime, we recommend additional control experiments to ensure experimental reliability.

Figure Legends

Figure 1. Samples for optogenetics. A. Mouse. B. Drosophila adult and C. Larva. D. Local delivery of light to a rodent brain region through an optical fiber. E. Wide-field illumination of a neuronal field. F. Patterned illumination of specific cells in the field, potentially in a given temporal program. G. 1-Photon light reaches cells along an input cone. 2-Photon light activates small voxels, potentially over a single cell body. H. Subcellular activation of optogenetic effectors.

Figure 2. Ion gradients and the effect of microbial opsins on them. A. In most excitable cells, pumps and transporters maintain high extracellular levels of Na^+, Ca^{2+}, and Cl^-, with high intracellular levels of K^+. Usually extracellular pH is neutral or slightly basic, but can differ dramatically. B. A subset of effects of ion channels and pumps. Channelrhodopsin-2 (ChR2) is a fairly non-specific cation channel, letting Na^+ and Ca^{2+} in, and K^+ out. However, flux of H^+ is ~6 orders of magnitude higher than other ions. Anion-conducting channelrhodopsin mutants (ACRs) have greater selectivity for Cl^-, but still conduct many other ions.

Figure 3. G protein-coupled receptors, opto-XRs, and soluble nucleotide-modifying enzymes. A. GPCRs signal through a heterotrimeric G protein complex (α, β, γ), as well as arrestins and accessory Regulator of G protein Signaling (RGS) proteins (not shown). Depending on the coupled G proteins and accessory elements, signaling can be predominantly through several different pathways. Many other variables (protein complement, cell state, *etc*.) can perturb G protein signaling as well as signaling through arrestins and other pathways ("GPCR bias", not shown). GPCR activation typically involves hydrolysis of GTP to GDP and phosphate, or GMP and pyrophosphate. B. Opto-XRs are chimeric molecules made from a light-sensitive opsin such as rhodopsin, with intracellular loops grafted from a GPCR. Fluorescent proteins are almost always fused to the C-termini of opsin constructs, both for visualization and improvement of membrane trafficking. C. Soluble light-gated nucleotidyl cyclases and phosphodiesterases. Most cyclases used are improved versions of naturally evolved enzymes. The most commonly used phosphodiesterases are derived from protein engineering (e.g., LAPD for cAMP activity).

Fig. 4. Optogenetic dimerizers and allosterically regulated proteins. A. Various optogenetic hetero- and homo-dimerizers. Cryptochromes have a second pterin chromophore, such as 5,10-methenyltetrahydrofolic acid (not shown). B. Allosteric modulation of enzymes and other proteins through propagated conformational changes from LOV2-Jα or other photoreceptors.

Fig. 5. Optogenetic ion channels and pumps derived from protein engineering. A. Light-gated glutamate receptor (LiGluR) engineered to have a cysteine at a site allosterically coupled to the glutamate-binding pocket of an iGluR. In one conformation (*trans* in this case), glutamate is unbound from the pocket and the channel is closed; in another (*cis* in this case), glutamate is bound and the channel opens. B. Optogenetic K^+ channel BLINK2 derived from a viral channel, Kcv (grey), LOV2-Jα (yellow), an N-terminal myristoylation motif to promote membrane reentry (black), and a 14-3-3 protein binding site (red), to improve membrane trafficking. C. Creation of

an optogenetic ion channel by fusing a soluble optogenetic adenylyl cyclase such as bPAC to a cyclic nucleotide-gated (CNG) channel such as OLF. plasma membrane trafficking signal; Ex, endoplasmic reticulum export signal.

Acknowledgements
We thank Misha Ahrens, Baljit Khakh, Lorena Benedetti, and members of the Looger lab for helpful discussions and critical reading of the chapter.

References

1. Sternson SM, Roth BL. Chemogenetic tools to interrogate brain functions. Annu Rev Neurosci. 2014;37:387-407.
2. Maresca D, Lakshmanan A, Abedi M, Bar-Zion A, Farhadi A, Lu GJ, et al. Biomolecular Ultrasound and Sonogenetics. Annu Rev Chem Biomol Eng. 2018;9:229-52.
3. Ben-Aroya S, Pan X, Boeke JD, Hieter P. Making temperature-sensitive mutants. Methods Enzymol. 2010;470:181-204.
4. Nimpf S, Keays DA. Is magnetogenetics the new optogenetics? EMBO J. 2017;36(12):1643-6.
5. Meister M. Physical limits to magnetogenetics. Elife. 2016;5: e17210.
6. Anikeeva P, Jasanoff A. Problems on the back of an envelope. Elife. 2016;5: e19569.
7. Sun L, Perakyla J, Kovalainen A, Ogawa KH, Karhunen PJ, Hartikainen KM. Human Brain Reacts to Transcranial Extraocular Light. PLoS One. 2016;11(2):e0149525.
8. Foster RG, Grace MS, Provencio I, Degrip WJ, Garcia-Fernandez JM. Identification of vertebrate deep brain photoreceptors. Neurosci Biobehav Rev. 1994;18(4):541-6.
9. Ronzitti E, Ventalon C, Canepari M, Forget BC, Papagiakoumou E, Emiliani V. Recent advances in patterned photostimulation for optogenetics. Journal of Optics. 2017;19(11):113001.
10. Pégard NC, Mardinly AR, Oldenburg IA, Sridharan S, Waller L, Adesnik H. Three-dimensional scanless holographic optogenetics with temporal focusing (3D-SHOT). Nature communications. 2017;8(1):1-14.
11. Lerman GM, Little JP, Gill JV, Rinberg D, Shoham S. Real-Time In Situ Holographic Optogenetics Confocally Unraveled Sculpting Microscopy. Laser & Photonics Reviews. 2019;13(9):1900144.
12. Packer AM, Peterka DS, Hirtz JJ, Prakash R, Deisseroth K, Yuste R. Two-photon optogenetics of dendritic spines and neural circuits. Nat Methods. 2012;9(12):1202-5.
13. Rost BR, Schneider-Warme F, Schmitz D, Hegemann P. Optogenetic Tools for Subcellular Applications in Neuroscience. Neuron. 2017;96(3):572-603.
14. Zhu M, Chen M, Lichtler AC, O'Keefe RJ, Chen D. Tamoxifen-inducible Cre-recombination in articular chondrocytes of adult Col2a1-CreER(T2) transgenic mice. Osteoarthritis Cartilage. 2008;16(1):129-30.
15. Li X, Du ZJ, Chen MQ, Chen JJ, Liang ZM, Ding XT, et al. The effects of tamoxifen on mouse behavior. Genes Brain Behav. 2020;19(4):e12620.

16. Sula A, Hollingworth D, Ng LCT, Larmore M, DeCaen PG, Wallace BA. A tamoxifen receptor within a voltage-gated sodium channel. Mol Cell. 2021.
17. Kristianto J, Johnson MG, Zastrow RK, Radcliff AB, Blank RD. Spontaneous recombinase activity of Cre-ERT2 in vivo. Transgenic Res. 2017;26(3):411-7.
18. Bohin N, Carlson EA, Samuelson LC. Genome Toxicity and Impaired Stem Cell Function after Conditional Activation of CreER(T2) in the Intestine. Stem Cell Reports. 2018;11(6):1337-46.
19. Banala S, Arvin MC, Bannon NM, Jin X-T, Macklin JJ, Wang Y, et al. Photoactivatable drugs for nicotinic optopharmacology. Nature methods. 2018;15(5):347-50.
20. Deisseroth K. Optogenetics: 10 years of microbial opsins in neuroscience. Nat Neurosci. 2015;18(9):1213-25.
21. Terakita A. The opsins. Genome Biol. 2005;6(3):213.
22. Zabelskii D, Alekseev A, Kovalev K, Rankovic V, Balandin T, Soloviov D, et al. Viral rhodopsins 1 are an unique family of light-gated cation channels. Nat Commun. 2020;11(1):5707.
23. Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci U S A. 2003;100(24):13940-5.
24. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. Nature neuroscience. 2005;8(9):1263-8.
25. Li X, Gutierrez DV, Hanson MG, Han J, Mark MD, Chiel H, et al. Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin. Proc Natl Acad Sci U S A. 2005;102(49):17816-21.
26. Zhang F, Wang L-P, Boyden ES, Deisseroth K. Channelrhodopsin-2 and optical control of excitable cells. Nature methods. 2006;3(10):785-92.
27. Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A. Light activation of channelrhodopsin-2 in excitable cells of Caenorhabditis elegans triggers rapid behavioral responses. Current Biology. 2005;15(24):2279-84.
28. Govorunova EG, Sineshchekov OA, Li H, Spudich JL. Microbial rhodopsins: diversity, mechanisms, and optogenetic applications. Annual review of biochemistry. 2017;86:845-72.
29. Kato HE, Zhang F, Yizhar O, Ramakrishnan C, Nishizawa T, Hirata K, et al. Crystal structure of the channelrhodopsin light-gated cation channel. Nature. 2012;482(7385):369-74.
30. Berndt A, Yizhar O, Gunaydin LA, Hegemann P, Deisseroth K. Bi-stable neural state switches. Nat Neurosci. 2009;12(2):229-34.
31. Schoenenberger P, Gerosa D, Oertner TG. Temporal control of immediate early gene induction by light. PLoS One. 2009;4(12):e8185.
32. Klapoetke NC, Murata Y, Kim SS, Pulver SR, Birdsey-Benson A, Cho YK, et al. Independent optical excitation of distinct neural populations. Nat Methods. 2014;11(3):338-46.
33. Lin JY, Knutsen PM, Muller A, Kleinfeld D, Tsien RY. ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. Nat Neurosci. 2013;16(10):1499-508.
34. Lin JY, Lin MZ, Steinbach P, Tsien RY. Characterization of engineered channelrhodopsin variants with improved properties and kinetics. Biophys J. 2009;96(5):1803-14.

35. Gunaydin LA, Yizhar O, Berndt A, Sohal VS, Deisseroth K, Hegemann P. Ultrafast optogenetic control. Nat Neurosci. 2010;13(3):387-92.
36. Bedbrook CN, Yang KK, Robinson JE, Mackey ED, Gradinaru V, Arnold FH. Machine learning-guided channelrhodopsin engineering enables minimally invasive optogenetics. Nat Methods. 2019;16(11):1176-84.
37. Gradinaru V, Zhang F, Ramakrishnan C, Mattis J, Prakash R, Diester I, et al. Molecular and cellular approaches for diversifying and extending optogenetics. Cell. 2010;141(1):154-65.
38. Han X, Chow BY, Zhou H, Klapoetke NC, Chuong A, Rajimehr R, et al. A high-light sensitivity optical neural silencer: development and application to optogenetic control of non-human primate cortex. Front Syst Neurosci. 2011;5:18.
39. Chuong AS, Miri ML, Busskamp V, Matthews GA, Acker LC, Sorensen AT, et al. Noninvasive optical inhibition with a red-shifted microbial rhodopsin. Nat Neurosci. 2014;17(8):1123-9.
40. Pan ZH, Ganjawala TH, Lu Q, Ivanova E, Zhang Z. ChR2 mutants at L132 and T159 with improved operational light sensitivity for vision restoration. PLoS One. 2014;9(6):e98924.
41. Kleinlogel S, Feldbauer K, Dempski RE, Fotis H, Wood PG, Bamann C, et al. Ultra light-sensitive and fast neuronal activation with the Ca(2)+-permeable channelrhodopsin CatCh. Nat Neurosci. 2011;14(4):513-8.
42. Wietek J, Prigge M. Enhancing Channelrhodopsins: An Overview. Methods Mol Biol. 2016;1408:141-65.
43. Brown J, Behnam R, Coddington L, Tervo DGR, Martin K, Proskurin M, et al. Expanding the Optogenetics Toolkit by Topological Inversion of Rhodopsins. Cell. 2018;175(4):1131-40 e11.
44. Mardinly AR, Oldenburg IA, Pegard NC, Sridharan S, Lyall EH, Chesnov K, et al. Precise multimodal optical control of neural ensemble activity. Nat Neurosci. 2018;21(6):881-93.
45. Mahn M, Gibor L, Patil P, Cohen-Kashi Malina K, Oring S, Printz Y, et al. High-efficiency optogenetic silencing with soma-targeted anion-conducting channelrhodopsins. Nat Commun. 2018;9(1):4125.
46. Lewis TL, Jr., Mao T, Svoboda K, Arnold DB. Myosin-dependent targeting of transmembrane proteins to neuronal dendrites. Nat Neurosci. 2009;12(5):568-76.
47. Grubb MS, Burrone J. Channelrhodopsin-2 localised to the axon initial segment. PLoS One. 2010;5(10):e13761.
48. Tkatch T, Greotti E, Baranauskas G, Pendin D, Roy S, Nita LI, et al. Optogenetic control of mitochondrial metabolism and Ca(2+) signaling by mitochondria-targeted opsins. Proc Natl Acad Sci U S A. 2017;114(26):E5167-E76.
49. Gobbo F, Cattaneo A. Neuronal Activity at Synapse Resolution: Reporters and Effectors for Synaptic Neuroscience. Front Mol Neurosci. 2020;13:572312.
50. Tutucci E, Vera M, Biswas J, Garcia J, Parker R, Singer RH. An improved MS2 system for accurate reporting of the mRNA life cycle. Nat Methods. 2018;15(1):81-9.
51. AzimiHashemi N, Erbguth K, Vogt A, Riemensperger T, Rauch E, Woodmansee D, et al. Synthetic retinal analogues modify the spectral and kinetic characteristics of microbial rhodopsin optogenetic tools. Nat Commun. 2014;5:5810.

52. Okitsu T, Yamano Y, Shen YC, Sasaki T, Kobayashi Y, Morisawa S, et al. Synthesis of One Double Bond-Inserted Retinal Analogs and Their Binding Experiments with Opsins: Preparation of Novel Red-Shifted Channelrhodopsin Variants. Chem Pharm Bull (Tokyo). 2020;68(3):265-72.
53. Khorana HG, Knox BE, Nasi E, Swanson R, Thompson DA. Expression of a bovine rhodopsin gene in Xenopus oocytes: demonstration of light-dependent ionic currents. Proceedings of the National Academy of Sciences. 1988;85(21):7917-21.
54. Zemelman BV, Lee GA, Ng M, Miesenböck G. Selective photostimulation of genetically chARGed neurons. Neuron. 2002;33(1):15-22.
55. Koyanagi M, Takano K, Tsukamoto H, Ohtsu K, Tokunaga F, Terakita A. Jellyfish vision starts with cAMP signaling mediated by opsin-G(s) cascade. Proc Natl Acad Sci U S A. 2008;105(40):15576-80.
56. Bailes HJ, Zhuang LY, Lucas RJ. Reproducible and sustained regulation of Galphas signalling using a metazoan opsin as an optogenetic tool. PLoS One. 2012;7(1):e30774.
57. Karunarathne WK, Giri L, Kalyanaraman V, Gautam N. Optically triggering spatiotemporally confined GPCR activity in a cell and programming neurite initiation and extension. Proc Natl Acad Sci U S A. 2013;110(17):E1565-74.
58. Koyanagi M, Saito T, Wada S, Nagata T, Kawano-Yamashita E, Terakita A. Optogenetic Potentials of Diverse Animal Opsins: Parapinopsin, Peropsin, LWS Bistable Opsin. Adv Exp Med Biol. 2021;1293:141-51.
59. Eickelbeck D, Rudack T, Tennigkeit SA, Surdin T, Karapinar R, Schwitalla JC, et al. Lamprey Parapinopsin ("UVLamP"): a Bistable UV-Sensitive Optogenetic Switch for Ultrafast Control of GPCR Pathways. Chembiochem. 2020;21(5):612-7.
60. Koizumi A, Tanaka KF, Yamanaka A. The manipulation of neural and cellular activities by ectopic expression of melanopsin. Neurosci Res. 2013;75(1):3-5.
61. Kim JM, Hwa J, Garriga P, Reeves PJ, RajBhandary UL, Khorana HG. Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops. Biochemistry. 2005;44(7):2284-92.
62. Airan RD, Thompson KR, Fenno LE, Bernstein H, Deisseroth K. Temporally precise in vivo control of intracellular signalling. Nature. 2009;458(7241):1025-9.
63. van Wyk M, Pielecka-Fortuna J, Lowel S, Kleinlogel S. Restoring the ON Switch in Blind Retinas: Opto-mGluR6, a Next-Generation, Cell-Tailored Optogenetic Tool. PLoS Biol. 2015;13(5):e1002143.
64. Li P, Rial D, Canas PM, Yoo JH, Li W, Zhou X, et al. Optogenetic activation of intracellular adenosine A2A receptor signaling in the hippocampus is sufficient to trigger CREB phosphorylation and impair memory. Mol Psychiatry. 2015;20(11):1339-49.
65. Oh E, Maejima T, Liu C, Deneris E, Herlitze S. Substitution of 5-HT1A receptor signaling by a light-activated G protein-coupled receptor. J Biol Chem. 2010;285(40):30825-36.
66. Masseck OA, Spoida K, Dalkara D, Maejima T, Rubelowski JM, Wallhorn L, et al. Vertebrate cone opsins enable sustained and highly sensitive rapid control of Gi/o signaling in anxiety circuitry. Neuron. 2014;81(6):1263-73.
67. Gunaydin LA, Grosenick L, Finkelstein JC, Kauvar IV, Fenno LE, Adhikari A, et al. Natural neural projection dynamics underlying social behavior. Cell. 2014;157(7):1535-51.
68. Siuda ER, Copits BA, Schmidt MJ, Baird MA, Al-Hasani R, Planer WJ, et al. Spatiotemporal control of opioid signaling and behavior. Neuron. 2015;86(4):923-35.

69. Zheng W, Zhou J, Luan Y, Yang J, Ge Y, Wang M, et al. Spatiotemporal Control of GPR37 Signaling and Its Behavioral Effects by Optogenetics. Front Mol Neurosci. 2018;11:95.
70. Keri D, Barth P. Reprogramming G protein coupled receptor structure and function. Curr Opin Struct Biol. 2018;51:187-94.
71. Gurevich VV, Gurevich EV. Biased GPCR signaling: Possible mechanisms and inherent limitations. Pharmacol Ther. 2020;211:107540.
72. Smith JS, Lefkowitz RJ, Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. Nat Rev Drug Discov. 2018;17(4):243-60.
73. Hu J, Stern M, Gimenez LE, Wanka L, Zhu L, Rossi M, et al. A G Protein-biased Designer G Protein-coupled Receptor Useful for Studying the Physiological Relevance of Gq/11-dependent Signaling Pathways. J Biol Chem. 2016;291(15):7809-20.
74. Costa WS, Liewald J, Gottschalk A. Photoactivated adenylyl cyclases as optogenetic modulators of neuronal activity. Methods Mol Biol. 2014;1148:161-75.
75. Stabel R, Stuven B, Hansen JN, Korschen HG, Wachten D, Möglich A. Revisiting and Redesigning Light-Activated Cyclic-Mononucleotide Phosphodiesterases. J Mol Biol. 2019;431(17):3029-45.
76. Iseki M, Matsunaga S, Murakami A, Ohno K, Shiga K, Yoshida K, et al. A blue-light-activated adenylyl cyclase mediates photoavoidance in Euglena gracilis. Nature. 2002;415(6875):1047-51.
77. Fluck RA, Jaffe MJ. Cholinesterases from plant tissues. VI. Preliminary characterization of enzymes from Solanum melongena L. and Zea mays L. Biochim Biophys Acta. 1975;410(1):130-4.
78. Stierl M, Stumpf P, Udwari D, Gueta R, Hagedorn R, Losi A, et al. Light modulation of cellular cAMP by a small bacterial photoactivated adenylyl cyclase, bPAC, of the soil bacterium Beggiatoa. J Biol Chem. 2011;286(2):1181-8.
79. Ohki M, Sugiyama K, Kawai F, Tanaka H, Nihei Y, Unzai S, et al. Structural insight into photoactivation of an adenylate cyclase from a photosynthetic cyanobacterium. Proc Natl Acad Sci U S A. 2016;113(24):6659-64.
80. Ryu MH, Moskvin OV, Siltberg-Liberles J, Gomelsky M. Natural and engineered photoactivated nucleotidyl cyclases for optogenetic applications. J Biol Chem. 2010;285(53):41501-8.
81. Gasser C, Taiber S, Yeh CM, Wittig CH, Hegemann P, Ryu S, et al. Engineering of a red-light-activated human cAMP/cGMP-specific phosphodiesterase. Proc Natl Acad Sci U S A. 2014;111(24):8803-8.
82. Tian Y, Gao S, von der Heyde EL, Hallmann A, Nagel G. Two-component cyclase opsins of green algae are ATP-dependent and light-inhibited guanylyl cyclases. BMC Biol. 2018;16(1):144.
83. Briggs WR. The LOV domain: a chromophore module servicing multiple photoreceptors. J Biomed Sci. 2007;14(4):499-504.
84. Pudasaini A, El-Arab KK, Zoltowski BD. LOV-based optogenetic devices: light-driven modules to impart photoregulated control of cellular signaling. Front Mol Biosci. 2015;2:18.
85. Lin C, Shalitin D. Cryptochrome structure and signal transduction. Annu Rev Plant Biol. 2003;54:469-96.
86. Rockwell NC, Su YS, Lagarias JC. Phytochrome structure and signaling mechanisms. Annu Rev Plant Biol. 2006;57:837-58.

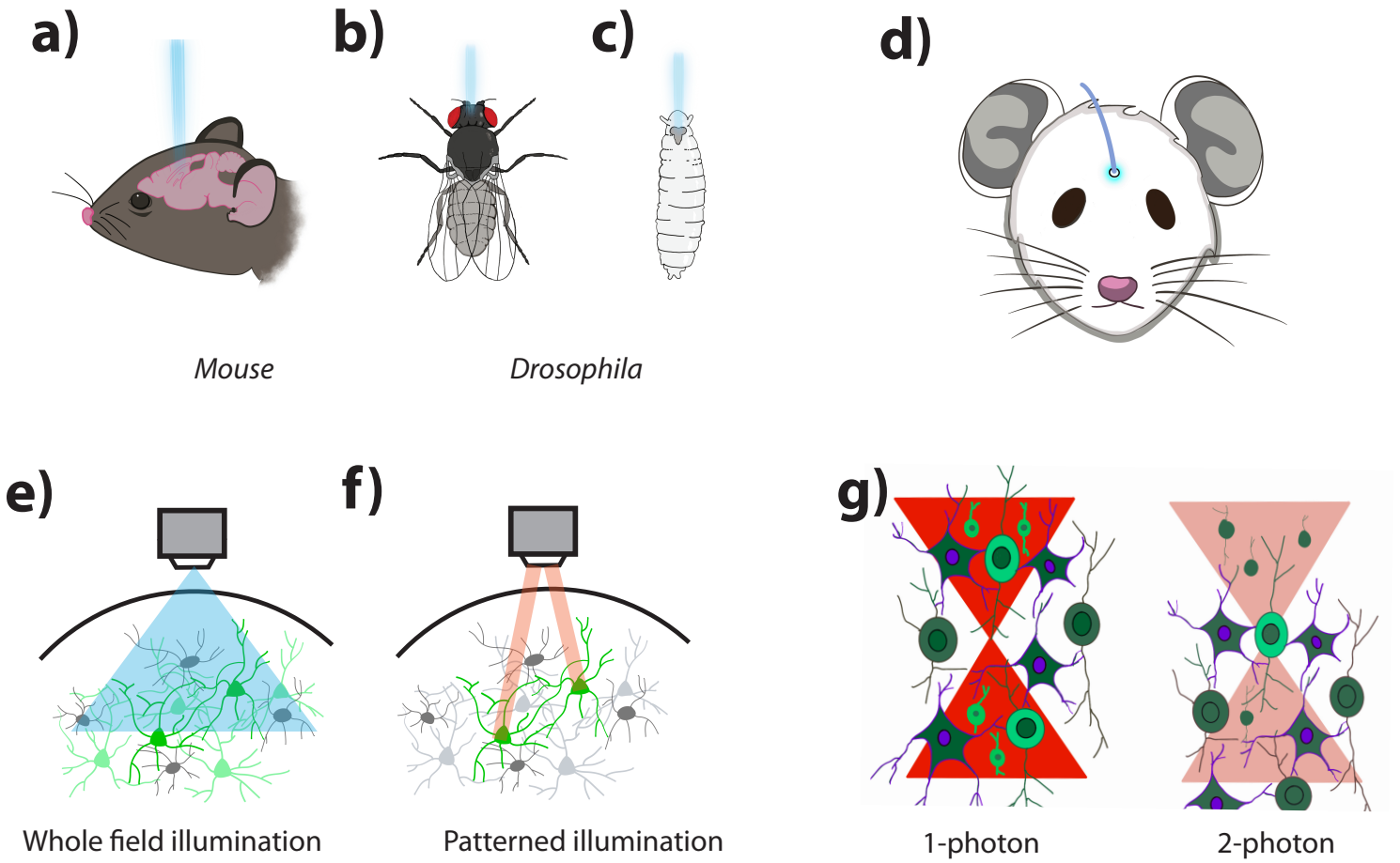
87. Jenkins GI. The UV-B photoreceptor UVR8: from structure to physiology. Plant Cell. 2014;26(1):21-37.
88. Masuda S. Light detection and signal transduction in the BLUF photoreceptors. Plant Cell Physiol. 2013;54(2):171-9.
89. Kim B, Lin MZ. Optobiology: optical control of biological processes via protein engineering. Biochem Soc Trans. 2013;41(5):1183-8.
90. Johr R, Bauer MS, Schendel LC, Kluger C, Gaub HE. Dronpa: A Light-Switchable Fluorescent Protein for Opto-Biomechanics. Nano Lett. 2019;19(5):3176-81.
91. Glantz ST, Carpenter EJ, Melkonian M, Gardner KH, Boyden ES, Wong GK, et al. Functional and topological diversity of LOV domain photoreceptors. Proc Natl Acad Sci U S A. 2016;113(11):E1442-51.
92. Zoltowski BD, Crane BR. Light activation of the LOV protein vivid generates a rapidly exchanging dimer. Biochemistry. 2008;47(27):7012-9.
93. Nash AI, McNulty R, Shillito ME, Swartz TE, Bogomolni RA, Luecke H, et al. Structural basis of photosensitivity in a bacterial light-oxygen-voltage/helix-turn-helix (LOV-HTH) DNA-binding protein. Proc Natl Acad Sci U S A. 2011;108(23):9449-54.
94. Bugaj LJ, Choksi AT, Mesuda CK, Kane RS, Schaffer DV. Optogenetic protein clustering and signaling activation in mammalian cells. Nat Methods. 2013;10(3):249-52.
95. Kaberniuk AA, Shemetov AA, Verkhusha VV. A bacterial phytochrome-based optogenetic system controllable with near-infrared light. Nat Methods. 2016;13(7):591-7.
96. Redchuk TA, Omelina ES, Chernov KG, Verkhusha VV. Near-infrared optogenetic pair for protein regulation and spectral multiplexing. Nat Chem Biol. 2017;13(6):633-9.
97. Demarsy E, Fankhauser C. Higher plants use LOV to perceive blue light. Curr Opin Plant Biol. 2009;12(1):69-74.
98. Kawano F, Suzuki H, Furuya A, Sato M. Engineered pairs of distinct photoswitches for optogenetic control of cellular proteins. Nat Commun. 2015;6:6256.
99. Benedetti L, Marvin JS, Falahati H, Guillen-Samander A, Looger LL, De Camilli P. Optimized Vivid-derived Magnets photodimerizers for subcellular optogenetics in mammalian cells. Elife. 2020;9: e63230.
100. Guntas G, Hallett RA, Zimmerman SP, Williams T, Yumerefendi H, Bear JE, et al. Engineering an improved light-induced dimer (iLID) for controlling the localization and activity of signaling proteins. Proc Natl Acad Sci U S A. 2015;112(1):112-7.
101. Strickland D, Lin Y, Wagner E, Hope CM, Zayner J, Antoniou C, et al. TULIPs: tunable, light-controlled interacting protein tags for cell biology. Nat Methods. 2012;9(4):379-84.
102. Hallett RA, Zimmerman SP, Yumerefendi H, Bear JE, Kuhlman B. Correlating in Vitro and in Vivo Activities of Light-Inducible Dimers: A Cellular Optogenetics Guide. ACS Synth Biol. 2016;5(1):53-64.
103. Benedetti L, Barentine AES, Messa M, Wheeler H, Bewersdorf J, De Camilli P. Light-activated protein interaction with high spatial subcellular confinement. Proc Natl Acad Sci U S A. 2018;115(10):E2238-E45.
104. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker CL. Rapid blue-light-mediated induction of protein interactions in living cells. Nat Methods. 2010;7(12):973-5.
105. Nihongaki Y, Kawano F, Nakajima T, Sato M. Photoactivatable CRISPR-Cas9 for optogenetic genome editing. Nat Biotechnol. 2015;33(7):755-60.

106. Han T, Chen Q, Liu H. Engineered Photoactivatable Genetic Switches Based on the Bacterium Phage T7 RNA Polymerase. ACS Synth Biol. 2017;6(2):357-66.
107. Polstein LR, Gersbach CA. Light-inducible spatiotemporal control of gene activation by customizable zinc finger transcription factors. J Am Chem Soc. 2012;134(40):16480-3.
108. Chen X, Wu YW. Tunable and Photoswitchable Chemically Induced Dimerization for Chemo-optogenetic Control of Protein and Organelle Positioning. Angew Chem Int Ed Engl. 2018;57(23):6796-9.
109. Liu Q, Sinnen BL, Boxer EE, Schneider MW, Grybko MJ, Buchta WC, et al. A Photoactivatable Botulinum Neurotoxin for Inducible Control of Neurotransmission. Neuron. 2019;101(5):863-75 e6.
110. Zhang W, Lohman AW, Zhuravlova Y, Lu X, Wiens MD, Hoi H, et al. Optogenetic control with a photocleavable protein, PhoCl. Nat Methods. 2017;14(4):391-4.
111. Wang H, Vilela M, Winkler A, Tarnawski M, Schlichting I, Yumerefendi H, et al. LOVTRAP: an optogenetic system for photoinduced protein dissociation. Nat Methods. 2016;13(9):755-8.
112. Harper SM, Neil LC, Gardner KH. Structural basis of a phototropin light switch. Science. 2003;301(5639):1541-4.
113. Wu YI, Frey D, Lungu OI, Jaehrig A, Schlichting I, Kuhlman B, et al. A genetically encoded photoactivatable Rac controls the motility of living cells. Nature. 2009;461(7260):104-8.
114. Lee J, Natarajan M, Nashine VC, Socolich M, Vo T, Russ WP, et al. Surface sites for engineering allosteric control in proteins. Science. 2008;322(5900):438-42.
115. Möglich A, Ayers RA, Moffat K. Design and signaling mechanism of light-regulated histidine kinases. J Mol Biol. 2009;385(5):1433-44.
116. Duan L, Che D, Zhang K, Ong Q, Guo S, Cui B. Optogenetic control of molecular motors and organelle distributions in cells. Chem Biol. 2015;22(5):671-82.
117. Nakamura M, Chen L, Howes SC, Schindler TD, Nogales E, Bryant Z. Remote control of myosin and kinesin motors using light-activated gearshifting. Nat Nanotechnol. 2014;9(9):693-7.
118. Moon J, Gam J, Lee SG, Suh YG, Lee J. Light-regulated tetracycline binding to the Tet repressor. Chemistry. 2014;20(9):2508-14.
119. Fukuda N, Matsuda T, Nagai T. Optical control of the Ca2+ concentration in a live specimen with a genetically encoded Ca2+-releasing molecular tool. ACS Chem Biol. 2014;9(5):1197-203.
120. Schmidt D, Tillberg PW, Chen F, Boyden ES. A fully genetically encoded protein architecture for optical control of peptide ligand concentration. Nat Commun. 2014;5:3019.
121. Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, et al. A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol. 2011;9(4):e1001041.
122. Lin JY, Sann SB, Zhou K, Nabavi S, Proulx CD, Malinow R, et al. Optogenetic inhibition of synaptic release with chromophore-assisted light inactivation (CALI). Neuron. 2013;79(2):241-53.
123. Gil AA, Carrasco-Lopez C, Zhu L, Zhao EM, Ravindran PT, Wilson MZ, et al. Optogenetic control of protein binding using light-switchable nanobodies. Nat Commun. 2020;11(1):4044.

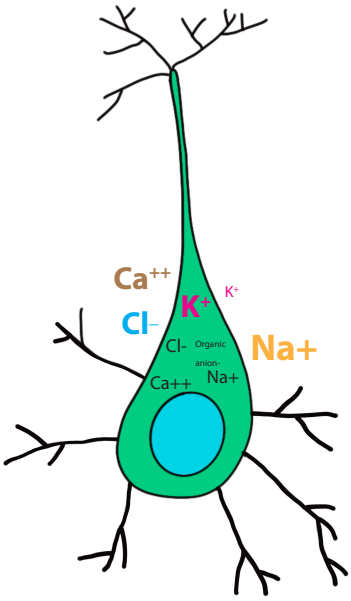
124. Grusch M, Schelch K, Riedler R, Reichhart E, Differ C, Berger W, et al. Spatio-temporally precise activation of engineered receptor tyrosine kinases by light. EMBO J. 2014;33(15):1713-26.
125. Strickland D, Moffat K, Sosnick TR. Light-activated DNA binding in a designed allosteric protein. Proc Natl Acad Sci U S A. 2008;105(31):10709-14.
126. Wang W, Wildes CP, Pattarabanjird T, Sanchez MI, Glober GF, Matthews GA, et al. A light- and calcium-gated transcription factor for imaging and manipulating activated neurons. Nat Biotechnol. 2017;35(9):864-71.
127. Lee D, Hyun JH, Jung K, Hannan P, Kwon HB. A calcium- and light-gated switch to induce gene expression in activated neurons. Nat Biotechnol. 2017;35(9):858-63.
128. Chambers JJ, Banghart MR, Trauner D, Kramer RH. Light-induced depolarization of neurons using a modified Shaker K(+) channel and a molecular photoswitch. J Neurophysiol. 2006;96(5):2792-6.
129. Szobota S, Gorostiza P, Del Bene F, Wyart C, Fortin DL, Kolstad KD, et al. Remote control of neuronal activity with a light-gated glutamate receptor. Neuron. 2007;54(4):535-45.
130. Caporale N, Kolstad KD, Lee T, Tochitsky I, Dalkara D, Trauner D, et al. LiGluR restores visual responses in rodent models of inherited blindness. Mol Ther. 2011;19(7):1212-9.
131. Lin WC, Davenport CM, Mourot A, Vytla D, Smith CM, Medeiros KA, et al. Engineering a light-regulated GABAA receptor for optical control of neural inhibition. ACS Chem Biol. 2014;9(7):1414-9.
132. Tochitsky I, Banghart MR, Mourot A, Yao JZ, Gaub B, Kramer RH, et al. Optochemical control of genetically engineered neuronal nicotinic acetylcholine receptors. Nat Chem. 2012;4(2):105-11.
133. Kienzler MA, Isacoff EY. Precise modulation of neuronal activity with synthetic photoswitchable ligands. Curr Opin Neurobiol. 2017;45:202-9.
134. Levitz J, Pantoja C, Gaub B, Janovjak H, Reiner A, Hoagland A, et al. Optical control of metabotropic glutamate receptors. Nat Neurosci. 2013;16(4):507-16.
135. Cosentino C, Alberio L, Gazzarrini S, Aquila M, Romano E, Cermenati S, et al. Optogenetics. Engineering of a light-gated potassium channel. Science. 2015;348(6235):707-10.
136. Alberio L, Locarno A, Saponaro A, Romano E, Bercier V, Albadri S, et al. A light-gated potassium channel for sustained neuronal inhibition. Nat Methods. 2018;15(11):969-76.
137. Beck S, Yu-Strzelczyk J, Pauls D, Constantin OM, Gee CE, Ehmann N, et al. Synthetic Light-Activated Ion Channels for Optogenetic Activation and Inhibition. Front Neurosci. 2018;12:643.
138. Lima SQ, Hromadka T, Znamenskiy P, Zador AM. PINP: a new method of tagging neuronal populations for identification during in vivo electrophysiological recording. PLoS One. 2009;4(7):e6099.
139. Petreanu L, Huber D, Sobczyk A, Svoboda K. Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. Nat Neurosci. 2007;10(5):663-8.
140. Miyashita T, Shao YR, Chung J, Pourzia O, Feldman D. Long-term channelrhodopsin-2 (ChR2) expression can induce abnormal axonal morphology and targeting in cerebral cortex. Frontiers in neural circuits. 2013;7:8.
141. Tojima T, Hines JH, Henley JR, Kamiguchi H. Second messengers and membrane trafficking direct and organize growth cone steering. Nat Rev Neurosci. 2011;12(4):191-203.

142. Patterson M, Yasuda R. Signalling pathways underlying structural plasticity of dendritic spines. Br J Pharmacol. 2011;163(8):1626-38.
143. Zimmermann D, Zhou A, Kiesel M, Feldbauer K, Terpitz U, Haase W, et al. Effects on capacitance by overexpression of membrane proteins. Biochem Biophys Res Commun. 2008;369(4):1022-6.
144. Feldbauer K, Zimmermann D, Pintschovius V, Spitz J, Bamann C, Bamberg E. Channelrhodopsin-2 is a leaky proton pump. Proc Natl Acad Sci U S A. 2009;106(30):12317-22.
145. Picot A, Dominguez S, Liu C, Chen I-W, Tanese D, Ronzitti E, et al. Temperature rise under two-photon optogenetic brain stimulation. Cell reports. 2018;24(5):1243-53. e5.
146. Cardozo Pinto DF, Lammel S. Hot topic in optogenetics: new implications of in vivo tissue heating. Nat Neurosci. 2019;22(7):1039-41.
147. Owen SF, Liu MH, Kreitzer AC. Thermal constraints on in vivo optogenetic manipulations. Nature neuroscience. 2019;22(7):1061-5.
148. Hirase H, Nikolenko V, Goldberg JH, Yuste R. Multiphoton stimulation of neurons. J Neurobiol. 2002;51(3):237-47.
149. Palumbo G, Caruso M, Crescenzi E, Tecce MF, Roberti G, Colasanti A. Targeted gene transfer in eucaryotic cells by dye-assisted laser optoporation. J Photochem Photobiol B. 1996;36(1):41-6.
150. Khan I, Tang E, Arany P. Molecular pathway of near-infrared laser phototoxicity involves ATF-4 orchestrated ER stress. Sci Rep. 2015;5:10581.
151. Cheng KP, Kiernan EA, Eliceiri KW, Williams JC, Watters JJ. Blue light modulates murine microglial gene expression in the absence of optogenetic protein expression. Scientific reports. 2016;6(1):1-11.
152. Rungta RL, Osmanski BF, Boido D, Tanter M, Charpak S. Light controls cerebral blood flow in naive animals. Nat Commun. 2017;8:14191.
153. Grossman N, Simiaki V, Martinet C, Toumazou C, Schultz SR, Nikolic K. The spatial pattern of light determines the kinetics and modulates backpropagation of optogenetic action potentials. J Comput Neurosci. 2013;34(3):477-88.
154. Lafferty CK, Britt JP. Off-Target Influences of Arch-Mediated Axon Terminal Inhibition on Network Activity and Behavior. Front Neural Circuits. 2020;14:10.
155. Vila CH, Williamson RS, Hancock KE, Polley DB. Optimizing optogenetic stimulation protocols in auditory corticofugal neurons based on closed-loop spike feedback. J Neural Eng. 2019;16(6):066023.
156. Perny M, Muri L, Dawson H, Kleinlogel S. Chronic activation of the D156A point mutant of Channelrhodopsin-2 signals apoptotic cell death: the good and the bad. Cell Death Dis. 2016;7(11):e2447.
157. Li N, Chen S, Guo ZV, Chen H, Huo Y, Inagaki HK, et al. Spatiotemporal constraints on optogenetic inactivation in cortical circuits. Elife. 2019;8: e48622.
158. Octeau JC, Gangwani MR, Allam SL, Tran D, Huang S, Hoang-Trong TM, et al. Transient, consequential increases in extracellular potassium ions accompany channelrhodopsin2 excitation. Cell reports. 2019;27(8):2249-61. e7.
159. Li T, Yang Y, Canessa CM. A method for activation of endogenous acid-sensing ion channel 1a (ASIC1a) in the nervous system with high spatial and temporal precision. J Biol Chem. 2014;289(22):15441-8.

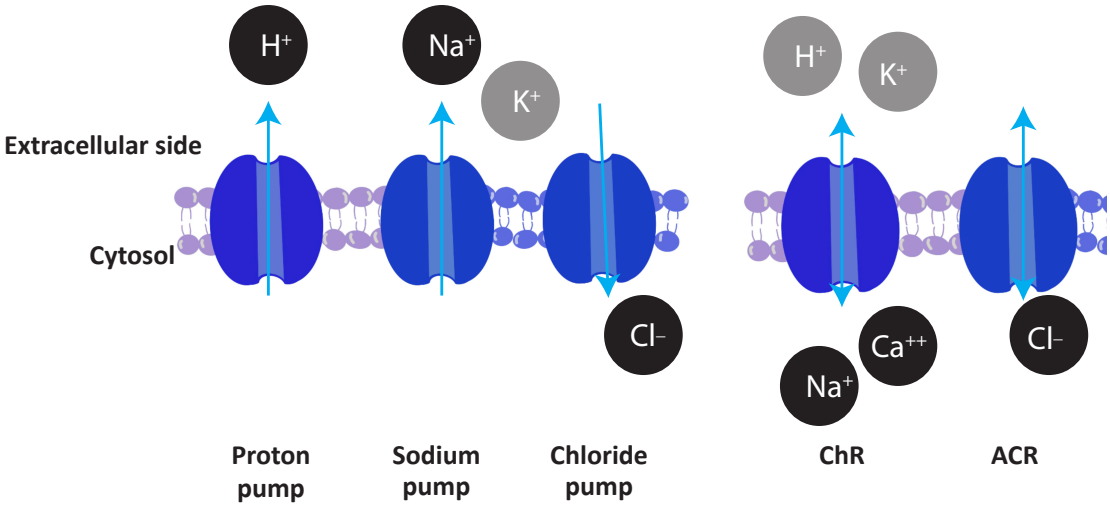
160. Poskanzer KE, Yuste R. Astrocytes regulate cortical state switching in vivo. Proceedings of the National Academy of Sciences. 2016;113(19):E2675-E84.
161. Chater TE, Henley JM, Brown JT, Randall AD. Voltage- and temperature-dependent gating of heterologously expressed channelrhodopsin-2. J Neurosci Methods. 2010;193(1):7-13.
162. Schneider F, Grimm C, Hegemann P. Biophysics of Channelrhodopsin. Annu Rev Biophys. 2015;44:167-86.
163. Jun NY, Cardin JA. Activation of Distinct Channelrhodopsin Variants Engages Different Patterns of Network Activity. eNeuro. 2020;7(1).
164. Adesnik H, Bruns W, Taniguchi H, Huang ZJ, Scanziani M. A neural circuit for spatial summation in visual cortex. Nature. 2012;490(7419):226-31.
165. Levesque M, Chen LY, Etter G, Shiri Z, Wang S, Williams S, et al. Paradoxical effects of optogenetic stimulation in mesial temporal lobe epilepsy. Ann Neurol. 2019;86(5):714-28.
166. Mahrach A, Chen G, Li N, van Vreeswijk C, Hansel D. Mechanisms underlying the response of mouse cortical networks to optogenetic manipulation. Elife. 2020;9: e49967.
167. Mahn M, Prigge M, Ron S, Levy R, Yizhar O. Biophysical constraints of optogenetic inhibition at presynaptic terminals. Nat Neurosci. 2016;19(4):554-6.
168. Seybold BA, Phillips EAK, Schreiner CE, Hasenstaub AR. Inhibitory Actions Unified by Network Integration. Neuron. 2015;87(6):1181-92.
169. Zhu H, Roth BL. DREADD: a chemogenetic GPCR signaling platform. Int J Neuropsychopharmacol. 2014;18(1).
170. Chen S, Weitemier AZ, Zeng X, He L, Wang X, Tao Y, et al. Near-infrared deep brain stimulation via upconversion nanoparticle-mediated optogenetics. Science. 2018;359(6376):679-84.
171. Allen BD, Singer AC, Boyden ES. Principles of designing interpretable optogenetic behavior experiments. Learn Mem. 2015;22(4):232-8.
172. Wolff SB, Ölveczky BP. The promise and perils of causal circuit manipulations. Curr Opin Neurobiol. 2018;49:84-94.
173. Otchy TM, Wolff SB, Rhee JY, Pehlevan C, Kawai R, Kempf A, et al. Acute off-target effects of neural circuit manipulations. Nature. 2015;528(7582):358-63.
174. Phillips EA, Hasenstaub AR. Asymmetric effects of activating and inactivating cortical interneurons. Elife. 2016;5: e18383.
175. Moore AK, Weible AP, Balmer TS, Trussell LO, Wehr M. Rapid Rebalancing of Excitation and Inhibition by Cortical Circuitry. Neuron. 2018;97(6):1341-55 e6.
176. Südhof TC. Reproducibility: Experimental mismatch in neural circuits. Nature. 2015;528(7582):338-9.
177. Guru A, Post RJ, Ho YY, Warden MR. Making Sense of Optogenetics. Int J Neuropsychopharmacol. 2015;18(11):pyv079.
178. Kravitz AV, Bonci A. Optogenetics, physiology, and emotions. Front Behav Neurosci. 2013;7:169.


a)
b)
c)
d)
Mouse
Drosophila
e)
f)
g)
Whole field illumination
Patterned illumination
1-photon
2-photon
h)

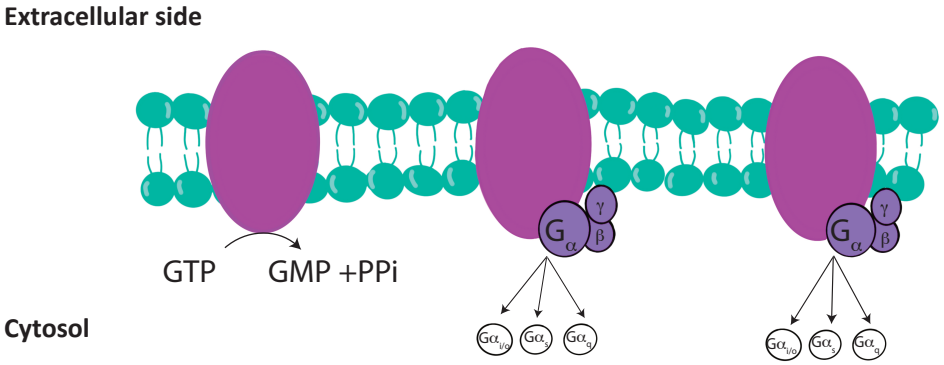
a)


Ca++
K+
K+
Cl-
Cl-
Organic anion-
Na+
Ca++
Na+

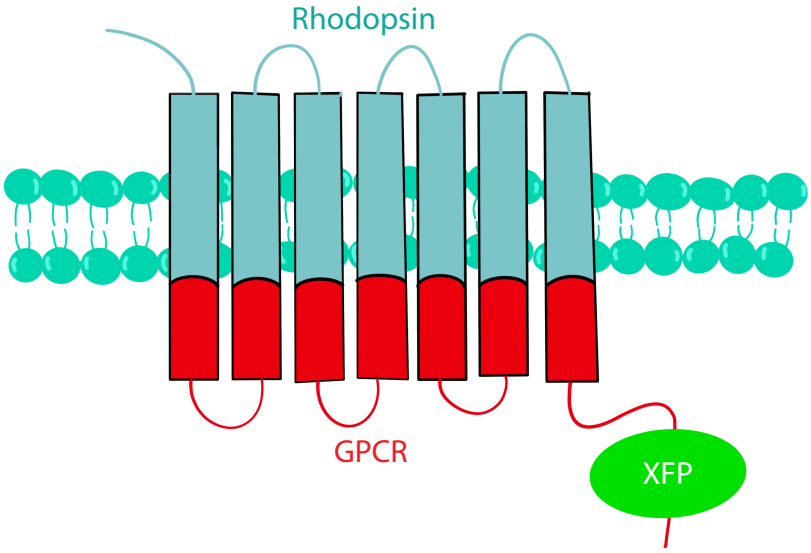
b)


H+
Na+
K+
Extracellular side
Cytosol
Cl-
H+
K+
Ca++
Na+
Cl-
Proton pump
Sodium pump
Chloride pump
ChR
ACR

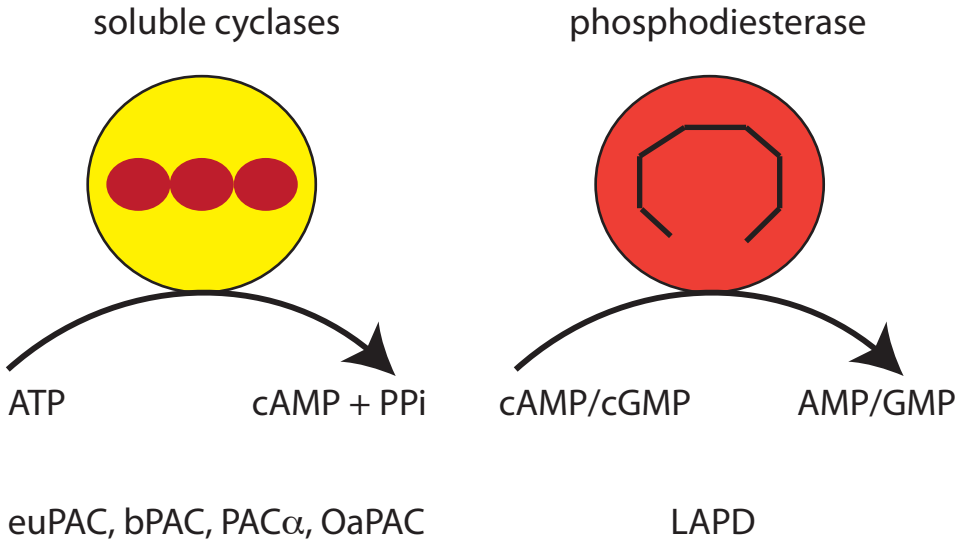
a)


Extracellular side
GTP
GMP +PPi
Cytosol

b)

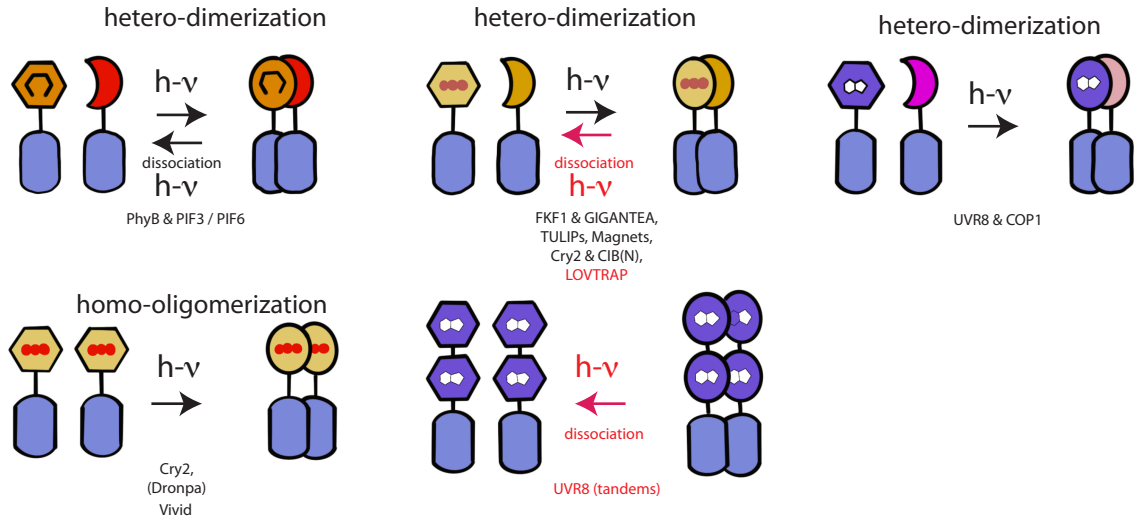

Rhodopsin
GPCR
XFP

c)


soluble cyclases
phosphodiesterase
ATP
cAMP + PPi
cAMP/cGMP
AMP/GMP
euPAC, bPAC, PACα, OaPAC
LAPD

a)

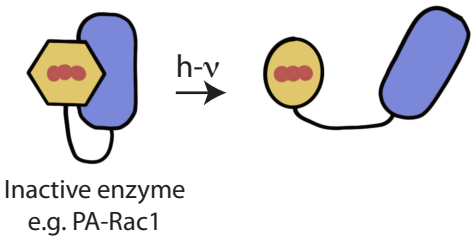
Dimerizers

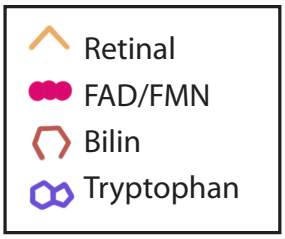


b)

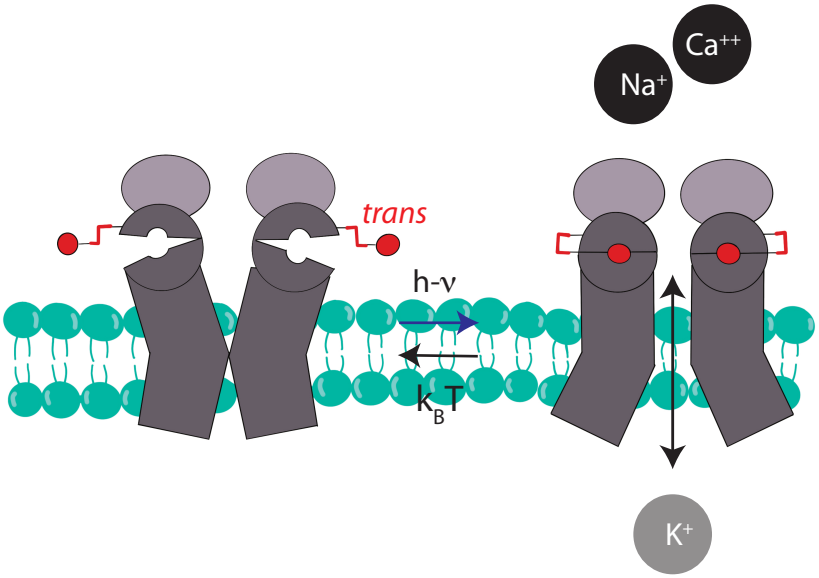
Allosteric modulation

Allosteric activation by LOV2 domain

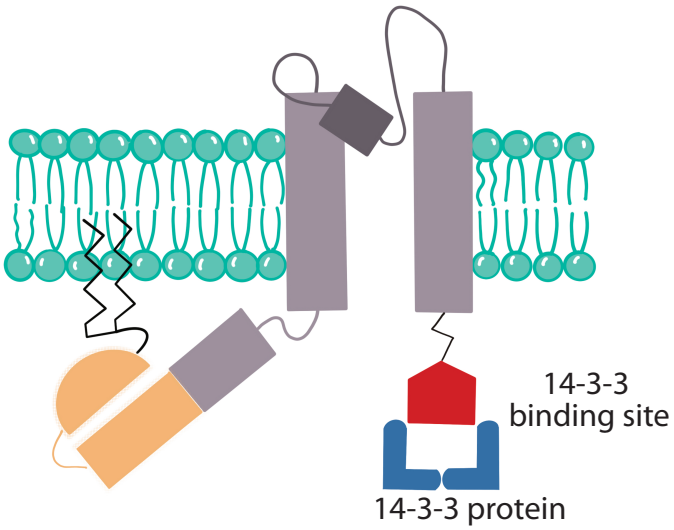




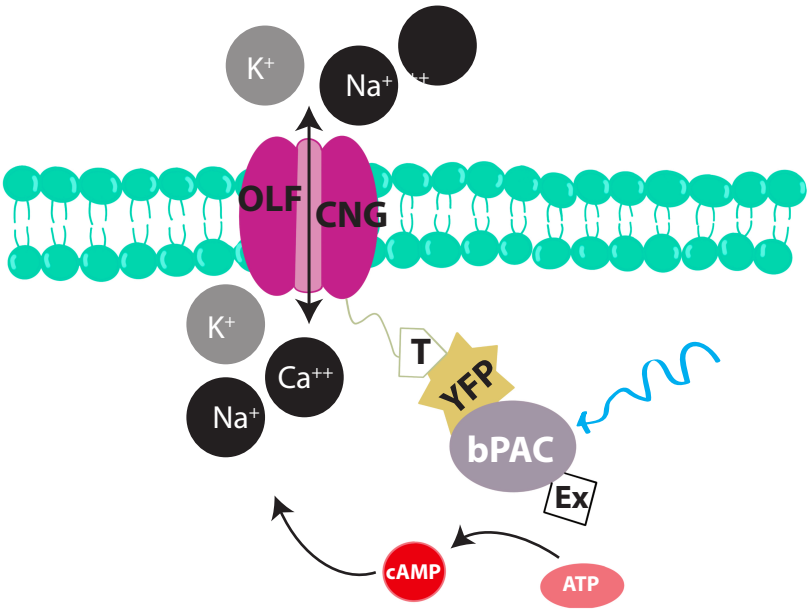
a)


Ca++
Na+
trans
h-ν
K_BT
K+

b)


14-3-3
binding site
14-3-3 protein

c)


K+
Na+
OLF
CNG
K+
Ca++
Na+
T
YFP
bPAC
Ex
cAMP
ATP