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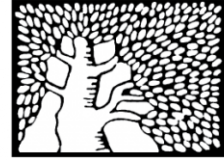

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# Optogenetics for light control of biological systems

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## Abstract:

Optogenetic techniques have been developed to allow control over the activity of selected cells within a highly heterogeneous tissue, using a combination of genetic engineering and light. Optogenetics employs natural and engineered photoreceptors, mostly of microbial origin, to be genetically introduced into the cells of interest. As a result, cells that are naturally light-*in*sensitive can be made photosensitive and addressable by illumination and precisely controllable in time and space. The selectivity of expression and subcellular targeting in the host is enabled by applying control elements such as promoters, enhancers and specific targeting sequences to the employed photoreceptor-encoding DNA. This powerful approach allows precise characterization and manipulation of cellular functions and has motivated the development of advanced optical methods for patterned photostimulation. Optogenetics has revolutionized neuroscience during the past 15 years and is primed to have a similar impact in other fields, including cardiology, cell biology and plant sciences. In this Primer we describe the principles of optogenetics, review the most commonly used optogenetic tools, illumination approaches and scientific applications and discuss the possibilities and limitations associated with optogenetic manipulations across a wide variety of optical techniques, cells, circuits and organisms.


## Introduction

Light-dependent processes are abundant in nature, occurring in diverse organisms from bacteria and algae to plants and animals and are used for energy capture and storage, to regulate developmental processes and to mediate orientation[1-3]. While the photoreceptors involved in light-sensing have been studied for decades, the use of such proteins for actuation of naturally light-insensitive cells began only in 2002 with the expression of the Drosophila rhodopsin and its associated signaling proteins in neurons[4]. The discovery of channelrhodopsin, identified in the same year in the green alga Chlamydomonas, in conjunction with the almost universal cellular availability of the chromophore all-trans retinal (Vitamin A) in most cells and organisms, accelerated the progress of this new technology. Almost in parallel with the initial application of Channelrhodopsin-2 (ChR2) in isolated

neurons in 2005[5] and brain slices in 2006[6], ChRs were rapidly adapted for use in living model organisms, including chicken embryos[7] and *C.elegans* in 2005[8], Drosophila in 2006 [9], freely moving mice in 2007[10], zebrafish in 2008[11] and even non-human primates in 2009[12]. The first experiments that pointed toward potential therapeutic applications were performed in 2006, pioneered by the expression of ChR in inner retinal cells to restore vision in blind mice[13]. Optogenetics is based on sensory photoreceptors sequences from microalgae, fungi or bacteria. But, only the combination of photoreceptor-encoding DNA with control elements like promoters and targeting sequences, typically derived from genes expressed selectively in target, allows the protein allows specificity not only in the choice of target cell population but also in the subcellular compartments to be manipulated. The DNA-constructs are incorporated into target cell populations, tissues or living organisms using vectors such as plasmids, viral vectors, or bacteria usingvia established transformation technologies as illustrated in Figure 1.

The robust function and revolutionary utility of ChR2 for the neuroscience field has led to an interdisciplinary research effort in the subsequent years, leading to a growth of the optogenetic technology at unprecedented pace. This resulted in the description and application of many photoreceptor subtypes, engineered or retrieved from genomic or cDNA databases, progress in protein expression and targeting, microelectrode **[G]** and optrode **[G]** technology, and finally the combination of optogenetic actuators with optical fluorescent reporter systems and high-resolution subcellular imaging, accelerated the interdisciplinary growth of the optogenetic technology with unprecedented pace. The need to control neuronal activity with increased spatial resolution, has in turn motivated the development of advanced optical methods for patterned photostimulation. Digital mirror devices (DMD) or liquid crystal spatial light modulators (LC-SLM) coupled to single or two-photon excitation have enabled in vitro and in vivo single and multi-target excitation with single spike precision and cellular resolution in head-restrained and freely-moving animals[14]. Optogenetics has developed as a basic science methodology for dissecting biological functions. While it has initially been adopted by neuroscientists to study brain function and dysfunction, its use is rapidly expanding into new research fields such as cardiology, microbiology, immunology, parasitology and plant science. These developments are culminating in highly-anticipated clinical applications, as envisioned in the early days of optogenetics, including multiple clinical trials currently in progress for selected human disorders. A crude timeline of key breakthroughs in optogenetic technology is displayed in Figure 2.

With the growth of optogenetic technology came an abundance of tools with diverse functional properties. This Primer is focused predominantly on rhodopsin-based optogenetic tools, which are the most widely used within the growing optogenetic toolbox. While the differences between tools can be subtle, their spectral sensitivity, kinetic properties and ion selectivity can have a major influence on the outcome of an optogenetic experiment. Understanding these features and careful design are therefore crucial for the success and interpretability of optogenetic experiments. As the technology matures and gains popularity across multiple fields of biology, this review aims to provide experimentalists with the most relevant knowledge needed to design, perform, and interpret optogenetic experiments.

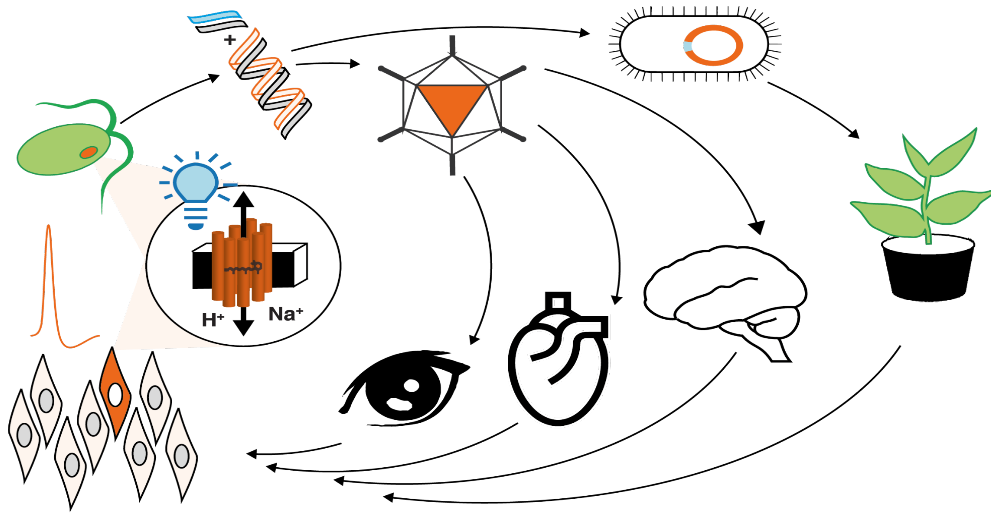


**Figure 1. Principles of Optogenetics**: DNA encoding a sensory photoreceptor derived from a microorganism, plant or animal (orange) is cloned under the regulation of control elements that allow the targeting of specific host cells (blue), packed into a vector such as a viral vector or bacteria and injected into the tissue, organ, or organism of interest. The targeted cell (orange) now expresses the light-sensitive protein and can be controlled with light in a variety of ways, depending on the specific photoreceptor expressed.

## Experimentation

Optogenetic experiments are based on the combination of several fundamental components: (i) a genetically-encoded actuator that after reconstitution with an organic molecule serving as chromophore responds to light and can be used to influence the function of the tool-expressing cell or tissue in a light dependent manner, (ii) a light source providing light at the appropriate wavelength and intensity; and (iii) a light-delivery system, which allows for illumination of targeted cells for temporally-precise activation of the optogenetic actuator. Together, these components allow the experimenter to modulate the biological system and interrogate its function.

### Selection of the right actuator

For the design of an optogenetic experiment, the first considerations should be the cellular parameter to be modulated and the optogenetic actuators **[G]** available for such an endeavor. An enormous number of light-switchable tools have been developed for the control of ion fluxes and membrane voltage, G-protein signaling, regulation of second messengers such as $Ca^{2+}$, cAMP, cGMP, IP3, receptor tyrosine kinases (TRKs), organelle repositioning, transcription, and translation (Fig. 2). Most actuators rely on photoreceptors or light sensing modules of natural origin, although photoswitchable synthetic organic compounds have also been employed. The latter technology has been termed chemooptogenetics or photopharmacology and the interested reader might consult related reviews[15,16]. Many light-moduated actuators have been described that do not rely on opsin proteins. While this review is focused on the opsin-based toolbox, the reader might find more information about non-opsin-based optogenetic tools in several excellent recent reviews[17,18].

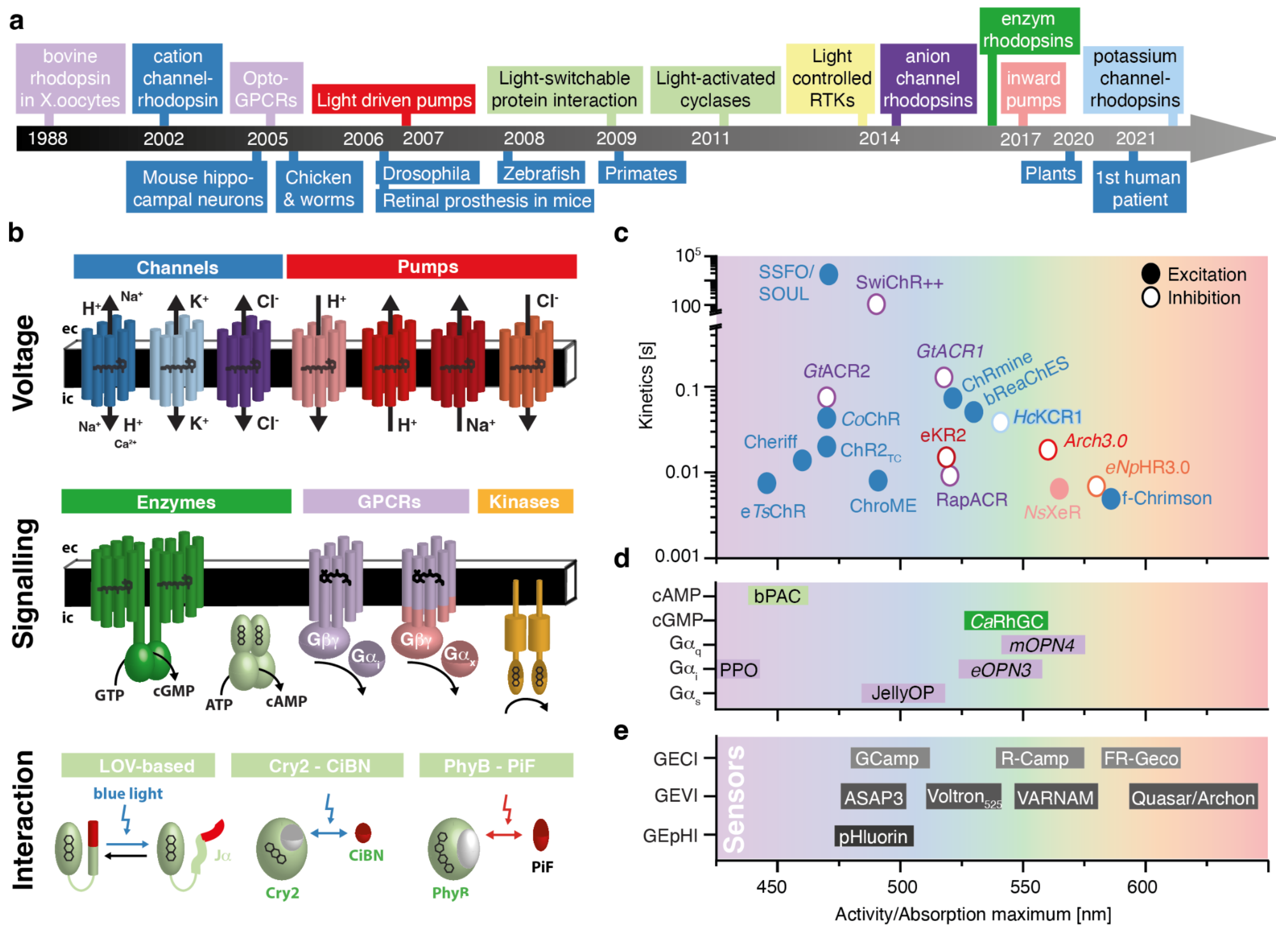


**Figure 2. The optogenetic actuator toolbox**

**a:** Key advances in the development of optogenetic tools. Note that not all available tools are highlighted here, but rather the major developments above the arrow and first application to model organisms including humans exemplified for channelrhodopsins are seen below the arrow.

**b:** Tools for optogenetic manipulation of membrane voltage and local ion concentrations (top), second-messenger-, G-protein- and kinase-signaling (middle) and the light-controlled interaction of photoreceptors with tethered partner proteins for subcellular application (bottom). Light is absorbed in rhodopsins with an *all-trans* or *11-cis* retinal chromophore, in LOV-domains and cryptochromes with flavin mono- or dinucleotide pigments and in phytochromes with a biliverdin tetrapyrrole. LOV-based dimerizers expose a “caged” signaling peptide after light-triggered unfolding of the Jα-Helix[19]. Cryptochrome 2 and Phytochrome B interact with CiBN or PIF-domains after blue or red light absorption respectively[20,21]. **c:** Commonly-used optogenetic tools for excitation or inhibition of neuronal activity, plotted according to their peak excitation wavelength and temporal kinetics. Neurons can be excited by cation conducting ChRs as for example e*Ts*ChR[22], Cheriff[23], *Co*ChR[24], *Cr*ChR2$_{TC}$[25], ChroME[26] and derivatives, SSFO/Soul[27,28], ChRmine[29], bReaChES[30] and f-Chrimson[31] and inward directed proton pumps (e.g. *Ns*XeR[32]) and inhibited by chloride and potassium conducting ChRs (e.g. GtACR1, GtACR2[33] and *Hc*KCR1[34]) as well as outward directed proton, sodium and chloride pumps (e.g. Arch3.0[35], eKR2[36], eNpHR3.0[37]), that differ in

their wavelength of peak activity and their photocurrent off-kinetics. **d:** The soluble enzyme bPAC[38] and the rhodopsin-guanyl-cyclase *Ca*RhGC[39] produce cAMP and cGMP following illumination whereas the non-bleaching opsins mOPN4[40], eOPN3[41], PPO[42] and JellyOP[43] activate different G-protein pathways. **e:** Genetically encoded sensors with diverse excitation spectra (depicted on the x axis) can be used to monitor changes in Ca2+ voltage, and pH, such as GCaMP and R-CaMP[44]and FRCaMP[45] for $Ca^{2+}$, ASAP3[46], Voltron[47], VARNAM[48], Quasar[23] and Archon[49] for voltage, and pHluorin[50] for pH. In experiments combining sensors and actuators, both need to be chosen carefully to minimize optical crosstalk (see Supplementary Figure 3).

## Light-activated ion channels

Until recently the most widely applied optogenetic photoreceptor was Channelrhodopsin-2 from the alga *Chlamydomonas reinhardtii* (named *Cr*ChR2 or simply C2) and its variant ChR2-H134R[8,51]. Today, almost 900 ChR sequences have been identified, including many with properties superior to those of the original prototypes (Figure 2)[52]. ChRs may be subdivided into cation or anion conducting channels, termed CCRs and ACRs respectively. CCRs typically conduct multiple types of cations with high preference for protons. $Na^{+}$ selectivity varies widely among different CCRs[53] and divalent cations are only poorly conducted under most physiological host conditions. Whereas there are no $Ca^{2+}$-selective CCRs available to date, continuous metagenomic screening recently revealed a new class of potassium selective channels, named KCRs[34]. ACRs are selective for a number of anions, similar to most human anion channels[54].

In host cells, $Na^{+}$ and $H^{+}$-conducting CCRs can be used as depolarizing actuators, whereas the action of ACRs depends on the chloride reversal potential in the targeted cells or subcellular compartment (see Box 1). ACRs may clamp the voltage to near the resting potential and inhibit action potential firing by shunting inhibition **[G]**. However, in cardiac cells, immature neurons and presynaptic terminals, chloride gradients are less pronounced and ACRs may depolarize the cell membrane [55,56]. In plants, the chloride gradient is always directed outward, and ACR activation will generally lead to membrane depolarization. Thus far, KCRs have been applied under highly controlled *in vitro* conditions, but - once established for *in vivo* experiments - hold major promise for optogenetic inhibition in all variants of cells and host model systems.

Today, our molecular understanding of ChRs mostly relies on *Cr*ChR2, which has been extensively studied and modified with respect to kinetics, ion selectivity, inactivation, and absorption wavelength [57,58] revealing principles that have been successfully transferred to other CCRs (Supplementary Figure S1). Recently discovered channelrhodopsins such as ChRmine and KCRs hold great promise but

belong to a new family of cation conducting ChRs and the molecular understanding of their mechanism is only beginning to emerge[59,60]. The maximal color sensitivity of known ChRs so far spans from 445 nm for *Ts*ChR to 610 nm for the ChrimsonSA mutant and Ruby-ACR[24,61,62] (Figure 2). Such distinct color sensitivity may allow the combination of different ChRs within the same experiment for activation and inhibition of the same or different cells. However, all rhodopsins absorb blue or UVA light to a certain extent due to transition to higher excited state levels. This has to be taken into consideration when combining multiple rhodopsins in a single or multiple cell populations (Supplementary Figure 3). For bidirectional voltage modulation **[G]** for example, the more potent actuator should be selected to absorb at the shorter wavelength (Figure 2) thereby allowing for lower light powers used in the blue range, which will in turn minimize the undesired activation of the red-shifted actuator. Another consideration is the reversal potential of the conducted ion. In nature, as well as in neuronal experiments, ACRs operate closer to the reversal potential than $Na^+$ or $H^+$ conducting, depolarizing CCRs. While it is possible to co-express two opsins using two separate viral vectors, this approach inevitably leads to incomplete co-expression in all cells. To overcome this draw back several constructs have been engineered which allow tandem expression of two opsins from the same vector. The most prominent examples are eNPAC which coexpresses eNpHR3.0 and ChR2(H134R) initially linked by a 2A selfcleaving peptite[63], and BiPOLES[64], which combines the red-shifted CCR Chrimson with the blue-shifted GtACR2 in a single targeting-optimized fusion contruct[65]. Due to the stoichiometric membrane expression, equal photocurrents near the cellular resting potential and comparable light sensitivities of both channel modules, BiPOLES outperforms previous bicistronic combinations of ChR2 with different ion pumps[59,60] and guarantees subcellular colocalization and selective red-light excitation for multicolor applications. A combination of optogenetics and chemogenetics has been exemplified by direct fusion of slow cycling step function rhodopsins (SFOs) with a luciferase that produces light upon peripheral injection of its small molecule substrate. These luminopsins allow direct light stimulation by optical fibers, while at the same time providing chemogenetic access in awake and anesthetized animals *in vivo*[61,62].

**Light-driven pumps**

The first application of optogenetics for neuronal silencing was achieved with the chloride pump halorhodopsin [66]. However, since the discovery of ACRs, the interest in optogenetic silencing of animal cells by light-driven pumps has decreased in animal cells because pumps require higher

expression levels and higher light intensities for sufficient ion turnover (Supplementary Figure 2). In contrast, in plants — which naturally hyperpolarize their membranes and drive secondary transporters via $H^+$ pumps — light-driven $H^+$ pumps are valuable tools. The advantage of light-driven pumps is their high ion specificity and robust electric response that depends less on the ionic composition of the surrounding buffers and the membrane voltage. Light-driven chloride pumps such as NpHR [67] or Jaws [68] allow reliable — although often weak — neuronal inhibition in synaptic terminals, where the action of ACRs is difficult to predict due to variable and elevated intracellular chloride concentrations [69]. Pumps may be successfully used in small compartments such as neuronal vesicles, lysosomes[70], mitochondria or thylakoids, where the action of ion channels is poorly defined due to the lack of free ions[71]. In the plasma membrane the use of light-driven ion pumps requires caution because both, proton and chloride pumps, can drive non-physiological ion concentrations in neurons and trigger off-target effects, including a transient increase of the chloride reversal potential, leading to excitatory actions of the inhibitory neurotransmitter GABA, and alkalization of presynaptic terminals, leading to increased spontaneous neurotransmission [69,72].

**Optogenetic control of biochemical signaling pathways** Animal rhodopsins are G-protein coupled receptors (GPCRs) and animal vision is the most studied G-protein signaling pathway. Gobind Khorana and colleagues demonstrated in a pioneering study that bovine rhodopsin expression may be used to activate G-protein signaling in Xenopus oocytes but without describing the signaling mechanism[73]. However, the off-response of rod-rhodopsins remained uncontrollable in the absence of rhodopsin kinase and Arrestin, and responses severely declined upon repetitive stimulation. The responses of $G_{i/o}$ activating cone rhodopsins [74-76] or Gs-specific box jellyfish opsins [43] declined faster, but were still not tightly controllable. The solution was approached by revitalizing melanopsin OPN4, which can be switched on and off with blue and yellow light, albeit incompletely due to substantial overlapping spectra of the dark- and signaling- states [40,77,78] and only the UV-sensitive Lamprey Parapinopsin (PPO) with its green-absorbing signaling state offered efficient on and off switching with a dual color light source [41,42,79,80].

However, since GPCR signaling depends on many properties of the receptors, including substrate binding kinetics, G-protein specificity and timing of activation, and receptor inactivation, which in total cannot be fully mimicked by rhodopsins, one way to more selectively mimic the activity of a specific GPCR is to engineer hybrids between structurally related opsins and GPCRs (optoGPCRs)

[81,82]. OptoGPCRs open new and possibly more specific routes for the analysis of intracellular signaling pathways compared to unmodified rhodopsins whereas the dynamics of G-protein coupling and pathway recruitment still has to be approached by testing various expression levels and light regimes. However, these optoGPCRs cannot be simply transferred to another cell type because G-protein promiscuity might activate unwanted pathways [40,83]. With OptoGPCRs the application of G-protein activation has enormously broadened the optogenetic actuator toolbox. These tools will be well-suited for temporally-defined modulation of non-excitable cells, potentially including glial cells in the brain and other non-neuronal cell types [84].

Receptor tyrosine kinases (RTKs) are another large family of cell surface receptors that sense growth factors and hormones to regulate a variety of cellular behaviors by target phosphorylation. Engineered light sensitive epidermal growth factor receptor (EGFR1) and the fibroblast growth factor receptor 1 (FGFR1) have shown robust light activation of both RTK-receptors and cellular signaling in human cancer and endothelial cells and faithful mimicking of complex mitogenic and morphogenic cell behavior [85]. Cobalamin-binding domain (CBD) and tropomyosin receptor kinase B (TrkB) have been fused to RTKs to yield light-sensitive receptors[86,87] . Fusions with TrkB have high specificity for the target proteins, although their application range is narrow and the constructs need to be optimized for every new application. Moreover, one drawback is that cobalamin-based light sensors or phytochrome-based light sensors generally require addition or cellular synthesis of the cofactor molecules, making their potential for in vivo applications more complex than the application of the retinal-based photoreceptors.

**Second messengers**

Photoactivated cyclases (PACs) have been employed for direct control of the second messengers cAMP and cGMP. The soluble bPAC from *Beggiatoa spp*. is a tandem of BLUF-type light sensors (blue-light sensors using FAD (Flavin adenine dinucleotide) (Figure 2) with C-terminal adenylyl cyclases. These optogenetic actuators show millisecond-range *on*-kinetics upon photostimulation and a second-range off-kinetics in the dark (bPAC $\tau_{off}$ = 12 s)[38]. Coexpression of bPAC with the small prokaryotic potassium-channel SthK (PAC-K silencer) in two-component optogenetic approaches has been exploited for long lasting neuronal hyperpolarization in cardiomyocytes as well as in fly, mouse and zebrafish neurons providing high operational light sensitivity but low time resolution [38,88-90].

However, color modification is only possible within a small range around 470 nm and occasional residual dark activity has been observed [91]. New spectral windows were opened by introducing Rhodopsin Guanylyl Cyclases (RGCs), which are cyclases with N-terminally linked rhodopsins. These rhodopsin-cyclases (RhCs) are characterized by low dark-activity, effective light absorption ($\varepsilon > 32000\ M^{-1}cm^{-1}$) and the promise of flexible color tuning [39,92,93]. RhCs show millisecond-range off-kinetics, are naturally GTP selective, and are convertible into ATP cyclases by genetic engineering. Some members of the fungal Chytridiomycota may use heterodimeric RhGCs, with one blue or green sensitive rhodopsin catalyst, and a second near infrared sensitive modulator (NeoR, $\lambda_{max}$ = 660 – 700 nm). These NeoRs might allow to extend the usable spectral range into the superior infrared spectral window [94].

**Protein abundance**

Control over the concentration of selected proteins within a cell has been a long-standing goal, and has stimulated the interest of protein engineers for decades. The most obvious point of intervention is the regulation of transcription. Previously explored concepts were based on the connection of DNA-binding proteins to a photoreceptor such as Phytochrome, FKF1 or VIVID (LOV-proteins), or CRY. Upon illumination, these photoreceptors bind to their signaling partner proteins PIF3, GIGANTEA/Tulips, or CIB respectively, with bound components of the transcription machinery as VP16 or VP64. In light, the transcription component is attracted to the promoter region of interest by photoreceptor and signal-protein interaction leading to the assembly of the transcription complex and initiation of transcription. But, the used GAL4-DNA binding domains have to be incorporated into the model organism (reviewed in[95,96]). To address any promoter of interest, programmable DNA-binding proteins zinc finger-DNA binding proteins[97], TALEs[98] and deactivated Cas9 have been functionalized as the second generation of transcription regulators[99,100]. The main caveat for Cas9 application is the prolonged occupancy of Cas9 at its DNA binding site, especially in situations where the DNA is not cleaved, which disturbs gene expression prior to the intended start of the experiment[101]. Inserting a LOV-domain into an anti-CRISPR protein like AcrIIA4 or AcrIIC3 (CASANOVAs) overcomes this problem and makes Cas9 binding better controllable. This approach works reliably in HEK cells, but has not been rigorously tested for non-embryonic cells such as neurons [104].

**Targeting strategies**

Optogenetics was first applied in neuroscience, driven by the complexity of neural circuits and the demand for improved selectivity in perturbational approaches for studying neural circuits. Genetic techniques, viral vector technology and optical methods have grown rapidly around the developing optogenetic toolbox. As a result, the tools and enabling technologies for optogenetic experimentation in neuroscience, as well as the fundamental understanding of the caveats and constraints of their application, are more advanced in neuroscience than in other fields. In the following section, we review some of the major targeting approaches for expression of optogenetic tools in neural circuits. One of the major benefits of the optogenetic paradigm is its selectivity to defined cells and circuits. In neuroscience applications, genetic targeting of optogenetic tools has advanced considerably, and has profited greatly from developments in viral vector technologies. Since optogenetic tools are genetically-encoded and mostly single-component actuators (requiring the introduction of only one gene to the target cell population), multiple delivery methods can be used to introduce them into the cells of interest. Targeting strategies are either based on promoter specificity directly, or through a combination of a conditional transgene expression cassette that can be switched on or off using a recombinase.

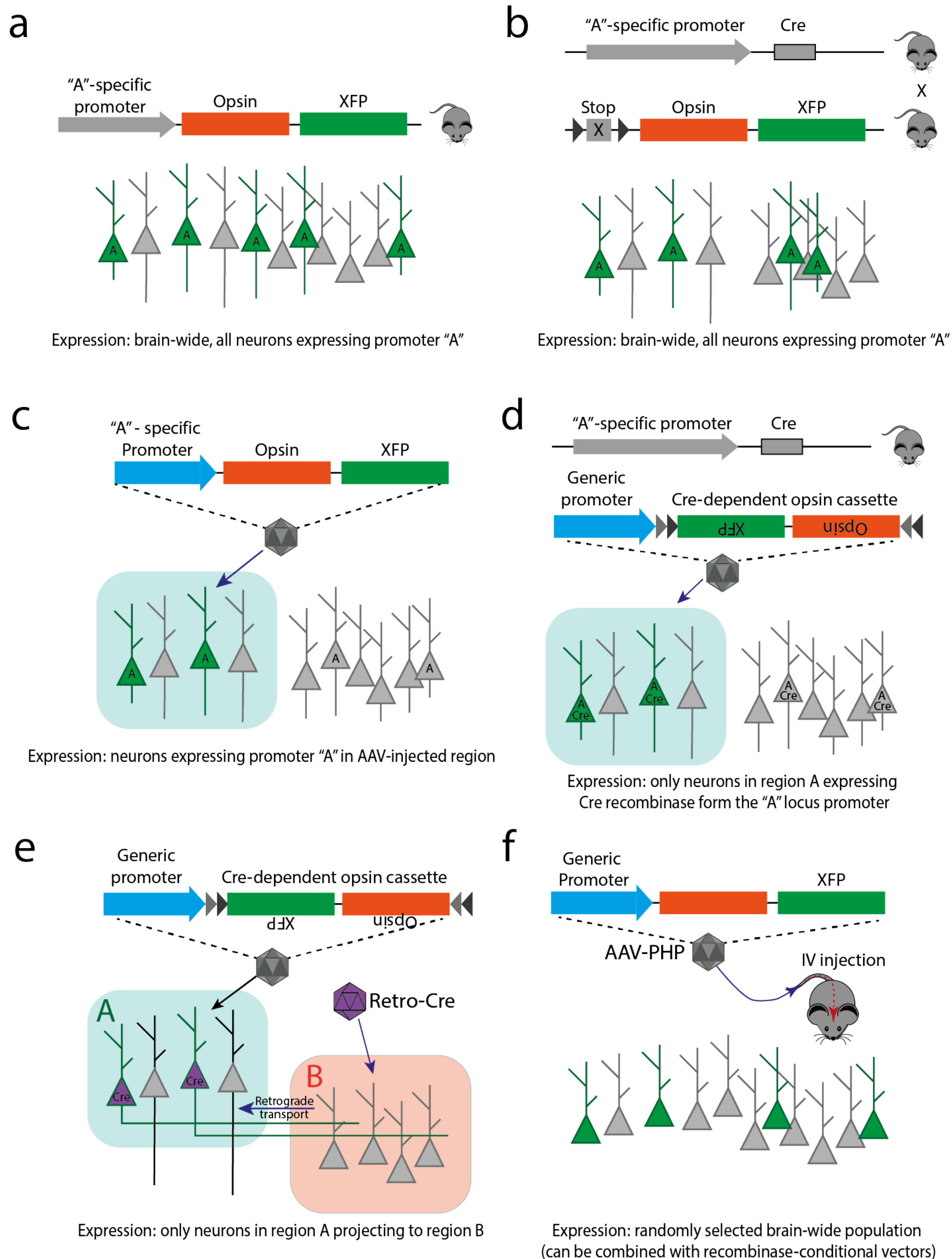


**Figure 3. Cell-type specific targeting of optogenetic tools**. **a**: Transgenic mice constitutively expressing an opsin gene from their genome allow simple experiments that only require the addition of a light-delivery apparatus. **b:** A transgenic animal expressing a recombinase such as Cre under the control of a cell-type specific promoter is crossed with a second line carrying a conditional expression cassette encoding the desired opsin. Dual transgenic offspring will then show

organism wide expression of the opsin in all cells that underwent promoter activation at any stage of development. **c:** Where a short minimal promoter sequence is available, targeted viral vector injections can be utilized to restrict expression spatially as well as by the cell's gene expression profile. A viral vector containing the specific minimal promoter sequence upstream of the opsin gene will lead to expression in specific cells expressing the promoter, only in the region targeted with the injection. **d:** Approaches a and b can be combined to achieve both spatial and gene expression specificity in cases where short specific promoters are not available, or where promoter activity is not specific during development. **e:** Projection neurons can be addressed by injection of an axon terminal-transducing, retrograde traveling viral vector encoding for the opsin or a recombinase into the target region. **f**: AAV capsids engineered for improved blood-brain barrier penetration allow brain-wide (mostly sparse) expression of an opsin through intravenous injection of the viral vector.

## Transgenic expression of optogenetic tools

Transgenic expression is the simplest approach to implement since it requires only the maintenance of an opsin-expressing animal strain (Figure 3a; [102] or the crossing of two strains of animals:a driver line — engineered to express a recombinase or transcription regulator like Cre and Flpo (in rodents) or a Gal4 driver (in zebrafish) in a particular cell population — and an animal strain expressing a conditional opsin gene under the control of the relevant driver (Figure 3b). The F1 progeny of such a cross will express the opsin gene in all cells in which the driver protein is expressed, and will therefore be amenable to optogenetic manipulation simply by illuminating the targeted brain region. The approach is simple to implement, but one should consider potential caveats, including the presence of axons from neurons in other brain regions, which might be activated along with the cell bodies in the illuminated region. In mice, expression of ChR2 or eNpHR3.0 from the ROSA26 locus [103] can be quite weak and not universally sufficient to drive activity in every neuron subtype. Expression of opsin genes from the TIGER locus [104] showed stronger opsin expression and might therefore be useful for some target neuron populations. However, this approach requires generation and/or breeding of a dedicated animal strain for every targeted neuron population and thus lacks the versatility and cost-efficiency of viral vector-based approaches. Another potential confound is unintentional targeting in some driver lines (see for instance [105], making a verification of driver lines advisable [106].

## Viral vector targeting

Lentiviral or adeno-associated viral vectors (AAVs) can be engineered to encode optogenetic actuators and delivered either directly to the brain parenchyma or through systemic injection to target either specific brain regions or brain-wide populations, respectively. Targeting of genetically-identified neuronal populations is achieved either by using the tissue tropism of the virus serotype and

a cell type-specific promoter or enhancer (Figure. 3c), or by injecting the viral vector into a transgenic recombinase-expressing animal strain (Figure 3d). Promoter-based viral vector targeting is attractive since it does not require the maintenance of a specific animal strain for every target neuron population, and can also be applied in non-genetic models. However, the limited viral payload size — particularly of AAVs — prohibits the use of most native promoters. The list of minimal promoter or enhancer sequences that have been validated to specifically express in defined neuron populations is quite restricted. However, this field is rapidly expanding[107,108] and is further diversified by synthetic approaches [109].

**Circuit-based viral vector targeting**

The most commonly used retrograde viral tracer is AAVretro[110], which can be taken up by presynaptic terminals and travel in retrograde to express at the soma of long-range projecting neurons (Figure 3e). The herpes simplex virus 1 (HSV1) and canine adenovirus 2 (CAV2) both have retrograde targeting abilities, but these are less readily available and have been shown to impair the health of targeted neurons, particularly over longer expression times of weeks to months [111].

**Systemic delivery of AAV-PHP capsids**

Targeting sparse brain-wide populations is beneficial for some experimental configurations. For example, structural imaging of dendritic spines in cortex or excitation of a randomly-selected sparse ensemble in a given brain region. For this purpose, AAV-PHP vectors have been engineered to cross the blood-brain barrier with high efficiency (Figure 3f). The AAV-PHP serotypes allow targeting of diverse central and peripheral nervous system neurons [112,113]. The same capsids can be used with Cre-dependent AAV expression plasmids to allow sparse brain-wide expression in a genetically-defined neuronal subtype. However, the efficiency of AAV-PHP serotypes in crossing of the blood brain barrier can vary in different mouse strains[114].

**Electroporation**

Concentrated DNA can also be injected into the cerebral ventricles followed by in utero electroporation [115-117], enabling the study of neural cell fate determination and migration or cortical layer specific expression.

**Compartment-specific functions**

The effective current resulting from a light-gated channel conductance can vary dramatically due to local ion concentration gradient differences. For neuroscience applications, this is particularly crucial for use of ACRs. At the somatic and dendritic compartments, this is an advantage, as they can be used for shunting inhibition. In contrast, ACRs can exert excitatory effects in axons and presynaptic terminals, in which the intracellular chloride concentration is higher (Box 1). Ion pumping rhodopsins on the other hand translocate the ion over the membrane in a predetermined direction, which can be an advantage due to the increased control of ion flux. However, the pumping-induced hyperpolarization and elevation in ion concentration can also have side effects, like the alkalinization of presynaptic boutons [118] or an artificial increase in intracellular chloride [72]. Similarly, the effects of G-protein coupled animal rhodopsins on neuronal activity strongly depend on the given second messenger cascade in the local compartment. For instance, in the soma and dendrite, $G_{i/o}$ signaling can activate G protein-coupled inward rectifying potassium (GIRK) channels whereas in the presynaptic compartment the $G_{i/o}$ pathway mainly acts through inhibition of voltage gated calcium channels (VGCC) and cAMP signaling [41].

**Optimization of expression and targeting**

Beyond single channel conductance, one of the main factors determining maximal photocurrent is the number of functional opsin molecules in the membrane, which in turn depends on expression level, protein-folding efficacy, retinal binding affinity, membrane trafficking and protein turnover rate. The expression level of a transgene can be controlled via promoter strength and transgene copy number. The opsin-folding efficacy and protein stability was shown to depend on the availability of retinal[119]. While retinal availability does not seem to be a limiting factor in mammalian tissues, it needs to be routinely supplemented in the food of invertebrate model systems and some cultured cell lines. In plants, the absence of retinal can be compensated for by its synthesis via expression of a bacterial ß-dioxygenase that facilitates rhodopsin expression. A common issue with unmodified opsin expression cassettes is aggregation of the synthetized protein in the endoplasmic reticulum (ER). To overcome this limitation, trafficking motifs involved in transport of membrane proteins along the secretory pathway to the cell surface were utilized to improve plasma membrane targeting (Supplementary Figure 4). The most widely-used trafficking motifs utilized were first described for the potassium channel $K_{ir}2.1$ – these motifs enhance ER export as well as Golgi-to-plasma membrane trafficking

[120], resulting in higher plasma membrane localization and increased photocurrents in animal [121] as well as plant cells [122].

Further optimization of functional expression can be achieved by adjusting the linkers between the opsin and the often co-expressed fluorophore, mutating potential ubiquitination sites, and screening random mutations in the opsin coding sequence [123]. Beyond improved photocurrents, targeting an opsin to a selected subcellular compartment can be used to investigate the function of the chosen compartment, e.g. mitochondria, synaptic vesicles, lysosomes or the endoplasmatic reticulum (Supplementary Figure 4), or to utilize the differential effects of ion channels discussed above. Somatic restriction has been successful in increasing the specificity of single cell stimulation by reducing inadvertent modulation of nearby neurites [26,124-128], as well as in reducing ACR-mediated axonal excitation [55]. Somatic restriction has the added effect of accelerating the effective photocurrent off-kinetics, due to the elimination of photocurrents arising from distal neurites in the illuminated tissue volume, as these are low-pass filtered while traveling along the neurite to the somatic compartment.

While targeting microbial rhodopsins to presynaptic vesicles is feasible[70], enrichment of rhodopsin abundance in the axonal plasma membrane has not been achieved. Cytosolic proteins can be enriched in the axon by mRNA shuttling motifs. However, local rhodopsin translation in the axon has not been successfully applied, potentially due to a lack of transmembrane protein synthesis in the vertebrate axon [129].

## Light delivery techniques

Although the vast majority of advanced light targeting approaches have been developed with the specific applications of neuronal and cardiac optogenetics in mind, these methods are generalizable and are beginning to be applied to other systems[130].Optogenetics is readily applicable to light-accessible preparations such as cultured cells, tissue slices, transparent organisms such as zebrafish larvae or to the cortical surface of the mammalian brain, allowing for extensive flexibility in light delivery. For whole circuit or brain region optogenetics, light needs to reach the target with sufficient irradiance to induce opsin activation. Ideally, light should be guided into the target structure with minimum damage to the tissue. In behaving animals, stimulation should also be conducted with minimal disruption to the measured behavior, limiting implantable weight and tether stiffness. Whole

circuit/region optogenetic stimulation is typically carried out using a multimode optical fiber, guiding the light from the source to the target (Figure 4a,b). Optical fibers targeting a deep brain region can be permanently implanted by attaching a fiberoptic implant to the skull using dental cement. The dimensions of the fiber and its optical properties strongly influence the spatial profile of light reaching the brain. Most commonly, flat-cleaved optical fibers are used. However, the high radiant flux density necessary at the fiber tip to achieve a sufficient irradiance within the targeted volume, can lead to heat-induced changes in neuronal activity and behaviour [131,132]. It is therefore advisable to consider tissue heating when planning the experiment and to use opsin-free light stimulated controls. One approach to minimizing the irradiance required in optogenetic experiments is to maximize the operational light sensitivity of the opsin used (Supplementary Figure 2). Another factor is wavelength, as absorption is higher for shorter wavelengths and therefore the peak temperature increase is lower for longer wavelengths at the same radiant flux density. Increased optical fiber diameter also reduces the peak light power density. However, wider fibers also cause more tissue damage and have a higher chance of illuminating blood vessels, which strongly absorb visible light and thus increase potential heating-related artifacts. This tradeoff can be at least partially mitigated by the use of tapered optical fibers (Figure 4c), which can be used to flexibly illuminate a large brain volume[133].

In these conventional optogenetic experiments, visible light is mostly delivered non-specifically to large tissue regions and genetic targeting strategies are used to express the optogenetic actuator in specific cell types. This approach has enabled tissue function to be mapped with unprecedented anatomical and cell-type specificity. However, widefield illumination synchronously activates or silences entire populations of all opsin-expressing cells, which does not replicate the physiological case: adjacent cells belonging to genetically defined classes have been observed to exhibit divergent activity patterns. To investigate complex population activity patterns, whole-region optogenetics is insufficient. DMD coupled to single-photon excitation have enabled single and multi-target excitation in head-restrained and freely-moving animals and found in situ applications in control of excitation waves underlying cardiac arrythmias[134,135] [136,137]. However, the use of visible light has limited these approaches to superficial brain layers or low scattering samples. Recent developments in opsin engineering, optical microscopy and multiphoton laser source development have given rise to circuit optogenetics[138], which allows modulation of neuronal activity deep in scattering tissue with single-spike precision and single-cell resolution (Figure 4d-f). Specifically, combining variants with enhanced kinetics [24] [31,139,140], higher conductance [139-141], or shifted absorption peaks [24,27,141] with

optimized targeting and expression strategies [26,125-127], enable neuronal control with single-cell, single-spike precision at millisecond temporal resolution and the generation of AP trains with high (50-100 Hz) spiking rates [142,143]. In parallel, advanced optical techniques, based on two-photon (2P) excitation (Box 2) have been developed to precisely guide light through tissue. The small single-channel conductance of commonly used optogenetic actuators such as ChR2 (40-90 fS)[144], and the limited number of channels or pumps recruited within a conventional 2P focal volume, mean that it is generally necessary to use spiral scanning or parallel light shaping using computer generated holography or the generalized phase contrast method (Supplementary Figure 5) combined with temporal focusing (Supplementary Figure 6) [14] to increase the portion of excited membrane [145,146] and to sufficiently depolarize a neuron to firing threshold or effectively silence it. Holographic light multiplexing with spiral scanning [147] or ad hoc spatiotemporal shaping approaches (Supplementary Figure 7) have been used to generate patterned illumination at multiple axially distinct planes [139,148,149]. Multiplexing divides the available laser power between targets and thus requires powerful lasers. Due to the higher peak photon density, amplified low-repetition rate (200 kHz -10 MHz) fiber lasers enable higher rates of 2P absorption compared to titanium:sapphire oscillators (at the same average power) and can therefore be used to reduce the necessary power to generate physiological signals [150]. Additionally, these sources deliver tens of Watts of power, facilitating the simultaneous photostimulation of hundreds of cells throughout $mm^3$ volumes. The combination of these technologies has recently led to the first demonstrations of multi-target neural circuit manipulation [26,140,151].

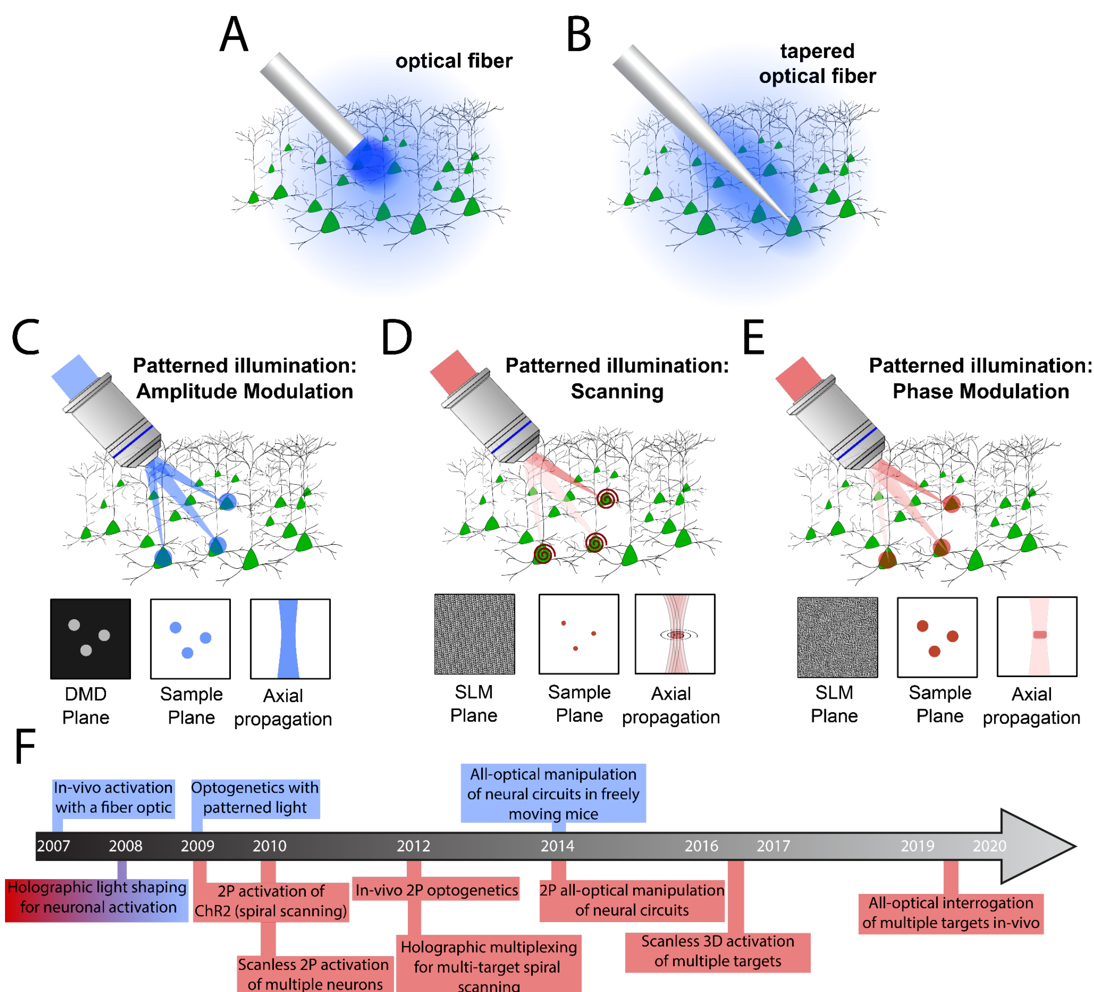


**Figure 4. Optical approaches for optogenetic stimulation. a-b)** Single-photon wide-field illumination (indicated in blue) of all genetically targeted opsin-expressing neurons using excitation through optical fibers. **a)** Illumination using a flat cleaved optical fiber causes high peak light power density at the fiber-tissue interface. **b)** A tapered fiber increases the optical fiber-tissue interface resulting in a reduced peak light power density. **c)** Single-photon multi-target patterned illumination by spatially shaping the intensity of the excitation beam by means of a DMD, placed in a plane conjugated with the sample plane. The light distribution at the DMD plane and at the sample plane only differ by a spatial scaling factor corresponding to the magnification of the optical system. The axial resolution is proportional to the square of the lateral spot dimensions. d-e) Two-photon multi-target illumination by holographic light shaping: **d)** a spatial light modulator (SLM) placed at a plane conjugated with the objective back aperture, generates a 3D distribution of holographic spots which are scanned with a spiral trajectory to cover the cell surface. The axial extension of the generated spot is optimized to cover the upper and lower cell membranes. **e)** An SLM is used to generate multiple extended spots with a size large enough to cover the whole cell soma. A grating for temporal focusing (TF) is used to maintain

micrometer axial resolution independently of the lateral spot size. **f)** A timeline indicating critical optical developments which have enabled new optogenetic experiments throughout the past fifteen years.

The ability to control neuronal activity with single cell precision and millisecond temporal resolution allows to functionally probe neuronal networks beyond the resolution of synchronous modulation of entire networks or genetically defined network components. For instance, using temporally precise single-cell excitation in visual cortex and olfactory bulb, the minimal number of coactivated cortical neurons necessary for visual perception [152] a and the dependence of olfactory perceptual detection on both the number of activated neurons and their relative spiking latency was characterized [153]. The requirement of high numerical aperture objectives has limited 2P-optogenetics to circuits in superficial (≤ 500 µm) cortical areas of mouse brain, transparent zebrafish larvae [154] or in-vitro applications. Micro-endoscopes are small optical probes that can be inserted into living tissues, and represent a promising solution to extend optical brain manipulation to deeper brain structures both in combination with holographic spiral scanning [155] or multi-temporally focused light shaping approaches [156]. Three-photon (3P) optogenetics, which relies on longer wavelengths and exhibits a cubic dependence of excitation efficiency on excitation power, could potentially be used to stimulate neuronal circuits in deeper brain regions (600 µm – 1 mm) with single cell resolution. However, to date, 3P photostimulation has only been demonstrated in vitro [157]. Collectively, these technical advancements have brought optogenetics into a new phase, where scientists can determine how single neurons, or neuronal ensembles, work together in an integrated fashion.

# Results

## Output analysis

When designing optogenetic experiments, one should take care to verify the impact of the optogenetic manipulation on the targeted cells. This can be achieved in a number of ways, including electrophysiological recordings in vitro or in vivo, optical recordings with genetically-encoded sensors, immediate early gene labeling and noninvasive imaging modalities. Below we outline the major techniques used in such experiments, and the considerations that should be taken into account when designing and performing such experiments.

## Electrophysiological recordings

To interpret the results of optogenetic manipulations, it is often necessary to determine the extent of optogenetic tool expression and its physiological effects on the targeted neurons. In the case of light gated ion channels or pumps, recording the electrophysiological changes induced by the optogenetic manipulation is the most direct way to characterize light-mediated effects (Figure 5a-c). As these effects can vary greatly between cell types, brain regions and even viral serotypes [158], it is crucial to validate the optogenetic effector in every new experimental system before proceeding to behavioral or other functional readouts. To describe effects on the level of spike rates and timing, whole-cell recordings are often not necessary. Instead, extracellular recordings are therefore often utilized (Figure 5d-f), given their higher throughput and minimal crosstalk with light delivery (see[159] for discussion of light-induced electrical artifacts). However, one should keep in mind that higher frequency spiking activity does not necessarily indicate increased synaptic transmission from the stimulated neurons[158]. Synaptic depression and depletion of neurotransmitter release can lead to erroneous interpretation and should be taken into account when performing optogenetic excitation experiments, particularly with neuromodulatory and neuropeptide-releasing neuronal populations.

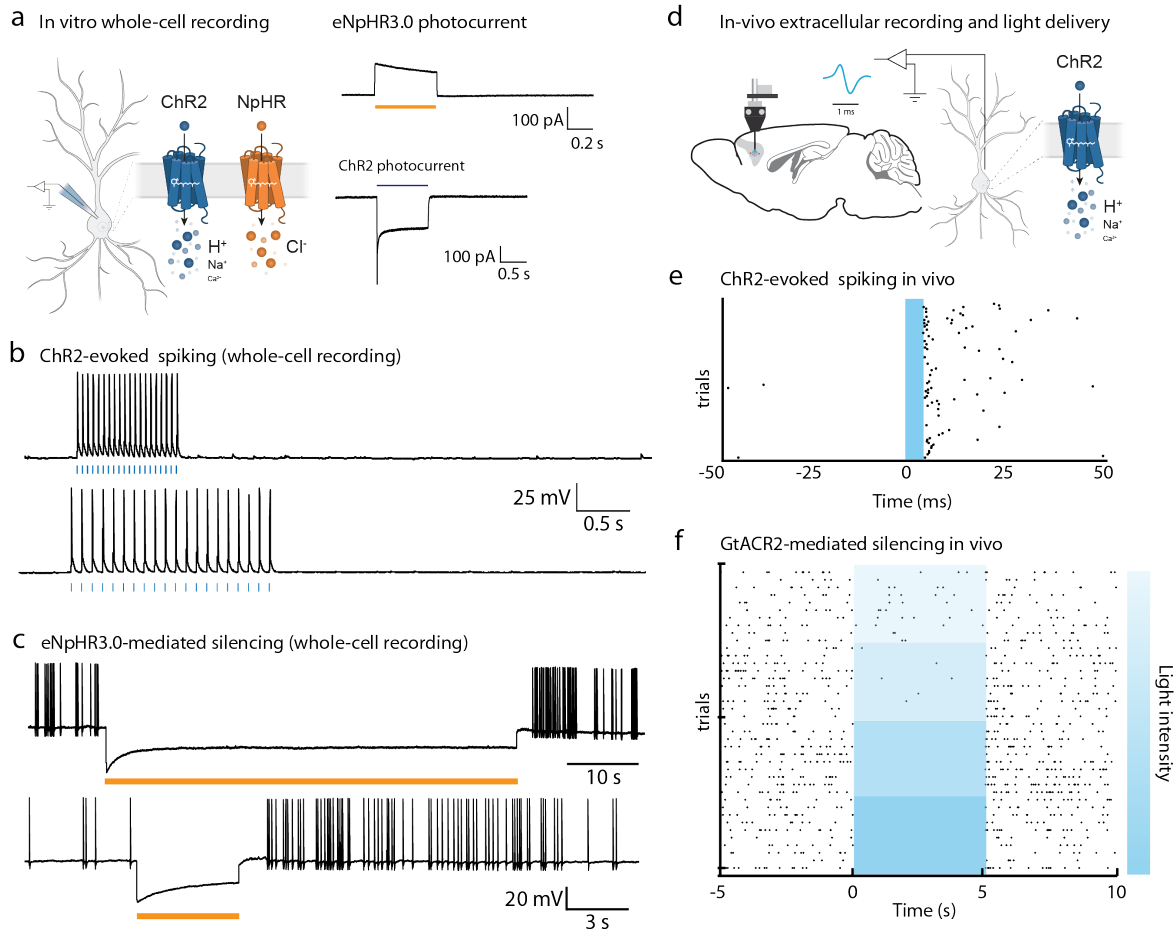


**Figure 5: Expected results in optogenetic experiments**

**a:** Expression of optogenetic actuators such as ChR2 or NpHR in neurons leads to the emergence of light-driven photocurrents, which can be recorded using the whole-cell patch clamp technique (left). Cells expressing the chloride-conducting NpHR will show an outward current (top right, voltage clamp recording with cell resting at -70 mV) while cells expressing the cation-conducting ChR2 will show an inward photocurrent (bottom right, voltage clamp recording with cell resting at -70 mV). **b:** Whole-cell current-clamp recordings in a neuron expressing the excitatory ChR2, showing action potentials evoked by brief light pulses (blue bars). **c:** Hyperpolarization and silencing of spontaneously-occurring action potentials in a neuron expressing eNpHR3.0. **d:** Extracellular recordings, coupled with local light delivery, used to reveal the activity of neurons in vivo, using the awake behaving "optetrode" configuration[160] **e:** Raster plot showing action potentials (represented as black dots) occurring briefly (milliseconds) after a 5-ms blue light pulse delivered into the target brain region. **f:** Raster plot showing the activity of neurons expressing the inhibitory anion-conducting GtACR2, showing increased inhibition of action potential firing with increasing light intensity.

**Optical recordings**

Fluorescent reporters are another common method for monitoring the effects of optogenetic manipulations. These techniques enable recording from the same cells over several recording sessions and the concurrent recording of high numbers of cells. However, given that optogenetics itself relies on light delivery, fluorescent reporters can be efficiently integrated only if a spectral or light power separation can be achieved to minimize the crosstalk between the recording and manipulation modalities (see Supplementary Figure 3). Additionally, when combining red-shifted $Ca^{2+}$ indicators with optogenetic actuators, extra care must be taken, as these can show blue-light-activated photoswitching behavior that can resemble $Ca^{2+}$ activity in their amplitude and kinetics [161].

While genetically encoded calcium sensors continue to be the state of the art in terms of optical activity readout, voltage indicators are gradually reaching a level of maturity that could allow for wider adoption by the field [162]. Novel fluorescent sensors for neurotransmitters, neuromodulators and other small molecules are continuously developed[163-165]. Another approach to read out gross neuronal firing rate changes is to characterize the expression of immediate early genes **[G]** , for instance via immunohistochemistry on the protein level [166] or on the mRNA level using quantitative PCR, in-situ hybridization, or single cell RNA sequencing [167]. Immediate early gene expression can be used to determine the relationship between the modulation of specific neuronal populations and global brain activity [168]. However, the temporal precision of this approach is limited to the average neuronal activity over minutes to hours and, unless combined with targeted recombination approaches [169], only a single manipulation can be characterized per animal.

Although channelrhodopsin variants with peak single-photon (1P) excitation wavelengths spanning the visible region of the electromagnetic spectrum have been engineered [57], performing crosstalk-free, multi-colour 2P experiments is not trivial. Ideally, spectrally orthogonal channelrhodopsins and activity reporters would be chosen, but, unfortunately, the 2P action spectra of commonly used opsins are extremely broad (Supplementary Figure 8) [57]. As previously introduced, opsins with red-shifted action spectra exhibit persistent activation in the blue range ("blue tails"), which coincide with wavelengths used for 2P imaging of commonly used activity reporters (920 – 980 nm). One approach to reduce crosstalk is to use opsins with fast kinetics. Although this approach does not eliminate sub-threshold network perturbation, the (relatively) fast repolarization of neurons expressing channelrhodopsins with fast off-kinetics means they are unlikely to fire action potentials due to

excitation by the imaging laser during scanning. Successful employment of this method requires careful titration of imaging parameters, including imaging power, frame rate and field of view. This is an interim approach until high efficiency blue-shifted opsins, red-shifted activity indicators, and amplified lasers in the appropriate spectral range become more widely available.

**Alternative recording modalities**

Electrophysiological and optical recording modalities both suffer from potential interactions with the light required to excite optogenetic actuators. The hemodynamic response **[G]** is an alternative physiological response to neural activity which can be exploited to report the impact of optogenetic modulation. For superficial brain areas such as the cortex, the hemodynamic response can be measured via intrinsic imaging [170,171], while functional magnetic resonance imaging[172] can be utilized to record brain-wide hemodynamics. Although the noninvasive nature and the ability to measure the hemodynamic response throughout the entire brain are major advantages, the main drawbacks are that the temporal resolution of this approach is fundamentally limited by the specificity and kinetics of the hemodynamic response itself and the limited spatial resolution of neurovascular coupling [173]. Heating should also be taken into account here as it can directly impact the hemodynamic response [174]. Functional ultrasound imaging is a rapidly developing technology which could be used to perform brain-wide detection of neural activity triggered by localized optogenetic stimulation. Although this method still relies on changes in neurovascular blood volume changes, it can be performed at a fraction of the cost of functional MRI recordings and is rapidly advancing to allow better spatio-temporal resolution and portability [175].

**Linking neural to behavioral readouts**

The exquisite spatial and temporal control of genetically defined cells with optogenetics are attractive features for experiments aiming at establishing links with causality between neural activity and behavior. The growing understanding of neuronal coding has also led to a nuanced understanding of the limits of interpretability of such experiments. However, when appropriately designed and controlled, optogenetic experiments can provide important information on how neural circuits drive behavioral processes.

Choosing the locus of intervention may be instructed by previous literature, lesion experiments and behavioral pharmacology. For example, we know that silencing the motor cortex with muscimol or baclofen causes motor impairment while optogenetic stimulation elicits muscle contraction [176]. While gain of function experiments may be a starting point, cell type-specific optogenetic inhibition of genetically-defined neurons in the motor cortex would provide a more complex picture, better dissociating physiological motor response from an artificial perturbation[177]. Another way to determine the brain region and cell types of interest is the use of activity markers such as the immediate early genes cFos or Arc. Finally, technological advances in wide-field optical monitoring of intracellular calcium may allow to visualize the activity of large cortical areas[44,173] and selectively silence defined cortical regions transcranially[178]. Alternatively, high density electrical recordings[179,180]can elucidate the activity of many neurons in deeper structures. This allows the experimenter to identify circuits with activity patterns that may be relevant to the behavior to be studied.

Next, observational experiments should be implemented to characterize the functional properties of the cell population to be modulated (Figure 6). This may be achieved using electrophysiology in vivo (for example, by tetrode recordings of photo-tagged neurons [181] or genetically encoded calcium sensor imaging [182]. The choice of the optogenetic intervention should ideally be instructed by these observational investigations and match the dynamic range of the activity observed. Additional selectivity can be achieved by aiming at axon terminals rather than cell bodies. Effectors aiming at hyperpolarizing terminals or creating shunting inhibition may not always be efficient or at times even perturb para-membranous ion concentrations such that the effect is difficult to predict[69]. With the advent of $G_{i/o}$-coupled effectors[41,42], presynaptic inhibition is more straightforward, but it remains good practice to validate the efficacy of inhibition, as well as its spatial selectivity, particularly with the highly light-sensitive effectors. It is particularly important to take into account the firing frequency of the cells under investigation as presynaptic inhibition is potentially less efficacious at higher firing rates.

There are two distinct approaches for optogenetic manipulations, one with an acute effect, the other with long-lasting effects. Acute manipulations require behavioral observations in real time. Ideally, a small set of optogenetic trials should be randomly interleaved with control trials. This allows us to assess not only the acute effects on optogenetic trials, but also to determine if there are longer-lasting changes to the subsequent control trials. Such laser on - off protocols can be used to control for adaptive behavioral changes throughout a given session. However, often the particular structure

of the behavioral paradigm does not allow for hundreds of trials. The timing of the optogenetic stimulation or inhibition should therefore occur in a behaviorally defined window, and be only as long as strictly necessary.

The long-term observation after optogenetic interventions is best suited when neural plasticity mechanisms are involved and the stimulation protocol can be derived from the literature (e.g., potentiation typically is achieved by high stimulation frequency, while depression requires sustained low frequency). This may apply to learned and adaptive behavior. The goal of such experiments is thus to induce synaptic plasticity at identified synapses and observe the effect on a behavior that is tested at a later time point when optogenetic stimulation is no longer active. For example, optogenetic stimulation has been used to restore baseline transmission in cortico-striatal synapses which have been potentiated by maladaptive plasticity mechanisms [183]. Similarly, daily optogenetic stimulation of corticostriatal axons for 10 minutes triggered long-term changes in synaptic strength and inhibited compulsion [184].

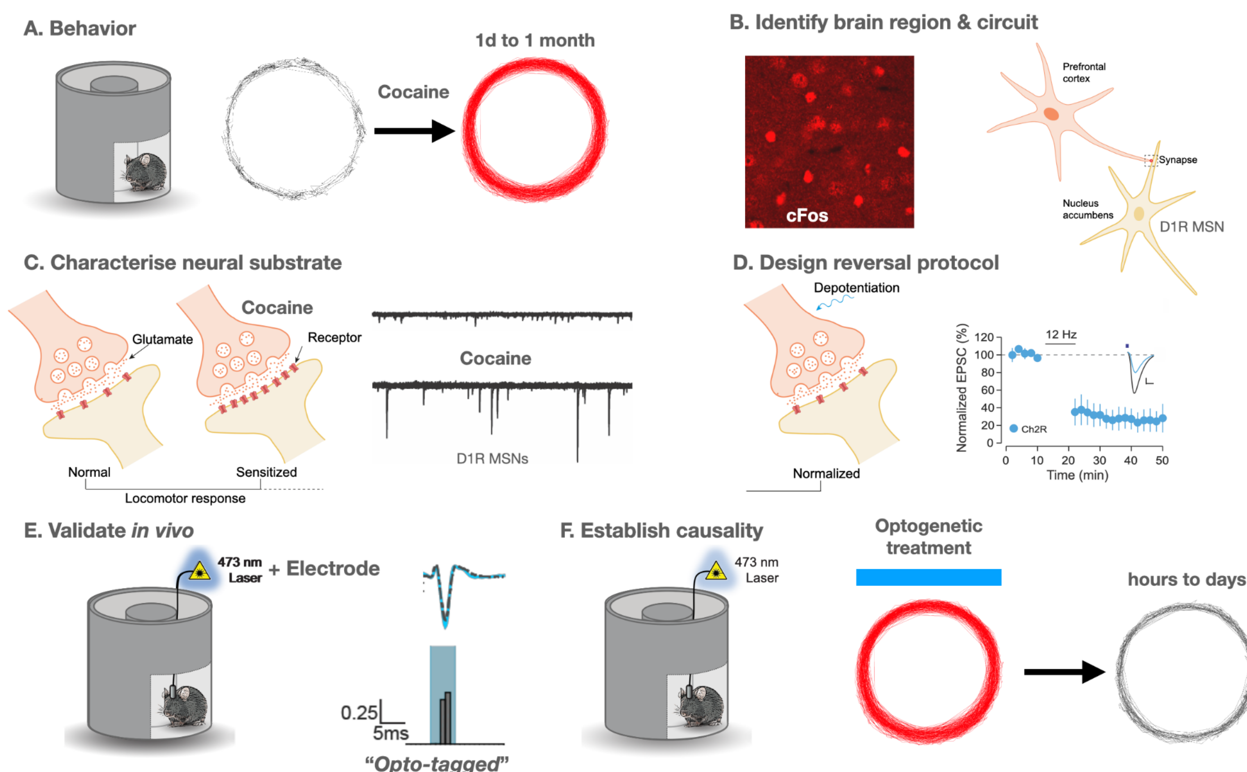


**Figure 6. Establishing links of causality with optogenetics.** Experimental road map based on identifying the neural correlate of behavioral sensitization to cocaine[185]. A. When injected, cocaine elicits a locomotor response quantified in a cyclotron. The response is enhanced upon a second injection of the same dose. B. c-Fos is an immediate-early gene

highlighting the neurons particularly active, which provided the entry point to identifying the mPFC to NAc projection as the behavioral relevant circuit. C. Slice electrophysiology allows to observe the selective potentiation of glutamate transmission onto D1R-MSNs[186]. D. A depotentiation protocol (LTD at 12Hz) validated in slices restores standard transmission. E. *In vivo* validation involves "opto-tagging", where spontaneously occurring spikes (grey, dashed trace) are compared to optogenetically-evoked spikes (blue trace). Waveform and latency are important parameters. F. The LTD protocol is eventually applied in vivo to reverse sensitization.

## Applications

The vast majority of applications of optogenetics have been in neuroscience and brain research. Many of the general principles and approaches can be extended to other organs, especially to those with excitable cells, e.g., skeletal muscle, heart, retina, and gut. Interesting newer areas of optogenetic applications include microorganisms and plants. In addition to common benefits and challenges, these other applications often present unique challenges and opportunities. Below, we illustrate some of these aspects with three select examples from visual, cardiac, and plant applications.

### Vision restoration

Retinal degenerative diseases, such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD), resulting in the loss of rod and cone photoreceptor cells leads to partial or complete blindness [187,188]. Rendering inner retinal neurons responsive to light is one of the most obvious medical applications for optogenetics (Figure 7I). The first proof-of-concept study involved the ubiquitous expression of ChR2 in retinal ganglion cells (RGCs) in retinal degenerated mice [189]. The approach since then has been reported by numerous studies using different optogenetic tools, retinal cell targeting strategies, and animal models (Suppl. Table 1).

Since 2015, multiple clinical trials using ChRs for treating blindness with RP have been initiated leading to very encouraging results (Supplementary Table 2). Recently, the first published case study reported the partial restoration of vision, such as perceiving, locating, and counting objects, in a blind RP patient [190]. Positive preliminary results were also reported in other clinical trials (see Supplementary Table 2). However, further efforts will be required to improve the outcome of optogenetic vision restoration, including the development of effective optogenetic tools and treatment strategies, and the improvement of gene delivery efficiency.

<u>Optogenetic tools:</u> The choice of optogenetic tool is the most critical factor that affects the outcome. Up to now, ChRs have been the mostly chosen optogenetic tools for vision restoration in animal models and clinical trials. With the increasing availability of optogenetic tools, especially ChR-based tools, the choice of optogenetic tools for vision restoration should consider two main issues. The first is its expression efficiency

and long-term safety in mammalian neurons. Unlike for basic science applications, an expression problem of an optogenetic tool is difficult to correct and usually results in cell toxicity in the long-term. The second issue is the low operational light-sensitivity of ChR-expressing retinal neurons in general caused by the small unitary conductance and substantial inactivation. The requirement of high light-intensity to activate the ChR-expressing retinal neurons would constrain this application and also raise the concern of tissue photochemical damage, especially for ChRs with blue spectral sensitivity. One solution to mitigate the potential photochemical damage is to use red-shifted ChRs, such as Chrimson, since the threshold of light intensity causing tissue photochemical damage is shifted to higher light-intensities for longer wavelengths[191-193]. Another solution is to improve the light sensitivity of a ChR expressing cell by slowing its closing kinetics or off rate with molecular engineering [35] (Supplementary Figure S1 and S2) combined with genome mining for more potent ChRs [24]. This strategy has been recently used to further optimize the more effective ChR variant CoChR. Functional vision is restored with improved CoChR mutants under ambient light conditions in a blind mouse model [194]. A third solution is to use G-protein coupled receptors (GPCRs), including animal opsins (e.g., rhodopsin, cone opsins) [195-197], or engineering of OptoGPCR chimeras [198], taking advantage of their high light sensitivity due to intracellular signal amplification. Further studies will need to evaluate the most effective optogenetic tools or develop better ones for this application.

Gene delivery: AAV vectors have been the current choice for transgene delivery in the retina both in animal studies and in clinical trials [199]. Intravitreal injection is a preferred route of viral vector administration due to its safe operation and the potential of achieving wide transduction throughout the whole retina. However, unlike in rodents, in non-human primates and also in the case of humans, virus transduction was mainly conferred in a narrow region surrounding the fovea or parafoveal region [200,201], due to the barrier of a thick limiting membrane in the retinal surface of primates [34]. This is one of the major factors that limits the outcome of the AAV-mediated optogenetic therapy. Further development of more efficient gene delivery vehicles or techniques is required.

Retinal cell targeting: Most animal studies and clinical trials have employed ubiquitous promoters to express depolarizing ChRs in RGCs. However, unlike the normal visual processing features in the retina including the segregation of ON and OFF signal pathways and the presence of antagonistic center-surround receptive fields (Figure 7I A), this treatment strategy converts all RGCs to ON cells (Figure. 7I B). Although useful vision could still be generated as demonstrated in animal studies and reported from clinical trials, it is commonly believed that restoration of vision to mimic the intrinsic visual processing features in the retina would result in a better outcome. To this end, one strategy is to target an optogenetic tool to distal retinal neurons. So far, targeting a depolarizing ChR to ON bipolar cells using the mGluR6 promoter has been the most employed strategy (Figure 7I C). Due to the unique rod pathway in the mammalian retina, this could lead to ON and OFF

responses at the level of RGCs [202-205], and possibly center-surround receptive fields. In addition, targeting surviving cone photoreceptors with a hyperpolarizing optogenetic tool, such as eNpHR, has also been reported [206]. As a limitation for this strategy, the distal retinal neurons are more susceptible to severe retinal deterioration or remodeling than RGCs after the death of photoreceptors[207]. Likely, multiple treatment strategies will need to be developed for treating patients with different retinal degenerative conditions.

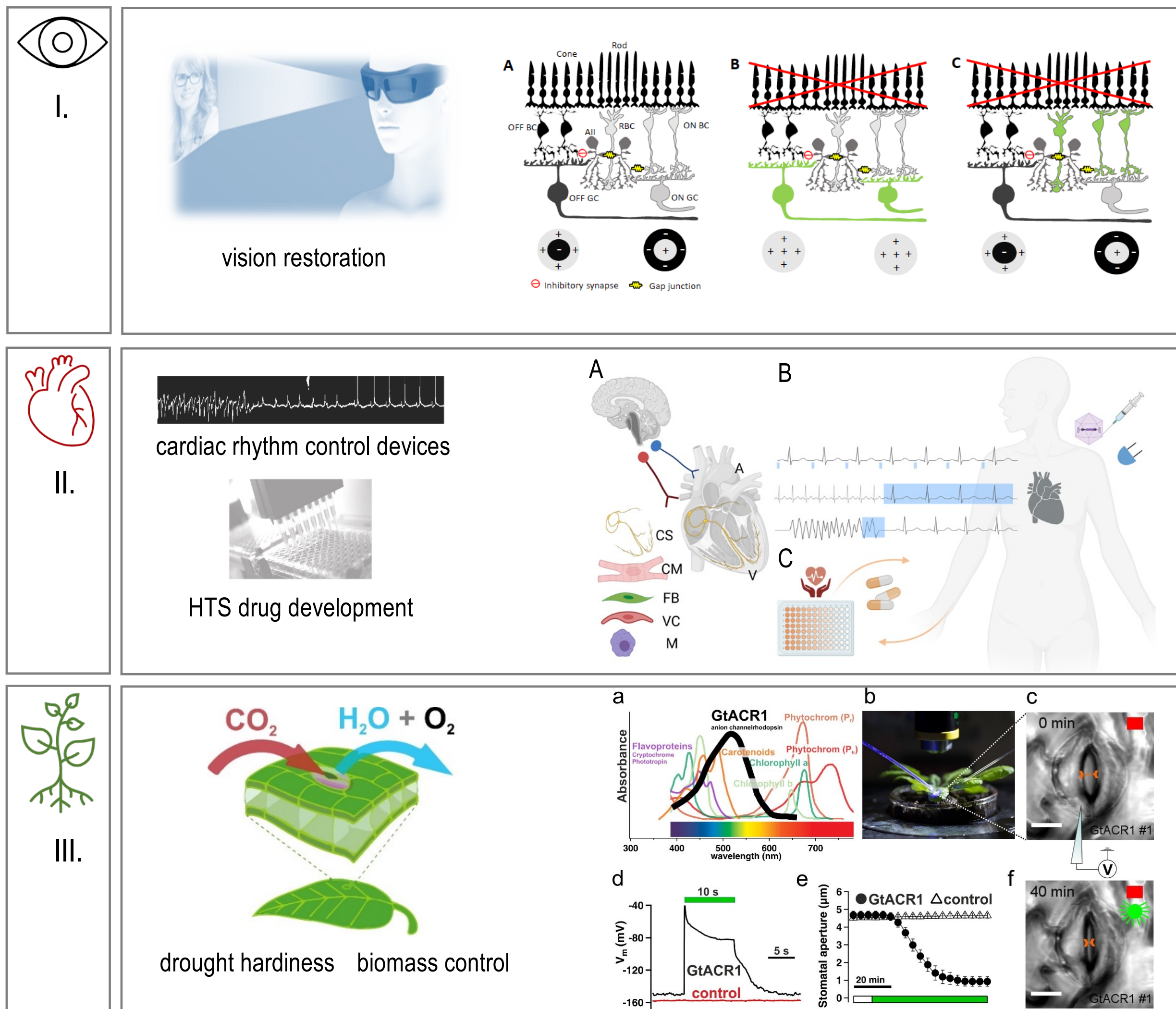


**Figure 7. Optogenetic application for vision restoration, cardiac research, plant modification**

I: **Vision:** Strategies for optogenetic restoration of vision following photoreceptor degeneration. (A) Visual processing pathways in the normal retina, illustrating the rod/cone, ON/OFF pathways, and the antagonistic center–surround receptive fields of retinal ganglion cells (RGCs). ON cells, including rod bipolar cells and AII amacrine cells, are shown in gray

tones, and OFF cells are shown in black. The ON and OFF regions of the receptive field of RGCs are indicated by + and –, respectively. (B) Ubiquitous expression of a depolarizing optogenetic tool (green) in all RGCs to convert them to ON cells. (C) Targeting a depolarizing optogenetic tool in ON bipolar cells to produce ON and OFF response in RGCs and possibly the center–surround receptive fields. Abbreviations: AII, AII amacrine cells; BC, bipolar cells; RGC, retinal ganglion cells; RBC, rod bipolar cells. (Modified from [208]).

**II: Cardiac research II: (**A). Cell-specific targeting is used for the sympathetic (red) and parasympathetic (blue) nervous control of the heart using TH and CHAT promoters; cardiomyocytes (CM) from the upper or lower chambers of the heart (atria, A, or ventricles, V) can be selectively light-sensitized; specific targeting of the fast conduction system (CS), cardiac fibroblasts (FB), vascular cells (VC) or macrophages (M) is also of interest. (B). Rhythm control can include optical pacemaking by short pulses (top trace), heart rate modulation by low-level constant (middle trace) or pulsed light by activating the sympathetic nervous system (increase) or the parasympathetic nervous system (decrease), and arrhythmias can be terminated to restore normal rhythm through a single long pulse (bottom trace), series of pulses and/or spatially-patterned light. (C). Cardiotoxicity testing, a required component in drug development, is enabled by high-throughput optogenetic platforms, which can integrate patient-derived iPSC-cardiomyocytes for personalized therapy. Biorender was used for the illustration.

**III: Plant:** The left side scheme displays carbon dioxide entering, while water and oxygen exiting leaves through stomata to support photosynthetic carbon gain of plants. The picture is from: https://evolution.berkeley.edu/ancient-fossils-and-modern-climate-change/leaves-with-microscopic-mouths/ with minor modifications. On the right side: Expression of rhodopsins to control plant cell behavior. (a) Absorbance spectra of anion channelrhodopsins GtACR1 (black) in relation to endogenous relevant plant photoreceptors. (b) Optical fiber illumination of a leaf from an Arabidopsis plant mounted in a microscope setup (picture from Sönke Scherzer with permission) for (c) simultaneous optical stimulation and electric recordings of guard cells embedded in the leaf epidermis . Images in (c) and (f) from Huang et al. 2021 with minor modifications. (d) A representative membrane voltage recording from tobacco wt (black) and stably GtACR1 expressing (black) guard cell (red) in response to 10 s 525 nm light pulse of 0.57 $mW/mm^2$ in the presence of background red-light (630 nm, 0.018 $mW/mm^2$), which usually causes stomatal opening. (e, f). Closure of the stomatal aperture is only induced in GtACR1-expressing cells in the presence of green light, indicated by the green bar in (e) and the green light spot in (f). Figure (e) from Huang et al., 2021 [209] with minor modifications.

## Cardiac research

The key benefits for clinical translation are sought in more versatile optogenetic pacing or suppression of wave propagation during arrhythmias, compared to currently used cardiac devices (pacemakers and cardioverter/defibrillators) [210-217], (Figure 7 II A-C). Strategies for rhythm control, enabled by optogenetic actuators, aim to lower the energy needed to power the devices, thus extending battery life, and to eliminate discomfort and pain during classic cardioversion/defibrillation for better quality

of life. Optogenetics addresses these challenges through the ability to deliver longer lower-energy light pulses — electrical pulse duration is limited due to electrochemical toxicity via Faraday effects — and through cell-specific genetic targeting to engage the fast conduction system [214], [218] or to specifically target myocytes and avoid unintended contractions of thoracic skeletal muscle, diaphragm and vocal cords like pain-inducing electrical defibrillation [219]. Computational modeling of the action of optogenetic tools in the heart has been developed to help explore strategies for control of arrhythmias, both with excitatory/depolarizing opsins and with inhibitory/hyperpolarizing opsins [213,214,220,221] [136]. Longer-term in vivo clinical applications face the challenges of genetic modification of the hard-to-access cardiac muscle, potential immune responses, and realizing embedded miniaturized light control devices that are reliable and safe [217]. Light penetration in the haemoglobin-rich heart muscle would require operation in the near-infrared and opsins excitable in that range, along with stabilization techniques to counter mechanical contractions. The atria are thinner (human atria are < 5mm) and along with more accessible autonomic nerves, e.g. the vagus nerve, present easier targets [222].

AAV9 is the most efficient serotype for targeting the ventricular myocytes in vivo when using an ubiquitous or a specific promoter, such as Myh6 [223]. The heart atria can be targeted optogenetically using the NPPA promoter and local viral gene delivery [216]. Cre-Lox transgenic mouse models with suitable promoters have been used to transform the fast conduction system cells (Cx40)[218], sympathetic neurons (tyrosine hydroxylase, TH) [224], and parasympathetic neurons (choline acetyltransferase, ChAT) [222,225] Figure 7 II-A. To translate the approaches from rodents (the current model of choice) to larger animals, more work is needed in finding minimally-invasive ways of transgene delivery to the heart, and in minimizing immune responses. Previous clinical trials on gene therapy for cardiac disorders found that a large portion of the patients had antibodies against the viral vectors used, thus reducing the efficacy of the therapy[226]. Most of the published studies have used ChR2-H134R as excitatory opsin. In general, more efficient and fast inhibitory opsins are desirable for arrhythmia control applications. There may also be a niche for step-function-like depolarizing [G] opsins that have fast recovery from inactivation as clamping tools in arrhythmia management. Bidirectional closed-loop control could make an all-optical approach, named optical clamp [G] at the whole organ level a reality. However, this will require spectral compatibility to accommodate not only for an excitatory and an inhibitory opsin, but also for the optical readout of a voltage indicator.

Overall, clinical applications of optogenetics to the heart face many challenges compared to the more accessible, immune-privileged applications to the eye that have seen translational advances. Considering the potential impact for control of arrhythmias, efforts should continue to improve the genetic targeting by more specific promoters, safer viral vectors, longer-wavelength opsins for better penetration and miniaturized distributed light sources. Basic science experiments with optogenetic tools provide invaluable insights for improvement of current cardiac devices and may yield new strategies for arrhythmia control [135,137,217,227,228]. These new strategies take advantage of the ability to produce complex space-time control patterns by light (unlike discrete signals from electrode arrays) to "steer" waves of excitation towards non-arrhythmic behavior at very low energy. In the meantime, optogenetics-empowered high-throughput systems can more immediately improve cardiotoxicity testing and drug development. All-optical cardiac electrophysiology, which combines optogenetic actuators and optical/optogenetic sensors [23,229,230], offers immediate adoption and translation (Figure 7 II-C). Cardiotoxicity testing is crucial in development of any new pharmaceutical, and high-throughput optogenetic methods with patient-derived cells (induced pluripotent stem-cell-derived technology) represent impactful technology for personalized medicine [231,232]. Optogenetic techniques (using hyperpolarizing opsins, e.g. ArchT) have been used to dynamically alter the action potential characteristics of induced pluripotent stem-cell-derived cardiomyocytes, iPSC-CMs, towards a more mature phenotype to better predict drug responses [233]. Furthermore, the maturity of tissue-engineered constructs of such patient derived iPSC-CMs can be improved through chronic optogenetic pacing [232] towards new regenerative solutions for the heart. For further details the reader might consult Supplementary Figure 9 with the related text Cardiac Opportunities.)

**Plants**

Plant growth and development is strictly dependent on light that is absorbed by chlorophyll. But, a large set of photoreceptors that control phototropism, diurnal rhythms and photomorphogenesis also play fundamental roles in plant growth and development. Blue-light absorbing phototropins and cryptochromes or red/far-red light absorbing phytochromes are found in almost all plant tissues (Figure 7 IIIa). Therefore, when using optogenetics tools, special care has to be taken with regard to light regimes used. The light required for plant growth will activate the optogenetic tools when light of the entire visible spectrum is used; this can be avoided by combining a blue light-regulated

transcriptional repressor with a red light-triggered switch [234], allowing plants to grow at ambient white light. Which and how flavoprotein-based optogenetic tools are applied in plants has been described in detail recently [235,236]. Based on the light-oxygen-voltage (LOV) domain, a synthetic light-gated $K^+$ channel called BLINK1 with a considerable dark activity was recently expressed in Arabidopsis guard cells allowing stomatal behavior to be controlled [237]. How light activation of BLINK1, which "clamps" the membrane potential to $E_K$, facilitated stomatal opening and closing in the same way, however, needs to be clarified in the future. A rather simple but valuable technique to avoid non-specific activation of rhodopsin-based optogenetic tools is to grow plants exclusively in red light [122,238]. Both chlorophyll a and chlorophyll b absorb red light (Figure 7 III a), and tobacco plants exclusively grown in red-light are hardly distinguishable from those grown in white light [122]. Green light is the least absorbed wavelength by endogenous plant photoreceptors, therefore, green light allows for optogenetic manipulation with only minimal crosstalk [122] (Figure 7 IIIa), especially with GtACR1.

To perform plant optogenetics with rhodopsins, retinal can be added externally [238] (Supplementary Figure 10 a-c), or retinal production can be empowered *in planta* [122] (Supplementary Figure 10 d-f). In the latter approach a ß-dioxygenase from a marine bacterium is expressed and targeted into the chloroplasts to synthesize retinal from carotenoids efficiently [122]. Special care must be taken when choosing the optogenetic tool because in contrast to animal cells, a plant cell's extracellular medium is low in ions and mostly moderately acidic. This may result in partly different electrical responses in plant and animal cells using the same rhodopsin (Supplementary Figure 11). Activation of ACRs in the soma of neurons leads to membrane hyperpolarization [33], while depolarization occurs in plant cells [122] due to the outward-directed anion gradient (Supplementary Figure 11). When expressed in leaves or pollen tubes, activation of GtACR1 by green light (530 nm) resulted in membrane depolarization by about 60-100 mV within milliseconds. Local GtACR1 activation on one side of the dome of apically growing pollen tubes has been used to demonstrate the involvement of an anion efflux in polar growth [122] (Supplementary Fig. 10 e, f), supporting earlier studies on the role of anion transport in polar growth [239,240]. In guard cells, native anion channel activity can be mimicked when GtACR1 is triggered by a series of light pulses (Figure 7 III c-f), demonstrating that anion channel driven depolarization is sufficient to close stomata [209]. Although plants do not have neuronal-like networks, voltage changes in the form of depolarization waves are transmitted between leaves or even between different organs [241-243]. The role of these long-range electrical signals can now be investigated

with the help of GtACR1. Through GtACR1 induced anion efflux, depolarizations of any shape and intensity can be optogenetically generated to mimic the voltage changes observed in plants such as variation potentials, system potentials or action potentials[244-246].

A wide-range of processes in plants are induced by changes in cytoplasmic $Ca^{2+}$ and $H^+$ levels [245,246]. For both ions, there is a strong inward gradient, in contrast to animal cells, where there are hardly any differences in intracellular and extracellular pH (Supplementary Figure 11). The slow cycling ChR2 variant XXL with high proton conductance[247] is excellent to impose light induced pH changes. It has already been used to feed the P-type plasma membrane $H^+$ pump with substrate and study its voltage dependence [238](Supplementary Fig. 10 b, c). The resting potential of plants is negative with respect to $E_K$ (-120 to -180 mV) due to the voltage dependent activity of P-type plasma membrane $H^+$ pumps. The latter hyperpolarize the membrane and acidify the cell wall space [248]. This voltage deflection is used by the plant to open hyperpolarization active Shaker type $K^+$ channels [249] and electrophoretically move $K^+$ ions into the cell [250]. The combined driving proton-motive-force PMF of the electrical gradient and that of the $H^+$ is used by solute transporters using protons as co-substrate. The plant optogenetics toolbox therefore needs to be complemented by light-driven $H^+$ pumps such as Arch3. Great potential for the study of $Ca^{2+}$ signaling is the ChR2 variant XXM with increased $Ca^{2+}$ conductivity and medium open state lifetime [251]. Combined with electrophysiology and $Ca^{2+}$ imaging, the molecular mechanisms for long-distance $Ca^{2+}$ signaling could be resolved. They can represent either single events or rhythmically recurring signals. Whether, and if so, which signatures control specific processes in plants is still largely unexplored. In the future, $Ca^{2+}$-permeable ChRs could be used to elicit defined $Ca^{2+}$ signatures.

## Reproducibility and data deposition

### Reproducibility of optogenetic tools

Reproducibility in optogenetics experiments depends on the consistency of the tools used, the organism/cell type, the genetic transformation procedures, and the light delivery. Adherence to

minimum reporting standards to include all relevant parameters of an experiment can help to increase reproducibility.

Optogenetic actuators are used in a diverse range of organisms, tissues and cell types. Because of differences in codon usage between the original host (e.g. algae, archaea) and the organisms in which the tools are applied, it is common to "codon-optimize" the coding sequence to facilitate translation in these heterologous systems. One should keep in mind, however, that new codon-optimized sequences should be tested for expression, membrane targeting and function before applying these novel constructs in optogenetic experiments. The sequences of codon-optimized constructs should be appropriately reported in publications to allow reproduction of findings in other labs. However, even with codon-optimization or adding traffic motifs, the intracellular aggregation of many optogenetic actuators can still pose a problem for their applicability, especially for translational applications. A thorough evaluation in targeted organism/cell type is needed because the intracellular aggregation not only reduces expression efficacy but also affects cell health or causes cell death.

Viral vectors have become a popular gene-delivery system for optogenetic tools. The quality of virus vectors, purity and viral titer, can profoundly affect the transduction efficiency and experiment outcome. One should be aware that the quality of viral vectors produced by different labs, centralized viral vector cores, and companies can vary widely. Variation can even occur from batch to batch produced at the same facility. Therefore, even when produced by centralized viral vector cores, service centers, and major labs, viral vector preparations can vary in quality and efficiency. To minimize the variation, standardized purification and titration methods should be used. Each batch needs to be verified before scaling up experiments in order to obtain reproducible results.

**Reproducibility of opsin expression**

If viral vectors are used for transgene delivery, evaluation of the necessary titer is needed to optimize viral vector spread, expression level, and to minimize overexpression-mediated off-target effects. Many opsin viral vectors were designed to co-express a fluorophore. Standard histological methods can be used to visualize the strength and spatial extent of viral vector expression. Characterizing viral expression for every experimental animal can increase interpretability by correlating the variability in the behavioral effect to the variability in expression area and, for instance, optical fiber placement. Even when an experiment is planned based on published work, the paradigm should be validated in each new experiment due to the potential variability of viral vector batches, optical hardware, and

mouse strain. When presenting results obtained using viral vectors, the source of the viral vector, its purification and titration methods, and the duration of expression should be reported.

Viral vector expression can impact cell health or change the electrophysiological properties of the targeted neurons. It is therefore necessary to include a control group injected with a titer matched virus that expresses a control transgene. Researchers often use a virus encoding the same fluorophore that is co-expressed with the opsin. This control group can be used to evaluate direct effects of the virus injection surgery and potential phototoxic or heating effects due to the light delivery paradigm. Strong opsin expression has been reported to affect cell physiology [252]. It is therefore advisable to include an opsin expressing group where no light is applied. In case the experiment allows for multiple repeats of the same manipulation, the light and no light conditions can be tested in the same group, which results in a within-animal control.

Transgenic animals for optogenetics research should be genotyped continuously to confirm suitability for the experiments. For in vivo optogenetics with viral delivery, even when using the same tools in the same organism type, variations in responses may be due to variations in the immune response of the subjects (animals or humans) to the viral capsid, or the cargo (opsin and/or fluorescent reporter). To obtain reproducible data with viral delivery, testing for neutralizing antibodies (against the viral vectors used) can be implemented [253]. Appropriate control groups, immunohistochemistry and histology should be done routinely in animal experiments to demonstrate consistency of the optogenetic transformation.

**Reproducibility of light delivery**

Activation of optogenetic tools depends on the photon irradiance [Watt per area] or photon exposure in case of short flashes [Joule per area] and the spectral profile of the delivered light. The spectral profile should be reported by listing the light source, all filters, and optical components used in the experiments. Insufficient irradiance may lead to failure to engage the optogenetic tools and therefore failure to reproduce the phenotypic changes; excessive irradiance may lead to adverse thermal effects and photoreceptor bleaching that also affect reproducibility. For single photon excitation, the spatial pattern of the delivered light is variable and highly depends on the positioning of the light source and the tissue properties. While total power is trivial to report, the normalized values (irradiance) are influenced by the uncertainties of area estimation and the non-uniform spatial profile of light delivery.

At a minimum, effort should be made to measure and report the irradiance at the point of entry into the tissue; whenever possible, light-tissue interactions can be simulated [254] to yield relevant estimates of irradiance at points of interest.

Under optimal conditions, two photon optogenetics is capable of stimulating individual neurons within a circuit with single spike and single cell resolution. Irrespective of the light sculpting method used (spiral scanning or parallel illumination), one must keep in mind that the effective spatiotemporal resolution of optogenetic stimulation depends on several factors, including the functional expression level of the opsin, the targeting specificity, and the photon density required for sufficient actuation. Once a reliable and reproducible experimental preparation has been established, and the average incident powers required identified, the "physiological resolution" should be measured experimentally rather than drawing any conclusions about the confinement of actuation based on the optical resolution of the light targeting method.

**Data and metadata sharing**

The data type and format from optogenetics experiments can be extremely diverse. The outputs may include spectra, ion channel recordings, functional recordings of responses by different measurement technologies, images of altered responses, behavioral analysis etc. For each sub-field where optogenetics is deployed, minimum standards of reporting and guidance of data sharing will help determine best practices. In general, specifics of the instruments used, the acquisition and the analysis software need to be included. Github, figshare and other general repositories for data and analysis tools can be used to increase reproducibility.

## Limitations and optimizations

### Tissue heating and photodamage - single-photon optogenetics

Optogenetic experiments based on illumination with visible light excitation (450 - 630 nm) typically use optical fibers coupled to lasers or high-power light-emitting diodes (LED) for large (~$mm^3$) field illumination, relatively long (0.5 – 60 s) exposure times and excitation powers on the order of milliwatts (0.5-20 mW). Under these conditions, the main cause for concern with respect to photodamage is heating due to light absorption. This has been investigated both theoretically, using Monte Carlo with finite-difference time-domain simulations[131], or the finite element method [255], and

experimentally using thermocouples[131,255], infrared cameras [256], or electrophysiological recordings [257]. Depending on the precise stimulation protocol used, these experimental and theoretical studies report a wavelength and power density dependent temperature increase between 0.3 – 6 K throughout the volume of illuminated tissue [256] [131]. Temperature variations on the order of only 2 K can affect ion channel kinetics and conductance [258], synaptic transmission [259] and neuronal firing rate [131], and led to a bias in turning behavior [257] across various brain regions. Importantly, changes in temperature can induce physiological changes in the absence of detectable changes in behavior [260]. It is extremely important to carefully design optogenetic experiments to minimize photon exposure and absorption, for instance by using short illumination duty cycles [256] and opsins with long open state lifetimes and red-shifted absorption peaks [24,26,261]. Simulations [131,256,262,263] can be used to guide experimental design, but, since the effects of heating vary between cell types and brain regions, opsin-negative controls should always be performed.

**Tissue heating and photodamage - multiphoton optogenetics**

Generating sufficient rates of multi-photon excitation requires the use of pulsed lasers with high peak energies, but since typical optogenetic stimulation protocols irradiate cells on millisecond timescales, the temperature rises induced by single cell multi-photon photostimulation are of the order of $10^{-1}$ K [263]. Much larger temperature rises are induced during multi-target excitation due to the diffusion of heat from each target into the surrounding tissue. The resultant temperature increase occurs over hundreds of milliseconds and can approach or even exceed the 2 K threshold for thermal damage [263]. This effect can be mitigated by ensuring that the separation between adjacent targets is larger than the thermal diffusion length.

The risk of non-linear photodamage increases with peak fluence and could be a dominant source of photodamage in the case of spiral scanning which typically requires higher photon density than parallel illumination. Non-linear photodamage can be reduced by increasing the repetition rate of the pulsed laser source although this will increase photo induced temperature rises [263]. In all-optical experiments which combine 2P optogenetics with 2P imaging, the possibility of thermal or nonlinear damage induced by the imaging laser should also be considered[262].

**Interpretation of optogenetic experiments**

Light delivery schemes based on single photon excitation are not generally capable of recapitulating physiological activity patterns. In most optogenetic gain and loss of function experiments, a set of cells is activated or silenced, and the effects of this manipulation are subsequently characterized by functional or behavioral readouts to probe causal dependencies. Light-delivery via an optic fiber can be precisely controlled in terms of output power and temporal pattern to influence neuronal functions like spike rate and spike pattern and may be restricted to specific short behavioral epochs. However, such optogenetic manipulations typically lead to highly synchronous activity patterns, and might drive the circuits to states that are outside of their physiological activity range, potentially confounding any causal inference regarding the natural functions of the circuit [272]. One major current effort aimed at overcoming these constraints is the development of tools for evoking naturalistic network activity patterns. Such manipulations would enable causal inference of the effects of an activity pattern on a given behavior.

Non-physiological activity patterns can occur at the single-cell level as well as at the broader circuit scale [264,265]. On the single cell level, ion pump mediated hyperpolarization for instance can lead to rebound excitation upon inhibition release [266] or to supra-physiological ion concentrations [72,118]. High-frequency light pulse trains or constant illumination of an excitatory pyramidal neuron expressing a CCR can, for instance, lead to depolarization block, effectively reducing rather than increasing its firing rate [267]. Whether such rebound excitation or depolarization block occurs and to what extent is hard to predict, as it depends on many experiment-specific parameters which can greatly vary between laboratories. While axonal stimulation can be used to effectively isolate the activity of an anatomically-defined projection pathway, optogenetic stimulation of axons can cause antidromic activation **[G]** of both neuronal cell bodies as well as collaterals to other brain regions, leading to reduced specificity which should be taken into account.

At the circuit level, particularly when a large portion of cells expresses ion translocation based optogenetic tools such as ion-channels or ion-pumps, the simultaneous activation of these tools can lead to transient but significant changes in the ion composition of the local extracellular space, thereby indirectly affecting nearby non-opsin expressing neurons (bystander effect) [268]. Electrophysiological characterization of the optogenetic manipulation can be performed to quantify the extent of such

unintended effects, allowing the optimization of light power and illumination paradigms. Optogenetic tools that modulate biochemical activity within the cells or ones that act on slower timescales, or only induce subthreshold depolarization, are less prone to the caveats imposed by highly synchronous neuronal activation [269]. Finally, optogenetic firing rate modulation experiments are mostly designed to acutely alter the firing rates of targeted cells, which can have different effects than chronic manipulations. Brain circuits regulate their overall activity to achieve a homeostatic equilibrium, such that when the firing rate of a circuit is transiently increased or decreased, it can acutely affect the independent functions of downstream circuits and lead to markedly different results compared with chronic (lesion) manipulations[269]. Acute effects are normally more severe, and could lead to overestimation of the roles of targeted regions in a given behavior. While chronic manipulations such as lesions do not suffer from this limitation, plastic changes during lesion recovery can also lead to an underestimation of the necessity of a given input to a local circuit. In summary, a sound experimental strategy should balance the use of acute and powerful optogenetic approaches with chronic experiments, pharmacological manipulations or lesions, and use caution in claims of causality based purely on manipulations that might suffer from any of the above-mentioned artifacts.

## Outlook

**Refinement of the optogenetic toolbox**

We anticipate a further optimization of existing tools in terms of kinetics, ion or substrate selectivity, and widening of the spectral range from UV to the near-infrared to enhance the use of optogenetics. Additional light-activated enzymes allowing for optogenetic control beyond cell excitability are still to be discovered. Efforts are currently directed at optogenetic control of translation, transcription, nucleotide modification and epigenetics, as well as protein degradation. We are also expecting better tools for the control of cellular mechanics, development and differentiation.

**Enhancement of basic research.**

Optogenetics will further advance the investigation of neural circuits. This will not only establish links of causality between neural activity and behavior but eventually generate sufficient knowledge for a theory of the brain to emerge. Empirically observed neural activity in optogenetic experiments taking into account the activity of individual neurons may eventually allow deriving the neural code,

which when integrated into a solid theoretical framework will bring the neurosciences at par with other fields of natural sciences.

Optogenetics may also drive basic knowledge in other fields of life science, from cardiac physiology to plant physiology. For plant optogenetics, which is still in its infancy, there is great potential through the recently introduced *in planta* retinal synthesis, which now allows access for many rhodopsin-based manipulations. Implementing optogenetic approaches in any system of excitable cells will allow for the investigation of so far intangible questions. This may apply for example to the control of muscle contraction in the heart as well as the insulin secretion in the pancreas.

**Open routes of translation.**

Beyond advancing basic science, optogenetics also has translational potential, either by inspiring novel protocols of existing therapies or as a therapy in humans. Several possible optogenetically-inspired medical interventions and therapies are already outlined in this review. Optogenetics can be used in vitro to analyze cellular processes in single cells, cultured tissue or brain slices. Optogenetic can also simulate clinically-relevant scenarios in animal models of brain diseases. These include optogenetics-informed electrical stimulation protocols, or closed-feedback control schemes. Possible indications are epilepsy, Parkinson’s disease or addiction. Some of these interventions may eventually be emulated in humans, for example with refined deep brain stimulation protocols or pharmacology (Supplementary Figure 12).

With the recent proof of principle of optogenetic vision restoration in humans suffering from retinitis pigmentosa, longer-term gene therapy options remain open for optogenetics. Several challenges however need to be addressed. Optimization of gene delivery vectors that are safe and produce a long-lasting expression and optimization of light delivery to the desired organ will be essential. Light delivery deep into the tissue, beyond the limited optical depth penetration minimizing the use of optical fibers is also needed. One such non-conventional solution would be in situ sources of biological light, such as luminopsins. Triggered by a chemical process, like simple substrate delivery, they do not require device implantation and can be tuned to control inhibitory or excitatory actuators. Further remote trigger methods involve energy-conversion schemes via mechanoluminescent nanoparticles. The energy could be provided by intermittent focused ultrasound, thus recharging light emitting materials that can deliver short opsin-engaging pulses. Mechanosensitive TRAAK K+

channel for example could be specifically activated by ultrasound with submillisecond kinetics [270] providing a new, orthogonal dimension for external non-invasive manipulation of neural circuits.

Clinical applications of optogenetics to the heart face many challenges compared to the more accessible, immune-privileged applications to the eye that have seen translational advances. Considering the potential impact for control of arrhythmias, efforts should continue to improve the genetic targeting by more specific promoters, safer viral vectors, longer-wavelength opsins for better tissue penetration and miniaturized distributed light sources. Basic science experiments with optogenetic tools provide invaluable insights for improvement of current cardiac devices and may yield new strategies for arrhythmia control [135,217]. In the meantime, optogenetics-empowered high-throughput systems can more immediately improve cardiotoxicity testing and drug development. Similarly, in vitro assays for drug development and personalized medicine may use humanized optogenetic tools, patient-derived cells and engineered tissues coupled with highly parallel all-optical electrophysiology techniques to yield a low-cost, faster and more efficient drug-development pipeline.

Likewise, scalable optogenetic control of living plants, as discussed here, or microbes can be leveraged to address problems related to energy, food, biotechnology and climate challenges. As these do not involve deployment in the human body, they can be implemented on a shorter time scale, with less technical and regulatory obstacles.

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## Definitions:

**Channelrhodopsins (ChRs):** Light gated rhodopsin-based ion channels. They are 7-transmembrane photoreceptors with retinylidene chromophors. They may conduct cations or anions and are named CCRs and ACRs respectively. CCRs are first of all $H^+$-conducting channels but show at mammalian physiological conditions (100 mM $Na^+$, 2 mM $Ca^{2+}$ and pH 7.5) quite variable voltage dependent selectivities and a variable degree of inactivation. Most ChRs originate from microalgae of fresh water or see water origin but ChRs have been found in other flagellates and algal viruses as well.

**Step-function rhodopsin (SFRs):** ChRs with slow on and off kinetics and long-lasting conductance after light activation. Dark state and conducting state exhibit different absorption maxima with the potential to switch SFRs on and off with light of different wavelengths. Most SFRs show modifications in the DC gate, two residues close to the retinal polyene chain.

**Enzyme Rhodopsins:** They are tandems of an N-terminal rhodopsin linked to a C-terminal enzyme which might by guanylyl-cylases (R-GC) or phosphodiesterases (R-PDE). The dynamic light activation for RGCs is high and might reach values of > 1000 whereas the light-activation is still low (<10 fold) for RPDEs and needs further engineering and improvement.

**LOV-Domains:** Canonical Light Oxygen Voltage sensors with Flavin mononucleotide (FMN) chromophores. They may form monomers of dimers and many of them change the oligomerization state upon light activation. This property can be used to mimic dimerization of HKRs type hormone receptors upon illumination after N-terminal (AtLOV2) or C-terminal fusion (AC-LOV).

**Photoactivated cyclases (PACs).** They are either soluble Blue Light Photoreceptors using FAD (BLUF) or membrane inserted rhodopsins both with C-terminally fused cyclase domains. BLUF-cyclases and rhodopsin-cyclases (RGCs) may be ATP or GTP selective producing cAMP or cGMP, respectively. Most PAC proteins function as homodimers but some RGC may also function as heterodimers with two rhodopsins of different color sensitivity.

**Operational light sensitivity:** Effective opsin photocurrent as a result of multitude of factors including innate properties (single channel conductance, kinetics of photocycling, and kinetics of switching off) as well as expression levels (density of functional opsins). Strictly speaking the cellular light sensitivity at a certain wavelength is the reciprocal light intensity where an expected light response can be detected. (see also Supplementary Figure 2).

**Optical clamp:** Using light and real-time feedback to keep membrane electrical parameters, such as voltage or action potential shape at set desired value.

**Shunting inhibition:** If the reversal potential of a channel is identical to the resting potential, its activation does not lead to a substantial current, but a shunting conductance. The input resistance of the neuron is reduced, causing a smaller amplitude of subsequent excitatory potentials. If the synaptic reversal potential lies between the resting potential and the action potential threshold, however, the effects of shunting inhibition are more complex. The cell will be depolarized but excitatory potentials still lead to a reduced depolarization, and, consequently, spike rate. Upon channel closing the input resistance increases but the membrane potential is still depolarized, leading to a transient increased excitability. If chloride channels are activated over extended periods of excitatory drive, Cl- can accumulate in the cell, and the depolarizing phase of shunting inhibition will become more accentuated, leading to activity-dependent effects of shunting inhibition.

**Gene therapy:** A technique that replaces a faulty gene or adds a new gene in living cells to treat or prevent disease.

**Single photon excitation:** The process by which a molecular transition to an excited electronic state is induced by the absorption of a single photon with sufficient energy. This process is linear in the sense that the excitation rate is proportional to the number of incident photons.

**Two-photon excitation:** The process by which a molecular transition to an excited electronic state is induced by the quasi-simultaneous absorption of two photons with sufficient combined energy. Two photon absorption is a nonlinear process - the excitation rate is proportional to the time averaged square of the intensity (number of photons per unit area).

**SLM:** Spatial light modulators can modulate the amplitude, phase or polarization of light. In optogenetics, phase modulation with SLMs is used to sculpt light into extended patterns that cover the desired membrane, or to multiplex the excitation beam and photostimulate multiple cells simultaneously.

**DMD:** Digital micromirror devices are most commonly used for amplitude modulation to illuminate specific regions of the field of view.

**Temporal focusing:** The group of techniques which use a dispersive element to increase the effective pulse length of light outside of the target plane (the temporal focus). Temporal focusing is used in two photon optogenetics to maintain the axial resolution of patterned excitation.

**Fluorescent activity reporters:** sensors that provide an optical readout of cellular activity due changes in fluorescence intensity in response to chemical or electrical transients. Calcium and voltage indicators are most commonly used in combination with optogenetic actuators in so-called "all-optical" experiments.

**Glossary:**

**antidromic activation:** Retrograde propagation of an action potential from the axon to the neuronal soma.

**bidirectional voltage modulation:** Changing the voltage in the depolarizing (excitatory) or hyperpolarizing (inhibitory) directions.

**optogenetic actuator:** a light sensitive protein that during illumination transiently modifies cellular properties.

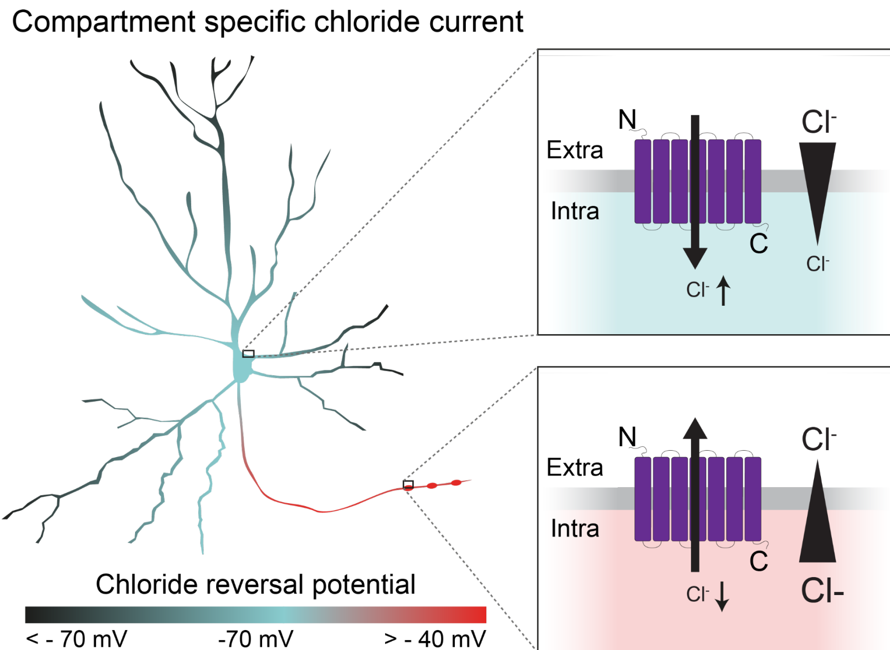
**electrode:** a wire, liquid-filled pipette or other conductive element, used to record electrical activity of cells or tissues.

**microelectrode:** an electrode with micrometer-sized tip, used to record single neuron activity.

**hemodynamic response:** a homeostatic adaptation to elevated neural activity, leading to increased blood flow to highly active brain regions.

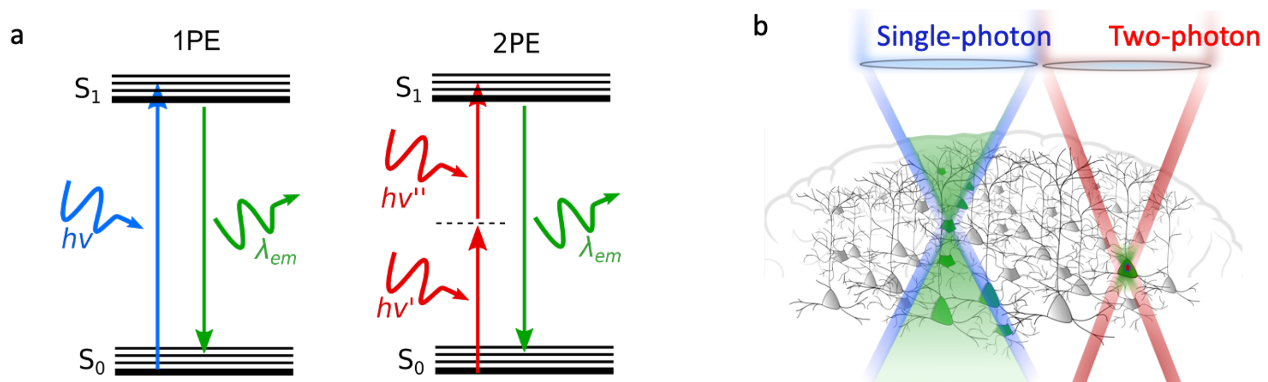
**immediate early genes:** genes that are rapidly induced by elevated neural activity, e.g. c-Fos.

**optrode:** an electrode coupled to an optical fiber, used to record and manipulate neural activity in cells expressing an optogenetic actuator.



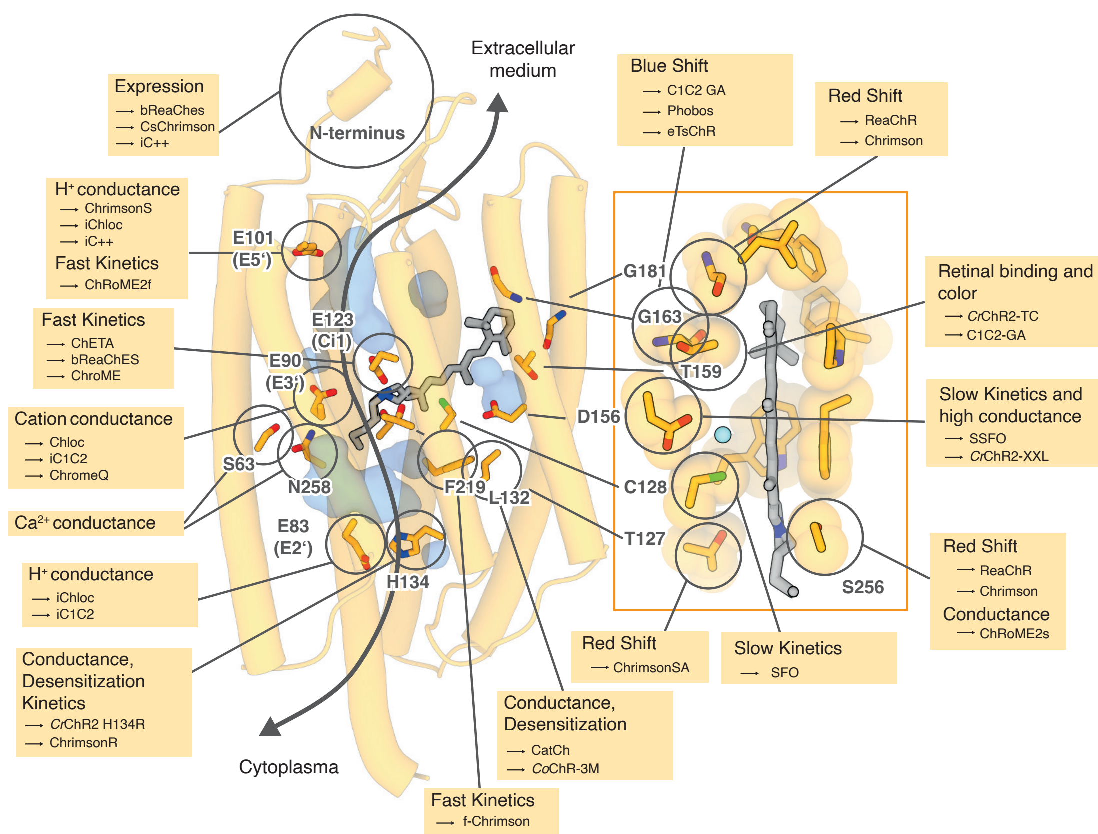

**Box 1: Compartment-specific functions of microbial rhodopsins.**

Left: schematic of a neuron colored according to the typical chloride reversal potential. Right: insets depicting the effects of the commonly used anion-conducting channelrhodopsins (e.g. GtACR2, iC++, iChloc) in the somatic compartment (top) and at the presynaptic terminal (bottom). Anion conducting channelrhodopsins can have a compartment specific effect due to variations in the chloride reversal potential. Optogenetic manipulations should thus take into account unique features of compartment-specific physiology.

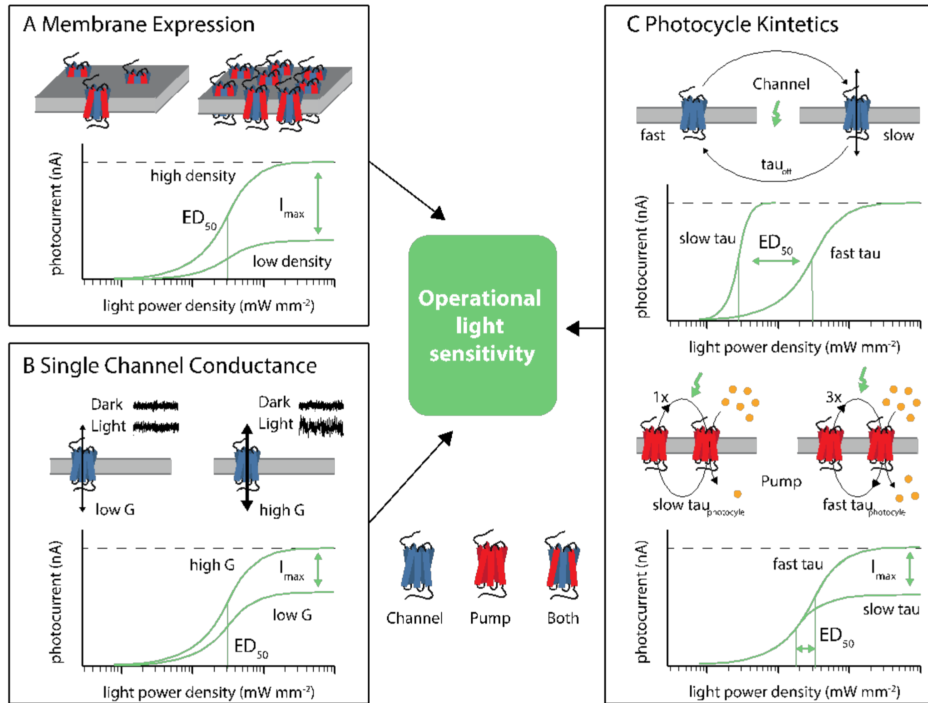



**Box 2: Single-photon vs. two-photon excitation, mechanism and focal volume**

In single-photon excitation (1PE) the absorption of a photon by a chromophore induces a molecular transition from the ground state (S0) to the excited electronic state (S1)(a, left), while in two-photon excitation (2PE), the same transition can be induced by the quasi-simultaneous absorption of two photons (2PE; a, right). Since 2P cross-sections are typically much smaller than for 1P, significantly higher photon fluxes are generally required to generate similar excitation rates, requiring more complex and expensive components such as ultrafast lasers. There are two main implications of two photon absorption in microscopy, firstly, since the probability of excitation is a quadratic function of the instantaneous photon density, targets at the focal plane are much more likely excited than out of focus targets, whereas in 1P excitation all targets throughout the light path can be excited (b). Secondly, the use of photons of lower energy and therefore of longer wavelengths (deep red and IR) can penetrate more deeply (~700 µm) in scattering tissue.

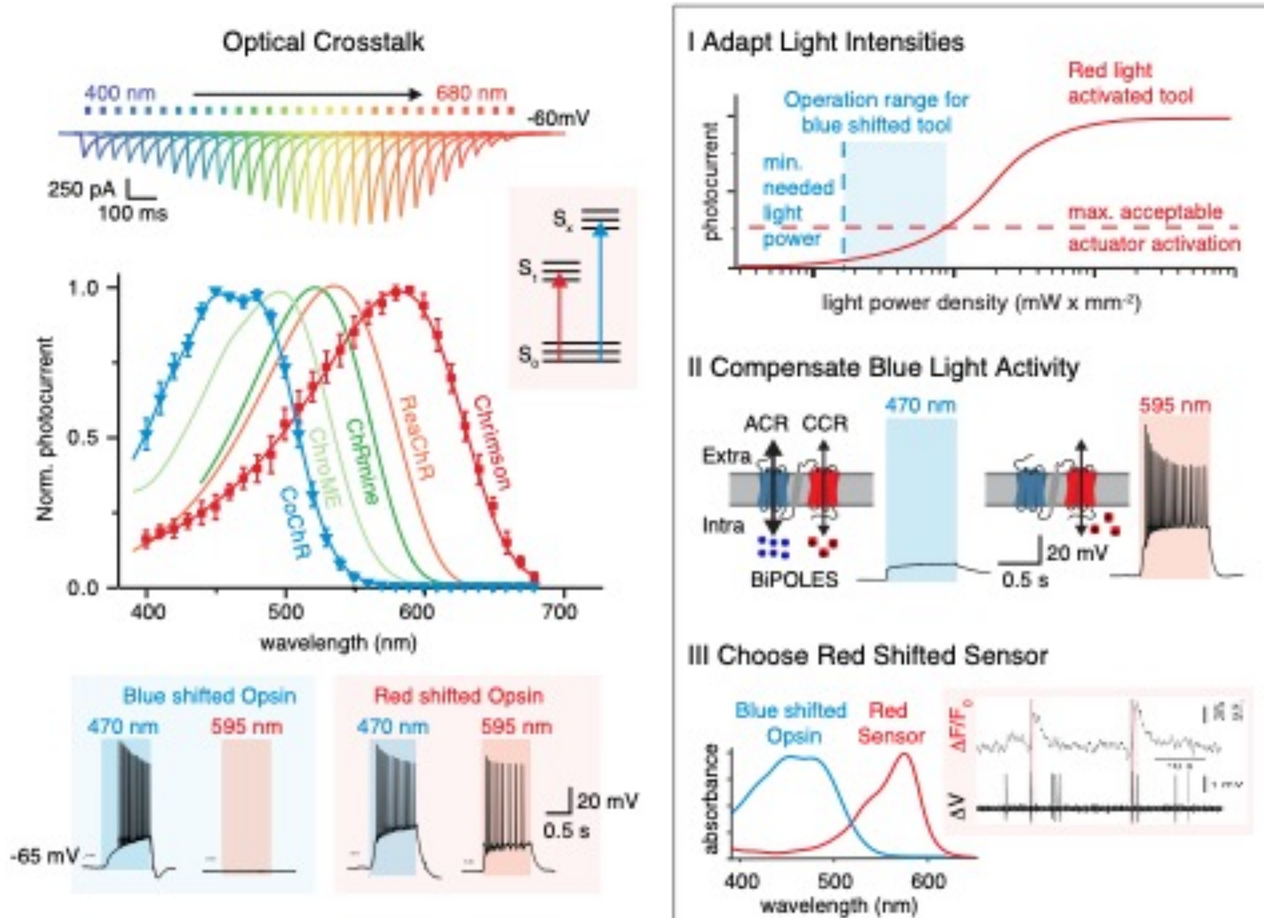



**Supplementary Figure 1. Mutational tuning of channelrhodopsin properties based on the ChR2 structure.** Cartoon of the CrChR2 (C2) structure with highlighted amino acids that have been mutated in C2 or related ChRs to successfully tune expression, selectivity, conductance, kinetics and degree of inactivation (selection). The blue regions depict high-probability locations of water molecules based on molecular dynamics calculations. N-terminal modification for improved expression: bReaChES[30], CsChrimson[271], iC++[272]; S63 and N258: $Ca^{2+}$-conductance[273]; E83 (E2′): $H^+$-conductance[274,275]; E90 (E3′): Cation conductance & selectivity: Chloc[276], iC1C2[274], ChromeQ[277]; E101: $H^+$-conductance: ChrimsonS[53], iChloc[275], iC++[272] and faster kinetics: ChRoME2f[278]; E123 (counter ion Ci1): Fast kinetics: CheTA[279], bReaChES[30], Chronos[24]; T127: Red Shift in ChrimsonSA[61]; C128: Slow kinetics in SFOs[280,281]; L132: Large conductance and reduced inactivation in CatCh [282,283]and CoChR-3M[194]; H134: Selectivity, desensitization, kinetics: *Cr*ChR2-H134R[8], ChrimsonR[24]; D156: Slow Kinetics in SFOs[284], SSFOs[27], *Cr*ChR2-XXL[247]; T159: Retinal binding and color: *Cr*ChR2-TC[25]; F219: Fast Kinetics: fChrimson[31]; S256: Red shift in ReaChR[285,286], Chrimson[61] and larger photocurrents: ChroME2s[278].




## Supplementary Figure 2: Operational light sensitivity

The operational light sensitivity (OLS) can be defined as the photocurrent induced in a rhodopsin expressing cell for a given photon irradiance or photon exposure in case of a short light flash. OLS depends on the probability of an opsins to enter the photocycle upon photon absorption and, in addition, on the number of opsin proteins residing in the plasma membrane (A), the single-channel conductance (B), and the kinetic properties (C) of the opsin. The consequences of kinetic changes may be very different for channels (blue) and pumps (red).




**Supplementary Figure 3: Combining optical techniques: dual-opsin manipulations and all-optical approaches.**

***Left*****:** Optical crosstalk: red shifted CCRs can also be activated by blue light but not vice versa. *Top)* Sample photocurrent traces for the red shifted CCR Chrimson showing significant depolarization in response to illumination with blue light. *Middle)* Action spectra of commonly used CCRs. *Inset:* Jablonski diagram. Y-axis represents the energy of the opsin bound retinal with possible energy states indicated by black bars. Absorption of a photon will result in an electron to be elevated from the ground state (S0) to an excited state (singlet state: S). In the case of red-light sensitive opsins, the energy needed for a transition to the first excitation state is reduced compared to blue shifted opsins. However, the higher energy of blue light can lead to the transition to a higher excitation state ($S_x$), making red-light sensitive opsins also sensitive to shorter wavelengths. *Bottom*) Current clamp recording of two neurons expressing the blue shifted opsin CheRiff (left) and the red shifted opsin Chrimson (right) respectively (adapted from [64]). In case of the red shifted opsin, both red and blue illumination leads to action potential firing.

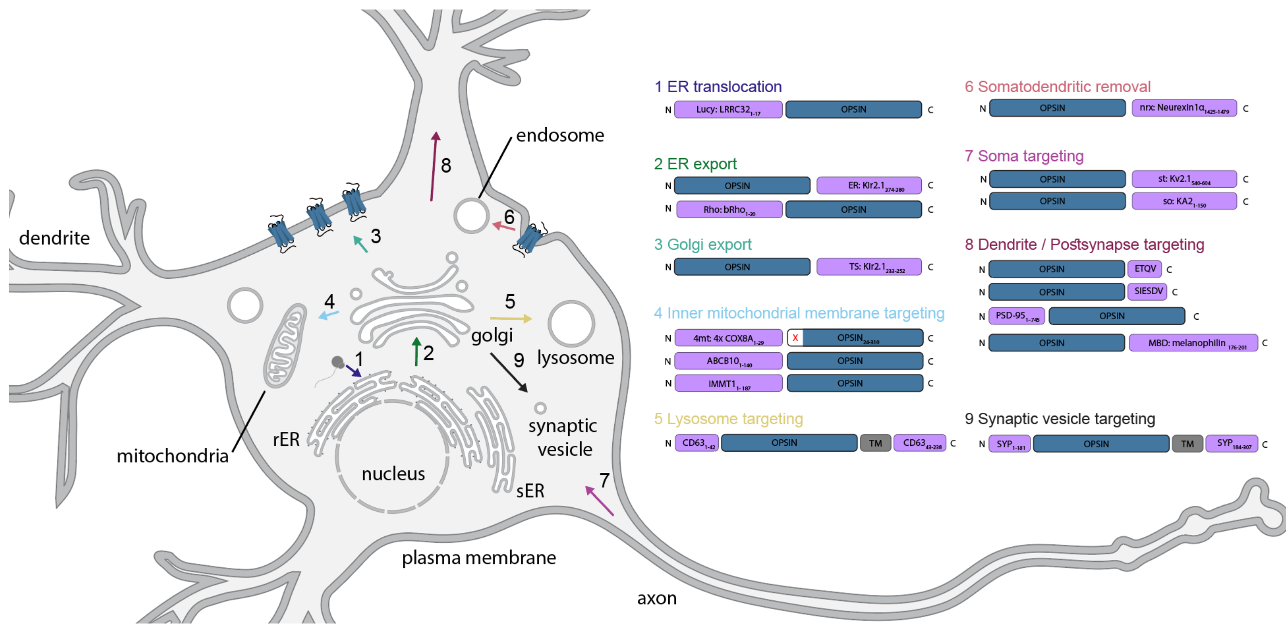
***Right*****:** Despite this crosstalk optical techniques can still be combined using one of the following strategies:

**Adapt light intensity:** In carefully controlled experiments a blue wavelength light power range that allows for sufficient activation of a blue light sensitive tool without activation of the red sensitive tool can be determined. We refer to this as the operational range (blue shading). An example is the combination of an indicator excited by blue light (e.g. GCaMP) with a red shifted ion-pump (e.g. NpHR), that will not significantly hyperpolarize the cell at the light power necessary for sensor imaging. However, especially when sub action potential threshold effects are taken into consideration, the definition of "acceptable activator activation" becomes non-trivial.

**Compensate blue light activity:** This can be achieved by matching two opsin actuators so that the activation of the red shifted tool by blue light is compensated by the blue light sensitive tool with opposite but stronger effect. A recent example is BiPOLES, where the blue light activated anion channel GtACR2 suppresses blue light activity of the fused red shifted Chrimson channel [64]. Blue light inhibition and changes of input resistance during blue light illumination of BiPOLES expressing cells might however require careful evaluation when using BiPOLES for optical multiplexing.

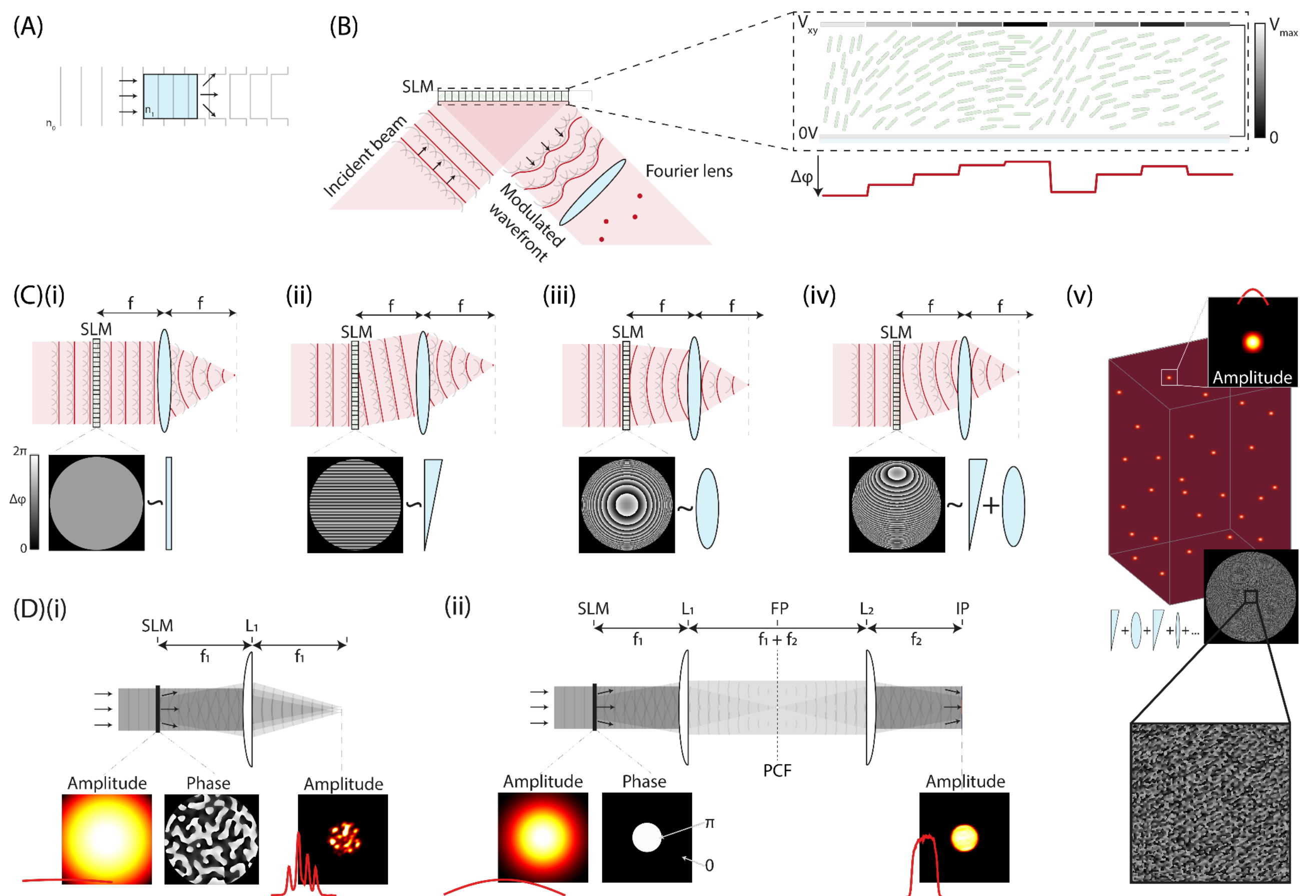
**Choose red shifted sensors:** The blue light excitation of red shifted sensors is usually acceptable as fluorescence decay is much faster than the fluctuations of the measured parameter such as intracellular calcium levels. The sensor can often not be measured during blue light illumination, but the measurement can be directly continued thereafter. *Inset*: Example measurements from a stCoChR and jRCaMP1a expressing neuron. Scanning at 1100 nm does not lead to apparent firing rate changes, while holographic single cell simulation using 920 nm light results in robust AP firing and jRCaMP1a fluorescence increase. *Top*) jRCaMP1a fluorescence read out by 1100 nm two-photon raster scanning. *Bottom*) Attached recording of the same neuron.

**Choose fast opsins:** when using two-photon scan imaging, the use of opsins with a fast-closing kinetics combined with fast scanning of a large field of view enables to reduce the imaging cross talk by reducing the accumulation of current between two successive laser passes. Considering that photostimulation with spiral scanning works better with slow opsins, this strategy is particularly indicated in combination with parallel photostimulation in which case the evoked photocurrent is independent of the opsin kinetics.




**Supplementary Figure 4. Sequence motifs used for directing subcellular targeting of optogenetic tools.**

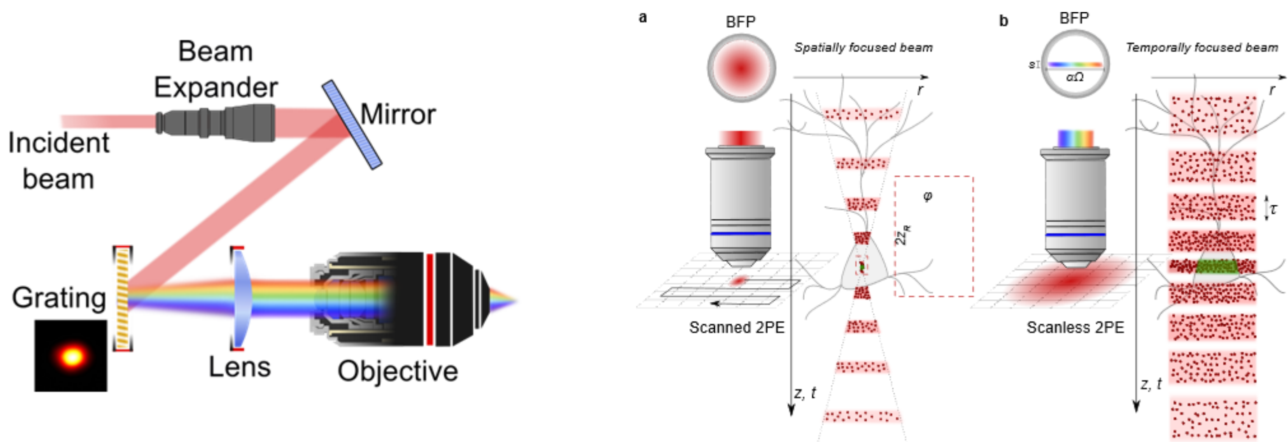
Left: Schematic of a neuron including subcellular compartments. Right: Commonly used amino-acid sequences to target transmembrane domain proteins. (1-3) Sequences employed to reduce intracellular accumulation by improved plasma-membrane targeting in plant (1) and mammalian cells (2-3). (4-9) Sequences utilized to target opsins to subcellular compartments.




**Supplementary Figure 5. Wavefront engineering: now**

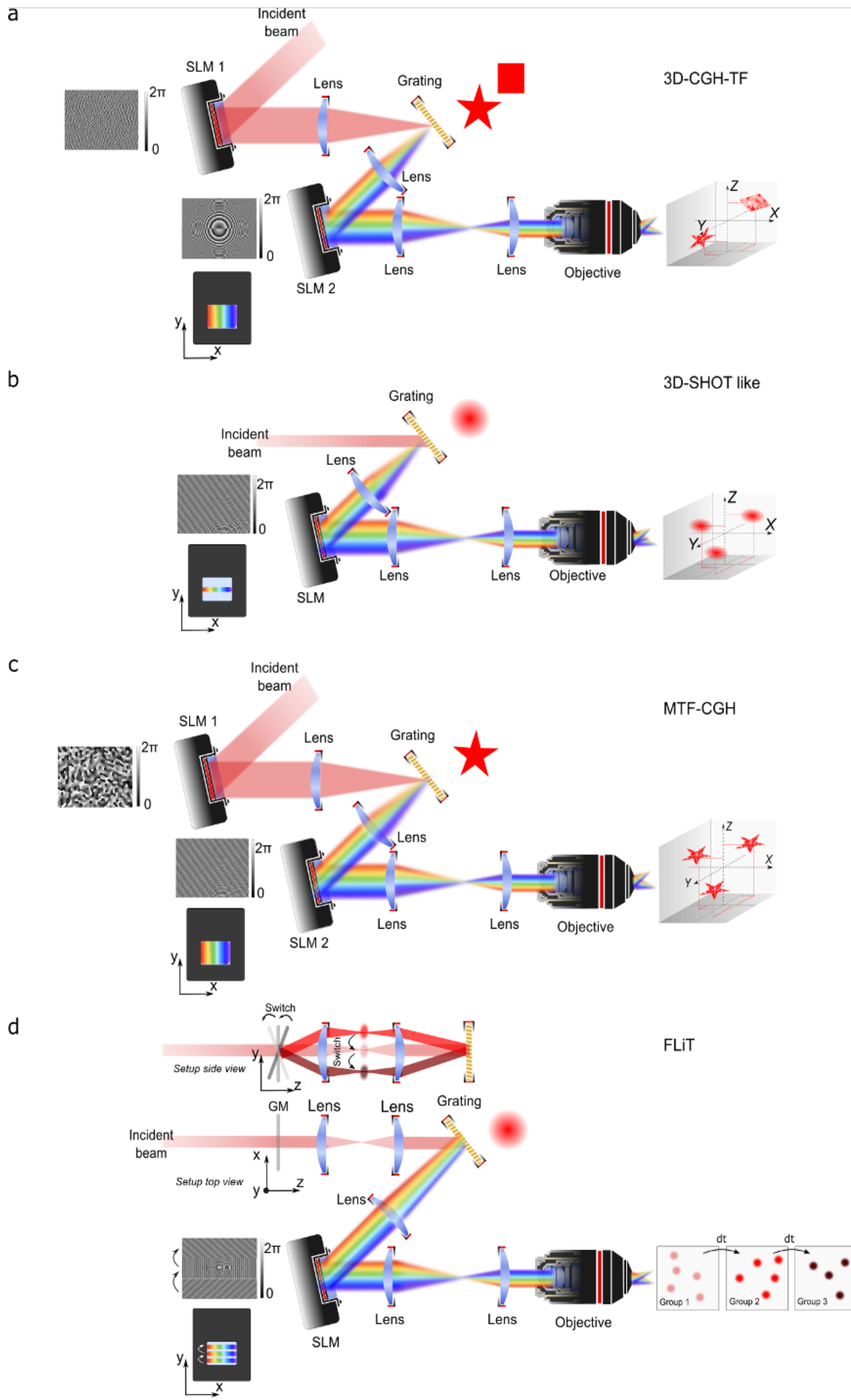
(A) In optogenetics, wavefront engineering is most commonly implemented using phase modulation since this approach maximizes light throughput. Phase modulation relies on the fact that light propagates at different velocities through media with different refractive indices (denoted n0 and n1 respectively). Propagation through a medium with a spatially varying refractive index introduces relative delays (known as phase shifts) between different parts of the wavefront. Liquid crystal on silicon spatial light modulators (LCOS-SLMs) are most widely used for implementing phase modulation. This relatively mature technology permits the dynamic generation of arbitrary wavefronts. Commercially available devices exhibit high pixel counts, minimal dead space between pixels and reasonably fast (tens of milliseconds) switching times. (B) The phase shift imparted by each pixel of an LCOS-SLM is determined by the voltage across the cell which corresponds to a particular orientation of the birefringent liquid crystal molecules and a consequent effective refractive index. (C) Simple wavefront shaping using Fourier holography and the most-intuitive “Gratings and Lenses” approach. In this configuration, the SLM is positioned at a focal length, f, prior to a lens. The output pattern is the Fourier transform of the modulated wavefront following the SLM and is generated in the focal plane (a distance f after

the lens). For clarity, transmissive SLMs are shown in the schematic diagrams but, in practice, reflective SLMs are used for most optogenetics applications. (i) Consider the ideal case of a plane wave incident on an SLM. When a constant voltage is applied to all SLM pixels, the profile of the wavefront is not distorted following propagation through the SLM, and hence is focused by the lens to a diffraction-limited spot in the focal plane. (ii) The diffraction limited spot can be displaced laterally throughout the focal plane by applying appropriate voltages to the SLM pixels such that the SLM imparts a linear phase ramp (grating) to the wavefront. (iii) Similarly, axial displacements can be obtained by imprinting the phase of a suitable lens profile on the wavefront using the SLM. (iv) Three-dimensional displacements can be obtained by a combination of phase ramps and lenses. (v) The simplest way to generate multiple spots with arbitrary three-dimensional positions is to apply voltages corresponding to a linear sum of the individual phase shifts to the SLM pixels. However, this approach generally results in an inhomogeneous, pattern-dependent, distribution of intensity between different spots since the amplitude of the complex field (LC-SLMs are usually only used to modulate phase) is neglected. This can be remedied by using an weighted iterative algorithm (typically a variant of the Gerchberg-Saxton approach) to optimize the hologram. (D)(i) Computer generated holography (CGH) can also be used to generate extended patterns. For optogenetics applications, CGH has been used to generate circular spots with diameter matched to the neuronal soma. (D)(ii)Schematic representation of the optical path used for generalized phase contrast (GPC), which is a different, interferometric, method used for wavefront shaping. In GPC, is a different, interferometric, method used for wavefront shaping for optogenetic applications. Schematic representation of the optical path used for generalized phase contrast. The beam is modulated using an SLM, which is used to impart a phase shift to the portion of the beam corresponding to the desired pattern. In case of binary GPC, the SLM usually imparts a π phase shift to the pixels within the pattern and 0 to those outside. A phase contrast filter (PCF) located in the Fourier plane (FP) after the first lens (L1) typically imparts a π phase shift to the "synthetic reference beam" (unmodulated field) with respect to the field that does not pass through the PCF. The different portions of the beam are recombined by L2 in the Image Plane (IP), where the modulated and synthetic reference fields interfere to form the desired, speckle-free, pattern.




**Supplementary Figure 6. Temporal focusing.**

In conventional 2P microscopy, a high numerical aperture objective is used to spatially focus light from an ultrafast (pulsed) laser source to a diffraction limited spot. In this configuration, 2PE is usually limited to a femtolitre volume in the vicinity of the focal point. However, for most optogenetic applications, this volume does not contain a sufficiently large number of actuators to adequately modulate the cellular parameter under investigation. Hence it is generally necessary to rapidly scan the excitation beam or to increase the lateral beam extent in order to excite actuators throughout the cell membrane. In general, increasing the lateral extent of the beam waist also increases its axial extent (due to diffraction) which results in a deterioration of axial resolution. Temporal focusing (TF) is widely used in 2P optogenetics to ameliorate the axial resolution of extended illumination patterns. TF is based on the use of a dispersive element (such as a diffraction grating) which introduces delays between different portions of the excitation beam, and increases the effective pulse duration at all planes outside of the temporal focus; the plane where the different frequency components are recombined **(b)**. For temporally focused 2PE, the axial resolution is independent of the lateral beam extent and can approach that of a diffraction limited spot [287]. Panel **a** depicts a schematic diagram to illustrate the simplest implementation of temporal focusing. An optical element (a diffraction grating) is used to spatially separate the spectral frequencies ("colors") of laser pulses. The different spatial frequencies are recombined at the focal plane of the microscope objective. (**b**) Spatial and temporal focusing maximize photon density, and, consequently, rates of two photon excitation at the focal plane. Spatial focusing (left) using a lens creates an axial gradient in photon density which decays quadratically with axial distance from the focal plane (inset). In temporal focusing (right), the duration of laser pulses increases with distance from the focal plane.


a
Incident beam
SLM 1
2π
0
Lens
Grating
3D-CGH-TF
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X
Y
y
x
b
Grating
3D-SHOT like
Incident beam
Lens
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SLM
Z
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Incident beam
SLM 1
MTF-CGH
Lens
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SLM 2
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Lens
Grating
Incident beam
Setup top view
Lens
2π
0
dt
dt
Group 1
Group 2
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Lens
Lens
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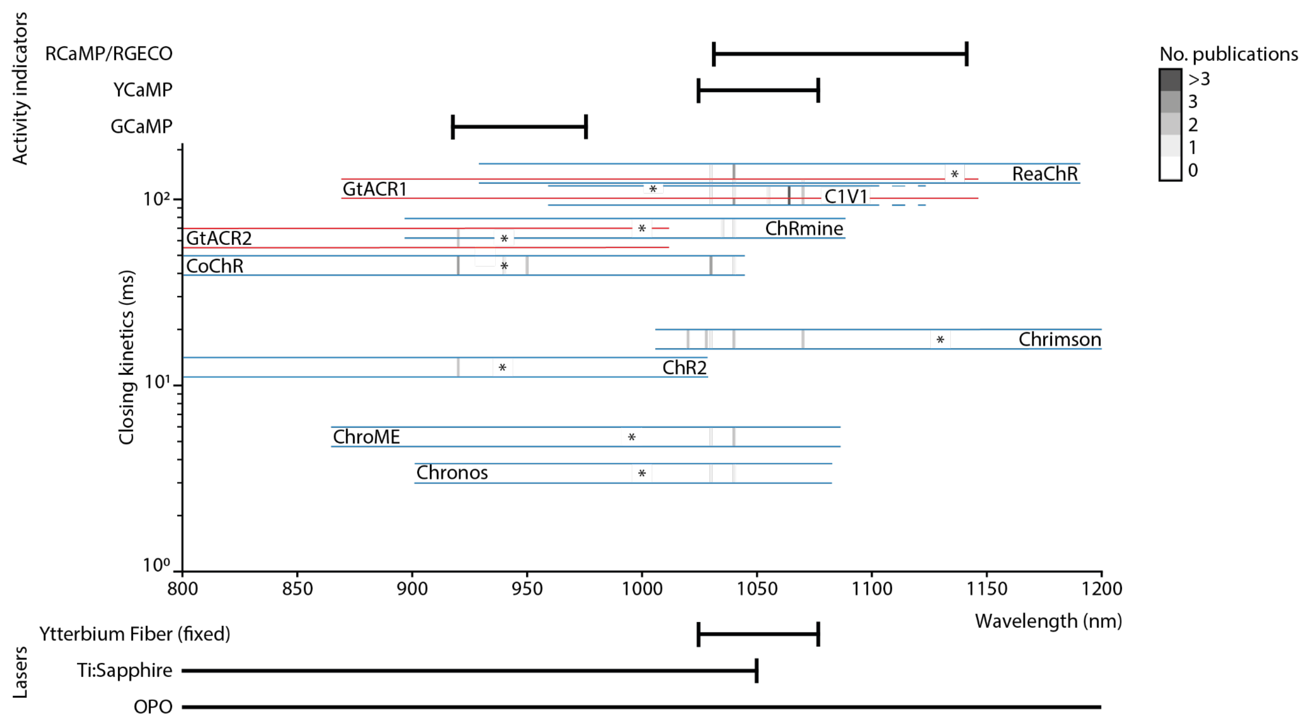
SLM

## Supplementary Figure 7. Comparison of current methods for 3-dimensional light shaping

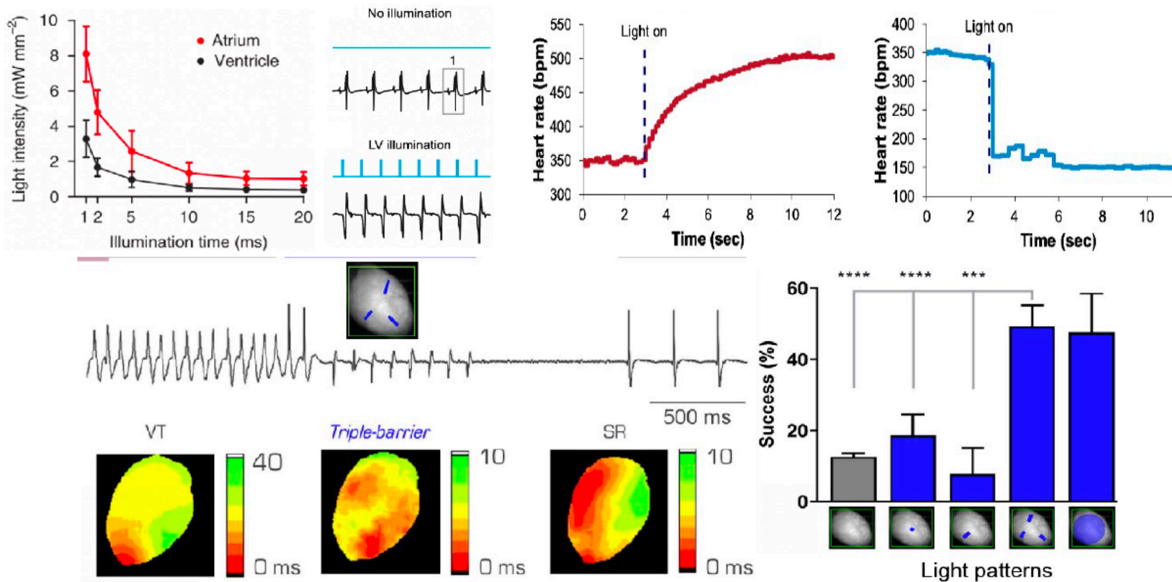
Generation of multiple extended temporally focused shapes is achieved in three-steps : (i) the beam amplitude shaping to control the shape and size of the illumination spot(ii) temporal focusing to axially confine the light absorption (iii) 3D spatial multiplexing to replicate the temporally focused shape in the excitation volume. This can be done in multiple ways depending on the characteristic of the generated pattern.

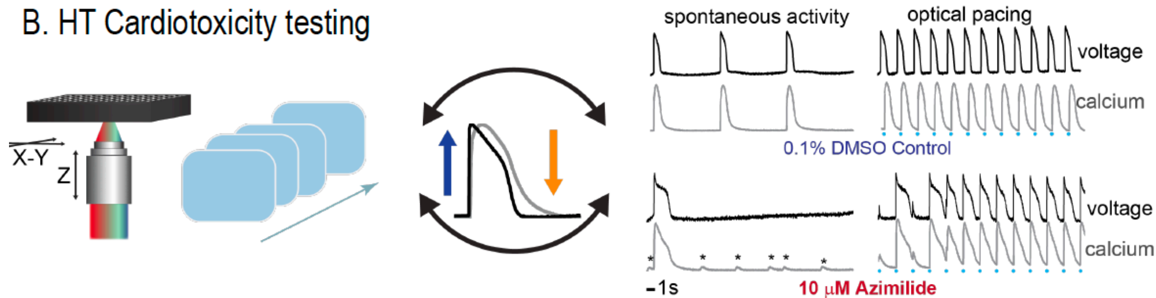
**a)** The generation of multiple shapes, temporally focused, in 3D requires 2 LC-SLMs, SLM1 and SLM2, addressed in vertical tiles (parallel to the orientation of the grating lines and orthogonal to the linear dispersion), each tile addresses a different pattern (defined in SLM1) at a defined axial plane (defined in SLM2). Precisely, on SLM1 each tile generates a 2D holographic shape (here illustrated by a star and a square), on the TF grating. SLM2 performs an independent axial displacement for each shape by modulating the corresponding beams with the corresponding lens effect[148].
**b)** The generation of multi temporally focused Gaussian beam (3DSHOT), requires 1 SLM which multiplexes in 3D the temporally focused gaussian spot generated at the TF grating. SLM2 is in this case illuminated with a temporally focused line or with an extended spot using an optical diffuser[139].
**c)** The generation of multiplexed temporally focused holographic spots of adjustable size and shape is achieved using a first LC-SLM (SLM1) which generates an holographic shape at the TF grating, and a second one, SLM2, uniformly illuminated[149], which multiplexes in 3D the temporally focused holographic shape.
**d)** For fast sequential light patterning, a collimated beam is reflected by a galvanometric mirror (GM) onto the diffraction grating and collimated onto the multiplexing SLM in the form of a horizontal (orthogonal to the orientation of the grating lines) line. A deflection of the beam by the GM results in a translation of the illuminating line on the SLM, addressed with *n* independent, tiled holograms. This leads to fast switching of different groups of (2D or 3D) light patterns.




**Supplementary Figure 8. Spectral ranges for two photon excitation of channelrhodopsins and genetically-encoded calcium sensors**.

A summary of the two-photon excitation spectra and closing kinetics of some of the most commonly used opsins. For each opsin, the horizontal bars span the wavelengths over which the peak normalized photocurrent is greater than 60% of the maximum value (indicated by an asterix) and are coloured according to whether the channelrhodopsin predominantly conducts cations (blue) or anions (red). The vertical grey lines indicate activation wavelengths previously used in pubmed-indexed articles. The number of articles for a given wavelength is indicated by the shade of grey (see legend). Notably, and unlike the single photon case, the wavelengths used for two photon excitation of channelrhodopsins rarely coincides with the spectral peak but instead are clustered at 920 nm and between 1030 – 1070 nm. These clusters correspond to commercially available fixed wavelength laser sources with sufficiently high peak pulse energies to permit the photoactivation of multiple cells. Whilst tunable sources such as Ti:Sapphire lasers, optical parametric oscillators (OPO's) and optical parametric amplifiers (OPA's) provide greater flexibility in terms of excitation wavelength, commercially available versions do not currently offer comparable average powers to the fixed wavelength ytterbium fiber sources. For reference, the excitation wavelength ranges of the most commonly used calcium activity indicators is also shown (top).




**Supplementary Figure 9. Cardiac applications of optogenetics.** A. Rhythm control can include optical pacemaking using atrial or ventricular sites (A. top left, with permission from [211]) or heart rate control (A. top right, with permission from [217]) by activating the sympathetic nervous system (increase) or the parasympathetic nervous system (decrease). Furthermore, restoration of normal rhythm can be achieved via cardioversion of ventricular tachycardia (VT) using patterned light in strategic shapes (A. bottom, with permission from [227]). B. High-throughput drug screening and cardiotoxicity testing is enabled by all-optical cardiac electrophysiology that allows high-content recordings and manipulation of the action potential by light. Voltage and calcium can be recorded optically under spontaneous conditions or under ChR2-mediated pacing in human iPSC-CMs (with permission from [230] and cardiotoxicity risk can be quantified for any drug.

**Cardiac Opportunities:**

A. Rhythm control:

Applications of optogenetics to the heart started about a decade ago [134,211] and are still lagging in scope and impact compared to neuroscience research [217]. The key benefits for clinical translation are sought in more versatile optogenetic pacing or suppression of wave propagation during arrhythmias, compared to currently used cardiac devices (pacemakers and cardioverter/defibrillators). Strategies for rhythm control, enabled by optogenetic actuators, aim to lower the energy needed to power the devices, thus extending battery life, and to eliminate discomfort and pain during classic cardioversion/defibrillation while being more effective in control of life-threatening arrhythmias and better quality of life. Optogenetics addresses these challenges through the ability to deliver longer lower-energy light pulses (electrical pulse duration is limited due to electrochemical toxicity via Faraday effects) and through cell-specific genetic targeting to engage the fast conduction system, e.g. His bundle or Purkinjie fibers for pacing [214], [218], or to target specifically the myocytes and avoid unintended contractions of thoracic skeletal muscle, diaphragm and vocal cords as pain-inducing electrical defibrillation does [219]. Computational modelling of the action of optogenetic tools in the heart has been developed to help explore strategies for control of arrhythmias, both with excitatory/depolarizing opsins and with inhibitory/hyperpolarizing opsins [136,213,214,220,221].

For optogenetic cardiac pacing to become a viable clinical alternative to the very successful and safe electronic pacemakers, it needs to offer much lower energy to essentially eliminate battery replacement. Optogenetic control of arrhythmias may be a more viable clinical application. Atrial arrhythmias, such as atrial flutter and atrial fibrillation, have increased over 3-fold in the last 50 years, affecting a large proportion of the ageing population. They are potential targets for optogenetic cardioversion due to smaller tissue thickness and better accessibility [210,216]. Ventricular arrhythmias are typically more complex, can be lethal and will require a spatially distributed strategy of light application to terminate [213] [227] [215]. Autonomic control of cardiac function, via the sympathetic and the parasympathetic system, can be an attractive target for the cell-specific optogenetic tools (Suppl. Figure XX A). Increasing parasympathetic tone or suppression of sympathetic activity have been recognized as anti-arrhythmic tools in various cardiac pathologies. Several recent studies have demonstrated the use of excitatory opsins (ChR2) targeting the parasympathetic system in transgenic

mice [222,225] or viral delivery of inhibitory opsins (ArchT) targeting the sympathetic system in dogs after acute ischemia [288]. Such neuro-cardiac applications may find quicker path to the clinic as the light delivery may be implemented through easier to implant subcutaneous small LED-based devices, similar to the electrical vagal stimulators.

B. High-throughput all-optical cardiac electrophysiology:

All-optical cardiac electrophysiology, combining optogenetic actuators and optical/optogenetic sensors [23,229,230], just like in neuroscience, offers immediate adoption and translation. Cardiotoxicity testing is particularly crucial in development of any new pharmaceutical and it is required as part of the drug development pipeline. High-throughput optogenetic methods with patient-derived cells enable new pursuits in personalized medicine [289-291], Suppl. Figure XX B.

Bidirectional voltage control, using for example ChR2 and ArchT, in conjunction with an optical voltage readout (Quasar1) can be used to explore drug effects on ion channels, similar to the classic voltage clamp [292]. Dynamic clamp, that reacts in real time to control the voltage applied to a cell, can be realized using optogenetic tools (ArchT) and computer-controlled LEDs to "inject" modifying current that yields a more mature phenotype in human induced pluripotent stem cell derived cardiomyocytes, iPSC-CMs [233].

High-content, high-throughput cell-based assays using optogenetic sensors and actuators and human iPSC-CMs represent the near-term translation of this technology to the cardiac area. They are more scalable and can provide more comprehensive information (about voltage, calcium and mechanical responses) compared to the automated planar-patch systems, due to the non-contact nature of interrogation by light. These assays can be deployed at the preclinical testing for all drugs to uncover electrophysiological and mechanical abnormalities beyond the currently used compound testing in heterologous systems on potassium voltage-gated channel subfamily H member 2 (also known as hERG) channel blocking, which a prime target for drugs. Regulatory agencies around the world are considering a shift to such more comprehensive cardiotoxicity assays with human iPSC-CMs [290]. Some high-throughput all-optical platforms have been already translated in the industrial setting for drug discover in cardiac and other applications [231,293,294].

To advance personalized medicine, optogenetic tools have been used for disease modelling with patient-derived iPSC-CMs, for chronic stimulation to improve maturity of these cells and obtain more adult-like phenotype [232]. Such approaches may help efforts towards regenerative medicine. Autologous sources of somatic cells, transduced with optogenetic tools, can be potentially used for in vivo cell delivery as alternative to classic gene therapy for tissue regeneration or rhythm control.

Specific Requirements and Challenges:

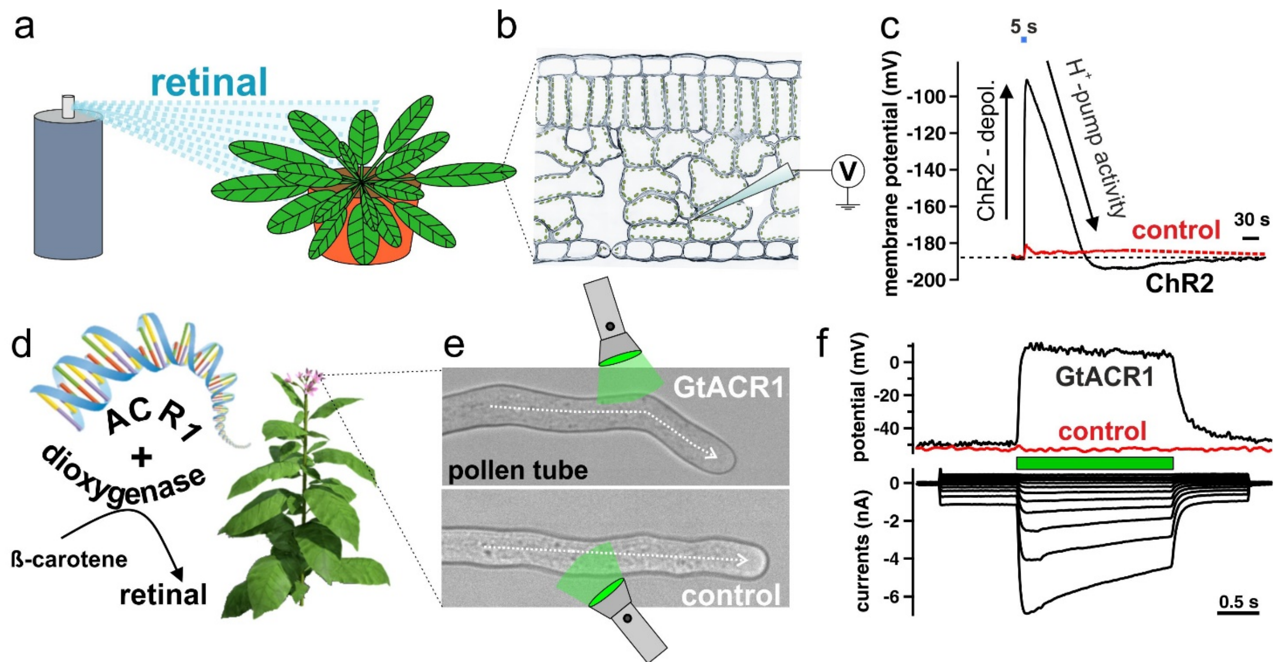
Longer-term in vivo clinical applications face the challenges of genetic modification of the hard-to-access cardiac muscle, potential immune responses, realizing embedded miniaturized light control devices that are reliable and safe [217].

The main working cell type in the heart are ventricular myocytes. AAV9 is the most efficient serotype for targeting these cells in vivo when using an ubiquitous or specific promoter, such as Myh6, [223]. The other cells of interest in the heart that have been targeted optogenetically include atrial myocytes using NPPA promoter and a "gene painting" technique with ReaChR-AAV9 [216], and targeting cardiac fibroblasts (WT1), cardiac macrophages (CX3CR1)[295], fast conduction system cells (Cx40) [218], sympathetic neurons (tyrosine hydrolase, TH)[224], and parasympathetic neurons (choline acetyltransferase, ChAT) [222,225] in Cre-Lox transgenic mouse models. Most studies thus far have been performed in rodents – transgenic mice and in mice and rats with AAV9 as a delivery vector topically or systemically using tail-vein injection. To translate the approaches to larger animals, more work is needed in finding minimally-invasive ways of delivery to the heart, and in characterizing immune responses. Previous clinical trials on gene therapy for cardiac disorders found that large portion of the patients had antibodies against the viral vectors used, thus reducing the efficacy of the therapy.

Most of the published studies have used ChR2 (the H134R variant) as excitatory opsin, with occasional ReaChR use. Inhibitory pump opsins, ArchT and Halo, have been successfully used to hyperpolarize the membrane in myocytes and to terminate activity, although they are less successful in termination of complex arrhythmias compared to the depolarizing (ChR2) opsins, likely due to anode-break re-excitation of the tissue. Cl- based opsins, GtACR1[296], are not trivially used in cardiac cells and tissues due to different Cl- gradient compared to neurons. In general, more efficient and fast inhibitory opsins are desirable for arrhythmia control applications. Also, there may be a niche for step-function-like depolarizing options that have fast recovery from inactivation as "clamping" tools in arrhythmia management. Bidirectional closed-loop control can make optical clamp [G] a reality at

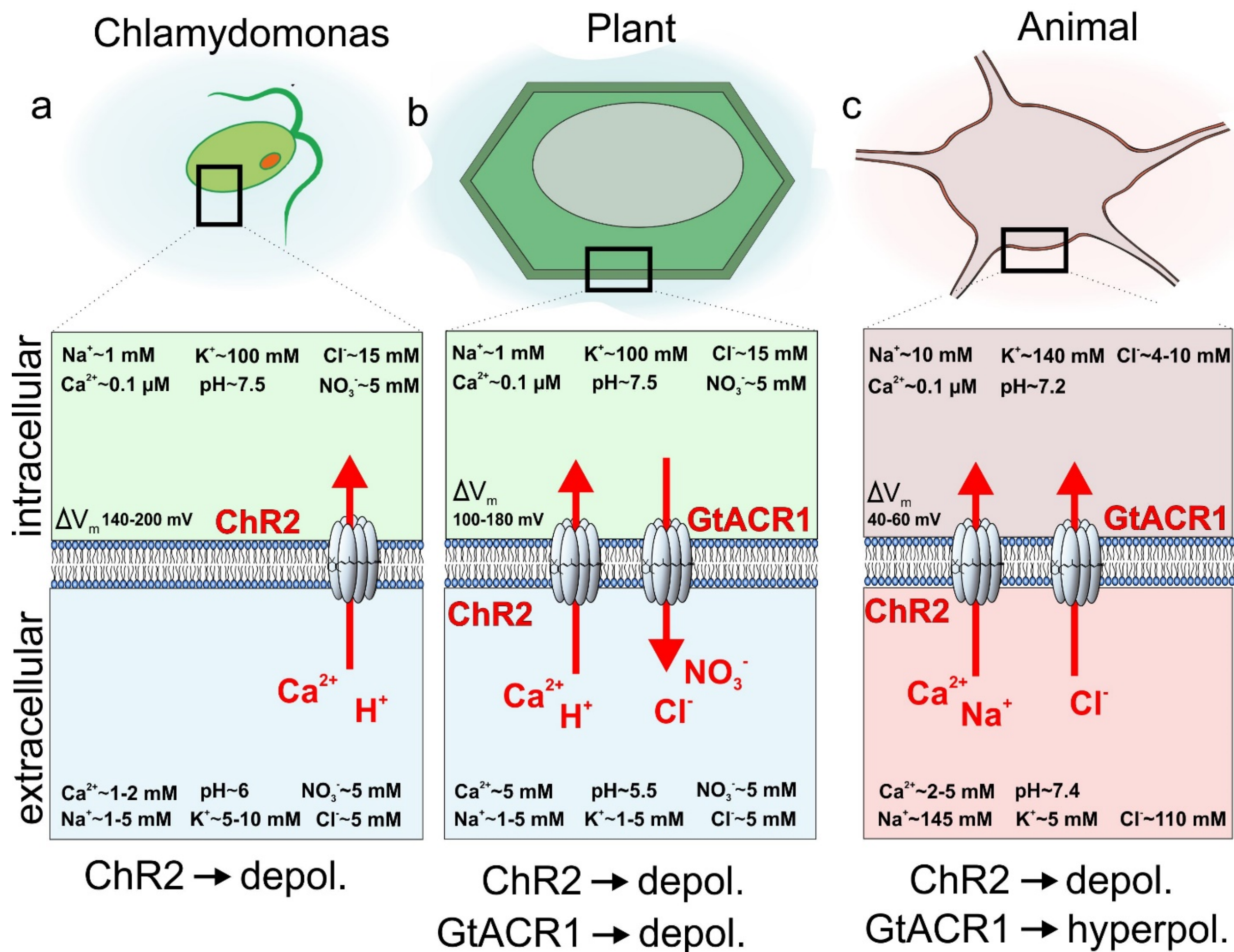
the whole organ level yet it will require spectral compatibility to accommodate excitatory, inhibitory opsins as well as means for optical readout of voltage.

Light delivery to the dense thick ventricular wall is non-trivial and presents even bigger challenges compared to neuroscience applications due to the vigorous contractions and difficulties to stabilize the devices. Light penetration in the haemoglobin-rich heart muscle would require operation in the near-infrared and the deployment of opsins excitable in that range. The atria are thinner (human atria are < 5mm) and along with autonomic nerves present easier targets.



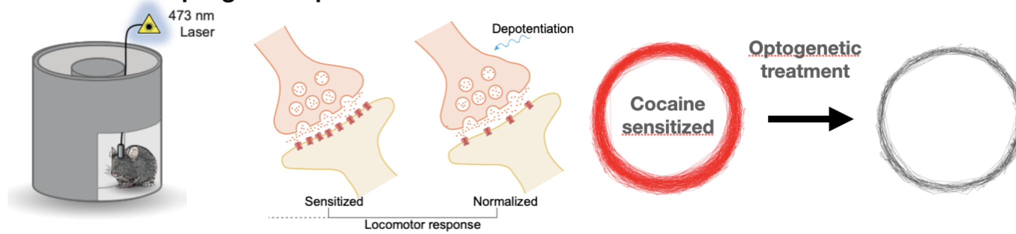

**Supplementary Figure 10. Feeding or *in planta* production of retinal enables rhodopsin-based plant optogenetics**

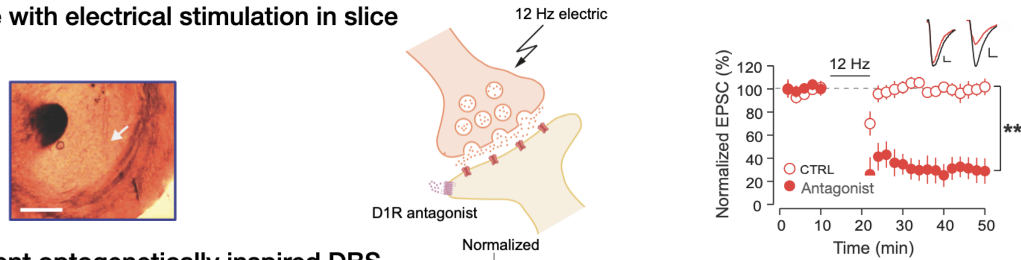
(a) Scheme of an Arabidopsis plant upon external feeding of retinal for (b) simultaneous optical stimulation and membrane voltage recordings of leaf mesophyll cells with intracellular electrodes as indicated. Scheme in (b) from Reyer et al., 2020 [238] with some minor modifications. (c) A representative membrane potential trace of a ChR2-XXL expressing (black) and wild type (red) *Arabidopsis thaliana* control leaf when extracellular retinal was added and a 5 s lasting blue-light pulse was applied. The ChR2-XXL-mediated depolarization is counteracted by the depolarization-activated $H^+$-pump activity at the plasma membrane when the blue-light pulse is terminated. Pictures and the example traces (from a to c) provided by courtesy of Dirk Becker and Antonella Reyer. (d) Stable introduction of the GtACR1 gene and that of retinal producing bacterial dioxygenase into the tobacco plant allows the functional expression of GtACR1. This allows to steer (e) pollen tube growth direction caused by (f) large directed membrane potential depolarizations (upper trace) induced by GtACR1-driven photocurrents (lower trace) of up to several nA amplitude in the presence of green light (532 nm, 0.18 mW $mm^{-2}$).

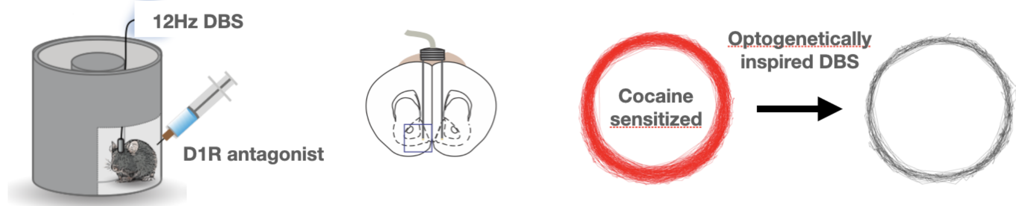



**Supplementary Figure 11. Ion gradients determine optogenetic response in plant and animal cell systems**

A scheme comparing the ion concentration gradients and direction of ion flow across the plasma membrane of Chlamydomonas as well as plant and animal soma cells when activating either ChR2 or GtACR1. In (a) Chlamydomonas and (b) plant cells the extracellular space facing the plasma membrane is low in ion concentration and pond water-like while (c) the extracellular space in most animal cells is rich in NaCl and sea water-like. The $Ca^{2+}$ inward gradient is comparable in most biological systems, but unlike animal cells, photosynthetically active cells have a steeper (2 pH units) inward $H^+$ gradient generating a steeper electric potential gradient (ΔVm). Therefore, ChR2 activation leads to depolarization, which in Chlamydomonas and plant cells is mostly based on an influx of $Ca^{2+}$ and $H^+$ but in animal soma cells on $Ca^{2+}$ and $Na^+$. Due to the reversed anion gradients in plant and animal cells, GtACR1 activation leads to depolarization in plant cells but hyperpolarisation in the soma of neurons; however cell specific conditions related to $Cl^-$ reversal potential with respect to the resting membrane potential determine GtACR1´s action in other cell types.




**Supplementary Figure 12. Optogenetically inspired DBS.** a. The process starts with a successful optogenetic reversal protocol, inducing LTD at mPFC to NAc synapses (see also Fig 6). b. Using electrical stimulation in the NAc (left), a protocol is established that emulates the optogenetic stimulation (middle). Here D1R needed to be blocked (antagonist) to allow the induction of LTD in D1R-MSNs (right). c. In vivo implementation combines DBS with systemic injection of the antagonist (left) to reverse locomotor sensitization (right).

## Supplemental Table 1: Studies of optogenetic vision restoration in animal models

| Targeted major retinal cell types | Optogenetic tools | Vectors and delivery route | Promoters | Animal models | Electrophysiological assessments | Threshold Light sensitivity (photons/cm$^2$s$^1$)* | Behavioral tests | Major results | References |
|---|---|---|---|---|---|---|---|---|---|
| **Ganglion cells** | ChR2 | AAV2; Intravitreal | CAG | *rd1* mice, wild-type mice and rats | WCR, MEA, VEP | At low $10^{15}$ | | Restoration of ChR2-mediated light response in the retina and VEP in the cortex | 13 |
| | ChR2 | AAV2; Intravitreal | CAG | RCS rats | VEP | | | Restoration of VEP in rats | 297 |
| | Melanopsin | AAV2; Intravitreal | CMV | *rd1* mice | WCR | ~$10^{13}$ (~100 Lux) | PLR Open-field avoidance, Water maze | Restoration of light response in the retina, PLR, light avoidance, and light/dark discrimination with melanopsin | 77 |
| | ChR2 | *Transgenic* | Thy-1 | *Rats, photo deplete* | WCR, VEP | At low $10^{15}$ | OMR | Restoration of optomotor response. | 298 |
| | ChR2, eNpHR | AAV2; Intravitreal | CMV CAG | *rd1* mice | WCR, MEA | ChR2: at low $10^{15}$; eNpHR: at low $10^{16}$ | | Restoration of OFF light response in the retina | 299 |
| | ChR2 | AAV2; Intravitreal | CAG | Normal C57BL/6J mice | | | | Report of long-term expression safety of ChR2 in the retina | 300 |
| | ChR2 | AAV2; Intravitreal | CAG | Marmoset | MEA | | | Functional expression of ChR2 and ChR2-mediated response in the non-human primate retina. | 200 |
| | ChR2 | ChR2; transgenic mice | Thy 1 | *rd1* mice | EXR, OIR | ~$10^{13}$ | OMR, Water maze | Restoration of light responses in the retina and cortex but failed to restore functional vision in *rd1* mice based on behavioral tests | 301 |
| | ChR2 | AAV2; Intravitreal | CAG | RCS rats | VEP | At low $10^{15}$ | OMR | Restoration of optomotor response | 302 |

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|---|---|---|---|---|---|---|---|---|---|
| | ChR2 | AAV2; Intravitreal 0.5 – 5 x$10^{10}$ GP | CAG | RCS rats | | | | Absence of immunologically harmful reactions of ChR2 in vivo | 303 |
| | ChR2/eNpHR | Electroporation | CaMKIIa | Rabbit | WCR, ECR | | | Creating ON and OFF antagonistic center-surround receptive field with motif targeting | 304 |
| | ChR2 | ChR2 transgenic mice | Thy 1 | *rd1* mice | MEA | | OMR | Restoration of vision with retina's neural code | 305 |
| | ChR2/eNpHR | AAV2; Intravitreal | CAG/ EF1α | C57BL/6J mice | MEA | | | AAV-mediated targeting with subcellular motifs to create ON and OFF center-surround antagonistic receptive field | 124 |
| | ChR2-C132C/T159S | AAV2; Intravitreal | CAG | *rd1* mice; | MEA | At low $10^{13}$ | | Creation of ChR2 mutants with improved operational light sensitivity | 306 |
| | mVChR1 (ChR1/vChR1 chimera) | AAV2; Intravitreal | CAG | RCS rats | VEP | | OMR | Creation of a red-shifted mVChR1 for vision restoration | 191 |
| | mVChR1 | AAV2; Intravitreal | CAG | RCS rats | | | | Absence of immunologically harmful reactions of mVChR1 *in vivo* | 307 |
| | ReaChR | AAV2; Intravitreal | hSyn 1 | *rd1* mice Macage Human retina | WCR, MEA | | Locomotion, Light avoidance | Restoration of vision using ReaChR for the toleration of high light intensity | 192 |
| | ChR2 | ChR2 transgenic mice | Thy-1 | *rd1* mice | MEA | ~$10^{15}$ | | Improvement of restored vision by the blockade of spontaneous activity | 308 |
| | ChR2 | ChR2 transgenic mice | Thy-1 | *rd1* mice | MEA | ~$10^{14}$ | | Improvement of restored vision by dampening spontaneous activity | 309 |
| | | AAV2/8(Y733F); Subretinal | CAG | *rd1* | MEA, OIR | ~$10^{13}$ | PLR, Light-dark box, | Restoration of image forming | 310 |

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|---|---|---|---|---|---|---|---|---|---|
| | Human melanopsin | | | | | | Object recognition | vision with melanipsin | |
| | ChR2 + mVChR1 | ChR2 transgenic rats; AAV2; Intravitreal | Thy-1 CAG | *Rats;* N-methyl-N-nitrosourea to induce retinal degeneration | WCR, VEP | | | Co-expression of ChR and mVChR1 improved wavelength sensitivity but not light sensitivity | 311 |
| | ChR2 and Melanopsin | AAV2; Intravitreal | hSyn 1 | Canine rd model | MEA | | | Report of long-term expression safety of ChR2 and melanopsin in dogs | 312 |
| | ChR2-L132C | AAV2; Intravitreal | SNCG | *rd1* mice | MEA, VEP | ~$10^{14}$ | | The use of RGC specific promoter improved light sensitivity | 313 |
| | ChR2-L132C/T159C | AAV2; Intravitreal | CAG | Macaques | | | | Characterization of expression of ChR2 in the macaque retina | 314 |
| | ChR2-mutants | AAV2; Intravitreal | CAG | Transgenic (Opn4$^{-/-}$ Gnat1$^{-/-}$ Cnga3$^{-/-}$) mice | | | PLR, OMR | Restoration of vision using a triple knock-out blind mouse model and optomotor assay | 315 |
| | CoChR-H94E/L112C/K264T | AAV2 or AAV2.7m8 (Y444F); Intravitreal | CAG | Transgenic (Opn4$^{-/-}$ Gnat1$^{-/-}$ Cnga3$^{-/-}$) mice | MEA | ~$10^{12}$ | OMR | Restoration of visual acuity and contrast sensitivity under ambient light condition. | 194 |
| | Human melanopsin | AAV2/8; subretinal | CMV | *rd1* mice | ERG, VEP | | Locomotion | Report of long-term expression safety of human melanopsin in the retina, but only transient restoration of visual response. | 316 |
| | Green cone opsin | AAV2/2-4YF; Intravitreal | hsyn-1 | *rd1* mice | MEA | ~$10^{11}$ | Light avoidance, Exploratory behavior | Restoration of vision with high light sensitivity, light adaptation, and pattern discrimination. | 197 |

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|---|---|---|---|---|---|---|---|---|---|
| | ReaChR; ChR2-L132C | AAV2; Intravitreal | hsyn-1 | Macaques | MEA | | | Restoration of visual acuity of 20/72 estimated based on MEA recordings | 317 |
| | ChrimsonR | AAV2.7m8; Intravitreal | CAG | Macaques | MEA | At low $10^{15}$ | | Restoration of visual acuity of 20/249 estimated based on MEA recordings | 193 |
| | ChrimsonR | AAV2; Intravitreal | CAG | Macaques | ROI | | | Response persistent over one year | 318 |
| | mVChR1/ChR2/CoChR chimera | AAV2; Intravitreal | CAG | RCS rats | VEP | $\sim 10^{13}$ | OMR | Report of improved light sensitivity than mVChR1 | 319 |
| **ON bipolar cells** | ChR2 | Electroporation | 200 bp mGluR6 enhancer | *rd1* mice | WCR, MEA, VEP | $\sim 10^{14}$ | OMR, Locomotion | Restoration of visual responses by targeting ChR2 to ON BCs | 202 |
| | ChR2 | Sc-AAV2/8; Subretinal | 200 bp mGluR6 enhancer | *rd1, rd10, rd16* mice | MEA | At low $10^{16}$ | Water maze | AAV-mediated targeting ChR2 to ON BCs with mGluR6 promoter | 320 |
| | ChR2(L132C) | AAV2/8BP2; Subretinal | *4x 200bp mGluR6 enhancer* | *rd1* mice | MEA | At low $10^{15}$ | | Improved transduction efficiency with AAV2/8BP2 in ON BCs targeting | 203 |
| | ChR2(H134R) | AAV2.7m8; Intravitreal | 200 bp mGluR6 enhancer | *rd1* mice | MEA , VEP | $\sim 10^{14}$ | Light avoidance | Improved transduction efficiency with AAV2.7m8 in ON BCs targeting | 204 |
| | Melanopsin/mGluR6 chimera | AAV2 and Transgenic mice | 200 bp mGluR6 enhancer | *rd1 x transgenic* mice | WCR, MEA, OIR | $\sim 10^{12}$ | OMR, Water maze | Creation of melanopsin/mGluR6 receptor chimera for vision restoration | 198 |
| | Rhodopsin | AAV2; Intravitreal | 200 bp mGluR6 enhancer & CAG | *rd1* mice | MEA | $\sim 10^{12}$ | Locomotion, Natural scenes stimulation | Restoration of vision with rhodopsin | 195 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Rhodopsin | AAV2; Intravitreal | *4x 200bp mGluR6 enhancer* | *rd1* mice | MEA, VEP | ~$10^{12}$ | Light avoidance, Mater maze, Fear conditioning | Restoration of vision with rhodopsin. | 196 |
| | ChR2 mutant + Chrimson | AAV2; Intravitreal | 200 bp mGluR6 enhancer | *rd10* mice | | | Water maze | Restoration of vision using dual wavelength ChRs | 321 |
| | CoChR-L112C | AAV2; Intravitreal | mGluR6 with intron and enhance; CAG | TKO (Opn4$^{-/-}$ Gnat1$^{-/-}$ Cnga3$^{-/-}$) mice | MEA | | PLR, OMR | RGC expression exhibited higher efficacy than ON BC targeting | 205 |
| | ChR2 mutant + Chrimson | AAV2; Intravitreal | 200 bp mGluR6 enhancer | *rd10* mice | WCR | ~$10^{15}$ | Water maze, OMR | Restoration of vision using dual wavelength ChRs | 322 |
| | Green cone opsin | AAV2.7m8; Intravitreal | 770En_454P (hGRM6) | *rd1* mice | | | OMR | Restoration of vision by targeting green cone opsin to ON BCs with improve human mGluR6 promoter | 323 |
| **Cones** | eNpHR | AAV2/8; Subretinal | hRho hRo | *rd1* and Cnga3$^{-/-}$ Rho$^{-/-}$ mice | WCR, MEA, VEP | At low $10^{13}$ | OMR, Light avoidance | Restoration of vision by targeting surviving cone photoreceptors | 206 |

WCR: whole-cell recording; ECR: extracellular recording; OIR: optical imaging recording; MEA: multi-electrode array recording; OMR: optomotor response; VEP: visually evoked potential; PLR: pupillary light reflex.
* The Threshold Light sensitivity is estimated based on retinal electrophysiological recordings.

**Supplemental Table 2: Clinical trials and pre-clinical studies of optogenetic therapies for vision restoration.**

| Company | Optogenetic tool | Targeted retinal cells | Vector construct & delivery route | Link to clinical trial | Status | Results and notes |
|---|---|---|---|---|---|---|
| RetroSense Therapeutics | ChR2 | RGCs | AAV2-CAG-ChR2; Intravitreal injection | https://clinicaltrials.gov/ct2/show/NCT02556736 | Phase 1/2a | RetroSense was acquired by Allergen in 2016. No result has been released. |
| GenSight Biologics | ChrimsonR | RGCs | AAV2.7m8-CAG-ChrimsonR-tdTomato; Intravitreal injection | https://clinicaltrials.gov/ct2/show/NCT03326336 | Phase 1/2a | Partial recovery of visual function in a blind patient using light-stimulating goggles (source: https://www.gensight-biologics.com/product/gs030-for-retinitis-pigmentosa/) |
| Bionic Sight | Chronos | RGCs | AAV-Chronos-FP; Intravitreal injection | https://clinicaltrials.gov/ct2/show/NCT04278131 | Phase 1/2 | Patients can see light and motion, and, in some cases, can detect the direction of motion after treatment (source: https://www.fightingblindness.org/research/bionic-sight-s-optogenetic-therapy-enables-blind-patients-to-detect-light-and-motion-in-early-trial-217). |
| Nanoscope Therapeutics | ChR2 mutant + Chrimson | ON BCs | AAV2-mGluR6-ChR2 mutant/Chrimson-FP; Intravitreal injection | https://clinicaltrials.gov/ct2/show/NCT04945772 | Phase 2 | Improvement of visual acuity and vision restoration in the ambient light environment (source: https://nanostherapeutics.com/2021/06/03/nanoscopes-optogenetic-gene-therapy-restores-clinically-meaningful-vision/) |

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