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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-insensitive 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

34 functions and has motivated the development of advanced optical methods for patterned
35 photostimulation. Optogenetics has revolutionized neuroscience during the past 15 years and is
36 primed to have a similar impact in other fields, including cardiology, cell biology and plant sciences.
37 In this Primer we describe the principles of optogenetics, review the most commonly used optogenetic
38 tools, illumination approaches and scientific applications and discuss the possibilities and limitations
39 associated with optogenetic manipulations across a wide variety of optical techniques, cells, circuits
40 and organisms.

41
42

43 **[H1] Introduction**

44

45 Light-dependent processes are abundant in nature, occurring in diverse organisms from bacteria and
46 algae to plants and animals and are used for energy capture and storage, to regulate developmental
47 processes and to mediate orientation¹⁻³. While the photoreceptors involved in light-sensing have been
48 studied for decades, the use of such proteins for actuation of naturally light-insensitive cells began
49 only in 2002 with the expression of the *Drosophila* rhodopsin and its associated signaling proteins in
50 neurons⁴. The discovery of channelrhodopsin, identified in the same year in the green alga
51 *Chlamydomonas*, in conjunction with the almost universal cellular availability of the chromophore
52 all-trans retinal (Vitamin A) in most cells and organisms, accelerated the progress of this new
53 technology. Almost in parallel with the initial application of Channelrhodopsin-2 (ChR2) in isolated
54 neurons in 2005⁵ and brain slices in 2006⁶, ChRs were rapidly adapted for use in living model
55 organisms, including chicken embryos⁷ and *C. elegans* in 2005 ref⁸, *Drosophila* in 2006 ref⁹, freely
56 moving mice in 2007 ref¹⁰, zebrafish in 2008 ref¹¹ and even non-human primates in 2009 ref¹². The
57 first experiments that pointed toward potential therapeutic applications were performed in 2006,
58 pioneered by the expression of ChR in inner retinal cells to restore vision in blind mice¹³. Optogenetics
59 is based on sensory photoreceptors sequences from microalgae, fungi or bacteria. But, only the
60 combination of photoreceptor-encoding DNA with control elements like promoters and targeting
61 sequences, typically derived from genes expressed selectively in target, allows the protein allows
62 specificity not only in the choice of target cell population but also in the subcellular compartments to
63 be manipulated. The DNA-constructs are incorporated into target cell populations, tissues or living

64 organisms using vectors such as plasmids, viral vectors or bacteria using established transformation
65 technologies (Fig. 1).

66 The robust function and revolutionary utility of ChR2 in neuroscience resulted in the
67 description and application of many photoreceptor subtypes, engineered or retrieved from genomic
68 or cDNA databases, progress in protein expression and targeting, **microelectrode [G]** and **optrode [G]**
69 technology, and finally the combination of **optogenetic actuators [G]** with optical fluorescent reporter
70 systems and high-resolution subcellular imaging, accelerating the interdisciplinary growth of
71 optogenetic technology with unprecedented pace. The need to control neuronal activity with increased
72 spatial resolution has in turn motivated the development of advanced optical methods for patterned
73 photostimulation. Digital mirror devices (DMD) or liquid crystal spatial light modulators (LC-SLM)
74 coupled to single or two-photon excitation have enabled single and multi-target excitation in vitro and
75 in vivo with single-spike precision and cellular resolution in head-restrained and freely-moving
76 animals¹⁴. Optogenetics has developed as a basic science methodology for dissecting biological
77 functions; while it has initially been adopted by neuroscientists to study brain function and
78 dysfunction, it has expanded into new research fields such as cardiology, microbiology, immunology,
79 parasitology and plant science. These developments are culminating in highly-anticipated clinical
80 applications, as envisioned in the early days of optogenetics, including multiple clinical trials
81 currently in progress for selected human disorders. A crude timeline of key breakthroughs in
82 optogenetic technology is displayed in Figure 2.

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

92

93 **[H1] Experimentation**

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

101

102 **[H2] Selecting the correct actuator**

103 When designing an optogenetic experiment, the first considerations should be the cellular parameter
104 to be modulated and the available optogenetic actuators for such an endeavor. An enormous number
105 of light-switchable tools have been developed for controlling ion fluxes and membrane voltage, G-
106 protein signaling, regulation of second messengers such as Ca^{2+} , cAMP, cGMP, IP3, receptor tyrosine
107 kinases (TRKs), organelle repositioning, transcription and translation (Fig. 2). Most actuators rely on
108 photoreceptors or light sensing modules of natural origin, although photoswitchable synthetic organic
109 compounds have also been employed¹⁵. The use of photoswitchable synthetic organic compounds is
110 also known as chemooptogenetics or photopharmacology, and the interested reader might consult
111 related reviews^{15,16}. Many light-modulated actuators have been described that do not rely on opsin
112 proteins. While this Primer is focused on the opsin-based toolbox, the reader might find more
113 information about non-opsin-based optogenetic tools in several excellent recent reviews^{17,18}.

114

115 **[H3] Light-activated ion channels**

116 Until recently the most widely applied optogenetic photoreceptor was Channelrhodopsin-2 from the
117 alga *Chlamydomonas reinhardtii* (known as CrChR2 or simply C2) and its variant ChR2-H134R^{8,19}.
118 Presently, almost 900 ChR sequences have been identified, including many with properties superior
119 to those of the original prototypes (Figure 2)²⁰. ChRs may be subdivided into cation or anion
120 conducting channels, termed CCRs and ACRs respectively. CCRs typically conduct multiple types of
121 cations with high preference for protons. Na^+ selectivity varies widely among different CCRs²¹ and
122 divalent cations are only poorly conducted under most physiological host conditions. Whereas there
123 are no Ca^{2+} -selective CCRs available to date, continuous metagenomic screening recently revealed a

124 new class of potassium selective channels (KCRs)²². ACRs are selective for a number of anions,
125 similar to most human anion channels²³.

126 In host cells, Na⁺ and H⁺-conducting CCRs can be used as depolarizing actuators, whereas the
127 action of ACRs depends on the chloride reversal potential in the targeted cells or subcellular
128 compartment (Box 1). ACRs may clamp the voltage to near the resting potential and inhibit action
129 potential firing by shunting inhibition (Box 1). However, in cardiac cells, immature neurons and
130 presynaptic terminals, chloride gradients are less pronounced and ACRs may depolarize the cell
131 membrane^{24,25}. In plants, the chloride gradient is always directed outward, and ACR activation will
132 generally lead to membrane depolarization. Thus far, KCRs have been applied under highly controlled
133 in vitro conditions, but — once established for in vivo experiments — hold major promise for
134 optogenetic inhibition in all variants of cells and host model systems.

135 Our current molecular understanding of ChRs mostly relies on *CrChR2*, which has been
136 extensively studied and modified with respect to kinetics, ion selectivity, inactivation and absorption
137 wavelength^{26,27} revealing principles that have been successfully transferred to other CCRs
138 (Supplementary Figure 1). Recently discovered channelrhodopsins such as ChRmine and KCRs
139 belonging to a new family of cation conducting ChRs hold great promise, but understanding of their
140 molecular mechanism is only beginning to emerge^{28,29}. The maximal color sensitivity of known ChRs
141 so far spans from 445 nm for *TsChR* to 610 nm for the ChrimsonSA mutant and Ruby-ACR³⁰⁻³²
142 (Figure 2). Such distinct color sensitivity may allow the combination of different ChRs within the
143 same experiment for activation and inhibition of the same or different cells. However, all rhodopsins
144 absorb blue or UVA light to a certain extent due to transition to higher excited state levels. This has
145 to be taken into consideration when combining multiple rhodopsins in a single or multiple cell
146 populations (Supplementary Figure 2). For **bidirectional voltage modulation [G]** for example, the
147 more potent actuator should be selected to absorb at the shorter wavelength (Figure 2) thereby
148 allowing for lower light powers used in the blue range, which will in turn minimize the undesired
149 activation of the red-shifted actuator. Another consideration is the reversal potential of the conducted
150 ion. In nature, as well as in neuronal experiments, ACRs operate closer to the reversal potential than
151 Na⁺ or H⁺ conducting depolarizing CCRs. While it is possible to co-express two opsins using two
152 separate viral vectors, this approach inevitably leads to incomplete co-expression in all cells. To
153 overcome this draw back, several constructs have been engineered which allow tandem expression of
154 two opsins from the same vector. The most prominent examples are eNPAC which coexpresses

155 eNpHR3.0 and ChR2(H134R) initially linked by a 2A selfcleaving peptide³³, and BiPOLES³⁴, which
156 combines the red-shifted CCR Chrimson with the blue-shifted GtACR2 in a single targeting-
157 optimized fusion construct³⁵. Due to the stoichiometric membrane expression, equal photocurrents near
158 the cellular resting potential and comparable light sensitivities of both channel modules, BiPOLES
159 outperforms previous bicistronic combinations of ChR2 with different ion pumps^{59,60} and guarantees
160 subcellular colocalization and selective red-light excitation for multicolor applications. A
161 combination of optogenetics and chemogenetics has been exemplified by direct fusion of slow cycling
162 step function rhodopsins (SFOs) with a luciferase that produces light upon peripheral injection of its
163 small molecule substrate. These luminopsins allow direct light stimulation by optical fibers, while at
164 the same time providing chemogenetic access in awake and anesthetized animals in vivo^{61,62}.

165

166 [H3] Light-driven pumps

167 The first application of optogenetics for neuronal silencing was achieved with the chloride
168 pump halorhodopsin³⁶. However, since the discovery of ACRs, the interest in optogenetic silencing
169 of animal cells by light-driven pumps has decreased in animal cells because pumps require higher
170 expression levels and higher light intensities for sufficient ion turnover (Supplementary Figure 3). In
171 contrast, in plants — which naturally hyperpolarize their membranes and drive secondary transporters
172 via H⁺ pumps — light-driven H⁺ pumps are valuable tools. The advantage of light-driven pumps is
173 their high ion specificity and robust electric response that depends less on the ionic composition of
174 the surrounding buffers and the membrane voltage. Light-driven chloride pumps such as NpHR³⁷ or
175 Jaws³⁸ allow reliable — although often weak — neuronal inhibition in synaptic terminals, where the
176 action of ACRs is difficult to predict due to variable and elevated intracellular chloride concentrations
177³⁹. Pumps may be successfully used in small compartments such as neuronal vesicles, lysosomes⁴⁰,
178 mitochondria or thylakoids, where the action of ion channels is poorly defined due to the lack of free
179 ions⁴¹. In the plasma membrane the use of light-driven ion pumps requires caution because both proton
180 and chloride pumps can drive non-physiological ion concentrations in neurons and trigger off-target
181 effects, including a transient increase of the chloride reversal potential, leading to excitatory actions
182 of the inhibitory neurotransmitter GABA and alkalization of presynaptic terminals, leading to
183 increased spontaneous neurotransmission^{39,42}.

184

185 [H3] Optogenetic control of biochemical signaling pathways

186 Animal rhodopsins are G-protein coupled receptors (GPCRs) and animal vision is the most studied
187 G-protein signaling pathway. A pioneering study demonstrated that bovine rhodopsin expression may
188 be used to activate G-protein signaling in *Xenopus* oocytes but without describing the signaling
189 mechanism⁴³. However, the off-response of rod-rhodopsins remained uncontrollable in the absence
190 of rhodopsin kinase and Arrestin, and responses severely declined upon repetitive stimulation. The
191 responses of $G_{i/o}$ activating cone rhodopsins⁴⁴⁻⁴⁶ or Gs-specific box jellyfish opsins⁴⁷ declined faster,
192 but were still not tightly controllable. The solution was approached by revitalizing melanopsin OPN4,
193 which can be switched on and off with blue and yellow light, albeit incompletely due to substantial
194 overlapping spectra of the dark-state and signaling-state⁴⁸⁻⁵⁰ and only the UV-sensitive Lamprey
195 Parapinopsin (PPO) with its green-absorbing signaling state offered efficient on and off switching
196 with a dual color light source⁵¹⁻⁵⁴.

197 GPCR signaling depends on many properties of the receptors, including substrate binding
198 kinetics, G-protein specificity and timing of activation and receptor inactivation, which in total cannot
199 be fully mimicked by rhodopsins. One way to more selectively mimic the activity of a specific GPCR
200 is to engineer hybrids between structurally related opsins and GPCRs (optoGPCRs)^{55,56}. OptoGPCRs
201 open new and possibly more specific routes for the analysis of intracellular signaling pathways
202 compared to unmodified rhodopsins whereas the dynamics of G-protein coupling and pathway
203 recruitment still has to be approached by testing various expression levels and light regimes. However,
204 these optoGPCRs cannot be simply transferred to another cell type because G-protein promiscuity
205 might activate unwanted pathways^{48,57}. With OptoGPCRs the application of G-protein activation has
206 enormously broadened the optogenetic actuator toolbox. These tools will be well-suited for
207 temporally-defined modulation of non-excitabile cells, potentially including glial cells in the brain and
208 other non-neuronal cell types⁵⁸.

209 Receptor tyrosine kinases (RTKs) are another large family of cell surface receptors that sense
210 growth factors and hormones to regulate a variety of cellular behaviors by target phosphorylation.
211 Engineered light sensitive epidermal growth factor receptor (EGFR1) and the fibroblast growth factor
212 receptor 1 (FGFR1) have shown robust light activation of both RTK-receptors and cellular signaling
213 in human cancer and endothelial cells and faithful mimicking of complex mitogenic and morphogenic
214 cell behavior⁵⁹. Cobalamin-binding domain (CBD)⁵⁹ and tropomyosin receptor kinase B (TrkB) have
215 been fused to RTKs to yield light-sensitive receptors^{60,61}. Fusions with TrkB have high specificity for
216 the target proteins, although their application range is narrow and the constructs need to be optimized

217 for every new application. Moreover, one drawback is that cobalamin-based light sensors or
218 phytochrome-based light sensors generally require addition or cellular synthesis of the cofactor
219 molecules, making their potential for in vivo applications more complex than the application of the
220 retinal-based photoreceptors.

221

222

223 [H3] Second messengers

224 Photoactivated cyclases (PACs) have been employed for direct control of the second messengers
225 cAMP and cGMP. The soluble bPAC from *Beggiatoa spp.* is a tandem of BLUF-type light sensors
226 (blue-light sensors using FAD (Flavin adenine dinucleotide) (Figure 2) with C-terminal adenylyl
227 cyclases. These optogenetic actuators show millisecond-range on-kinetics upon photostimulation and
228 a second-range off-kinetics in the dark (bPAC $\tau_{\text{off}} = 12$ s)⁶². Coexpression of bPAC with the small
229 prokaryotic potassium-channel SthK (PAC-K silencer) in two-component optogenetic approaches has
230 been exploited for long lasting neuronal hyperpolarization in cardiomyocytes as well as in fly, mouse
231 and zebrafish neurons providing high operational light sensitivity but low time resolution ⁶²⁻⁶⁵.
232 However, color modification is only possible within a small range around 470 nm and occasional
233 residual dark activity has been observed ⁶⁶. New spectral windows were opened by introducing
234 Rhodopsin Guanylyl Cyclases (RGCs), which are cyclases with N-terminally linked rhodopsins.
235 These rhodopsin-cyclases (RhCs) are characterized by low dark-activity, effective light absorption (ϵ
236 $> 32000 \text{ M}^{-1}\text{cm}^{-1}$) and the promise of flexible color tuning ⁶⁷⁻⁶⁹. RhCs show millisecond-range off-
237 kinetics, are naturally GTP selective and are convertible into ATP cyclases by genetic engineering.
238 Some members of the fungal Chytridiomycota may use heterodimeric RhGCs, with one blue or green
239 sensitive rhodopsin catalyst, and a second near infrared sensitive modulator (NeoR, $\lambda_{\text{max}} = 660\text{--}700$
240 nm). These NeoRs might allow to extend the usable spectral range into the superior infrared spectral
241 window ⁷⁰.

242 [H3] Protein abundance

243 Control over the concentration of selected proteins within a cell has been a long-standing goal, and
244 has stimulated the interest of protein engineers for decades. The most obvious point of intervention is
245 the regulation of transcription. Previously explored concepts were based on the connection of DNA-
246 binding proteins to a photoreceptor such as Phytochrome, FKF1 or VIVID (LOV-proteins), or CRY.
247 Upon illumination, these photoreceptors bind to their signaling partner proteins PIF3,

248 GIGANTEA/Tulips or CIB respectively, with bound components of the transcription machinery as
249 VP16 or VP64. In light, the transcription component is attracted to the promoter region of interest by
250 the photoreceptor and signal-protein interaction leading to the assembly of the transcription complex
251 and initiation of transcription. But, the used GAL4-DNA binding domains have to be incorporated
252 into the model organism (reviewed in^{71,72}). To address any promoter of interest, programmable DNA-
253 binding proteins zinc finger-DNA binding proteins⁷³, TALEs⁷⁴ and deactivated Cas9 have been
254 functionalized as the second generation of transcription regulators^{75,76}. The main caveat for Cas9
255 application is the prolonged occupancy of Cas9 at its DNA binding site, especially in situations where
256 the DNA is not cleaved, which disturbs gene expression prior to the intended start of the experiment⁷⁷.
257 Inserting a LOV-domain into an anti-CRISPR protein like AcrIIA4 or AcrIIC3 (CASANOVAs)
258 overcomes this problem and makes Cas9 binding better controllable. This approach works reliably in
259 HEK cells, but has not been rigorously tested for non-embryonic cells such as neurons¹⁰⁴.

260

261 [H2] Targeting strategies

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

277

278 [H3] Transgenic expression of optogenetic tools

279 Transgenic expression is the simplest approach to implement since it requires only the maintenance
280 of an opsin-expressing animal strain (Figure 3a)⁷⁸ or the crossing of two strains of animals. The latter
281 involves a driver line — engineered to express a recombinase or transcription regulator like Cre and
282 Flpo (in rodents) or a Gal4 driver (in zebrafish) in a particular cell population — and an animal strain
283 expressing a conditional opsin gene under the control of the relevant driver (Figure 3b). The F1
284 progeny of such a cross will express the opsin gene in all cells in which the driver protein is expressed,
285 and will therefore be amenable to optogenetic manipulation simply by illuminating the targeted brain
286 region. The approach is simple to implement, but one should consider potential caveats, including the
287 presence of axons from neurons in other brain regions, which might be activated along with the cell
288 bodies in the illuminated region. In mice, expression of ChR2 or eNpHR3.0 from the ROSA26 locus
289⁷⁹ can be quite weak and not universally sufficient to drive activity in every neuron subtype.
290 Expression of opsin genes from the TIGER locus⁸⁰ showed stronger opsin expression and might
291 therefore be useful for some target neuron populations. However, this approach requires generation
292 and/or breeding of a dedicated animal strain for every targeted neuron population and thus lacks the
293 versatility and cost-efficiency of viral vector-based approaches. Another potential confound is
294 unintentional targeting in some driver lines (see for instance⁸¹), making the verification of driver lines
295 advisable⁸².

296 [H3] **Viral vector targeting**

297 Lentiviral or adeno-associated viral vectors (AAVs) can be engineered to encode optogenetic
298 actuators and delivered either directly to the brain parenchyma or through systemic injection to target
299 either specific brain regions or brain-wide populations, respectively. Targeting of genetically-
300 identified neuronal populations is achieved either by using the tissue tropism of the virus serotype and
301 a cell type-specific promoter or enhancer (Figure. 3c), or by injecting the viral vector into a transgenic
302 recombinase-expressing animal strain (Figure 3d). Promoter-based viral vector targeting is attractive
303 since it does not require the maintenance of a specific animal strain for every target neuron population,
304 and can also be applied in non-genetic models. However, the limited viral payload size — particularly
305 of AAVs — prohibits the use of most native promoters. The list of minimal promoter or enhancer
306 sequences that have been validated to specifically express in defined neuron populations is quite
307 restricted. However, this field is rapidly expanding^{83,84} and is further diversified by synthetic
308 approaches⁸⁵.

309 [H3] **Circuit-based viral vector targeting**

310 The most commonly used retrograde viral tracer is AAVretro⁸⁶, which can be taken up by presynaptic
311 terminals and travel in retrograde to express at the soma of long-range projecting neurons (Figure 3e).
312 The herpes simplex virus 1 (HSV1) and canine adenovirus 2 (CAV2) both have retrograde targeting
313 abilities, but these are less readily available and have been shown to impair the health of targeted
314 neurons, particularly over longer expression times of weeks to months⁸⁷.

315 [H3] Systemic delivery of AAV-PHP capsids

316 Targeting sparse brain-wide populations is beneficial for some experimental configurations. For
317 example, structural imaging of dendritic spines in cortex or excitation of a randomly-selected sparse
318 ensemble in a given brain region. For this purpose, AAV-PHP vectors have been engineered to cross
319 the blood-brain barrier with high efficiency (Figure 3f). The AAV-PHP serotypes allow targeting of
320 diverse central and peripheral nervous system neurons^{88,89}. The same capsids can be used with Cre-
321 dependent AAV expression plasmids to allow sparse brain-wide expression in a genetically-defined
322 neuronal subtype. However, the efficiency of AAV-PHP serotypes in crossing of the blood brain
323 barrier can vary in different mouse strains⁹⁰.

324 [H3] Electroporation

325 Concentrated DNA can also be injected into the cerebral ventricles followed by in utero
326 electroporation⁹¹⁻⁹³, enabling the study of neural cell fate determination and migration or cortical
327 layer specific expression.

328

329 [H2] Compartment-specific functions

330 The effective current resulting from a light-gated channel conductance can vary dramatically due to
331 local ion concentration gradient differences. For neuroscience applications, this is particularly crucial
332 for use of ACRs. At the somatic and dendritic compartments, this is an advantage, as they can be used
333 for shunting inhibition. In contrast, ACRs can exert excitatory effects in axons and presynaptic
334 terminals, in which the intracellular chloride concentration is higher (Box 1). Ion pumping rhodopsins
335 on the other hand translocate the ion over the membrane in a predetermined direction, which can be
336 an advantage due to the increased control of ion flux. However, the pumping-induced
337 hyperpolarization and elevation in ion concentration can also have side effects, like the alkalization
338 of presynaptic boutons⁹⁴ or an artificial increase in intracellular chloride⁴². Similarly, the effects of
339 G-protein coupled animal rhodopsins on neuronal activity strongly depend on the given second

340 messenger cascade in the local compartment. For instance, in the soma and dendrite, $G_{i/o}$ signaling
341 can activate G protein-coupled inward rectifying potassium channels whereas in the presynaptic
342 compartment the $G_{i/o}$ pathway mainly acts through inhibition of voltage gated calcium channels and
343 cAMP signaling ⁵⁴.

344

345 [H2] Optimizing expression and targeting

346 Beyond single channel conductance, one of the main factors determining maximal photocurrent is the
347 number of functional opsin molecules in the membrane, which in turn depends on expression level,
348 protein-folding efficacy, retinal binding affinity, membrane trafficking and protein turnover rate. The
349 expression level of a transgene can be controlled via promoter strength and transgene copy number.
350 The opsin-folding efficacy and protein stability was shown to depend on the availability of the
351 chromophore retinal ⁹⁵. While retinal availability does not seem to be a limiting factor in mammalian
352 tissues, it needs to be routinely supplemented in the food of invertebrate model systems and some
353 cultured cell lines. In plants, the absence of retinal can be compensated for by its synthesis via
354 expression of a bacterial β -dioxygenase that facilitates rhodopsin expression. A common issue with
355 unmodified opsin expression cassettes is aggregation of the synthesized protein in the endoplasmic
356 reticulum (ER). To overcome this limitation, trafficking motifs involved in transport of membrane
357 proteins along the secretory pathway to the cell surface were utilized to improve plasma membrane
358 targeting (Supplementary Figure 4). The most widely-used trafficking motifs utilized were first
359 described for the potassium channel $K_{ir}2.1$ — these motifs enhance ER export as well as Golgi-to-
360 plasma membrane trafficking ⁹⁶, resulting in higher plasma membrane localization and increased
361 photocurrents in animal ⁹⁷ as well as plant cells ⁹⁸.

362 Further optimization of functional expression can be achieved by adjusting the linkers between the
363 opsin and the often co-expressed fluorophore, mutating potential ubiquitination sites, and screening
364 random mutations in the opsin coding sequence ⁹⁹. Beyond improved photocurrents, targeting an opsin
365 to a selected subcellular compartment can be used to investigate the function of the chosen
366 compartment, such as the mitochondria, synaptic vesicles, lysosomes or ER (Supplementary Figure
367 4), or to use the differential effects of ion channels discussed above. Somatic restriction has been
368 successful in increasing the specificity of single-cell stimulation by reducing inadvertent modulation
369 of nearby neurites ¹⁰⁰⁻¹⁰⁵, as well as in reducing ACR-mediated axonal excitation ²⁴. Somatic

370 restriction has the added effect of accelerating the effective photocurrent off-kinetics, due to the
371 elimination of photocurrents arising from distal neurites in the illuminated tissue volume, as these are
372 low-pass filtered while traveling along the neurite to the somatic compartment.

373 While targeting microbial rhodopsins to presynaptic vesicles is feasible⁴⁰, enrichment of rhodopsin
374 abundance in the axonal plasma membrane has not been achieved. Cytosolic proteins can be enriched
375 in the axon by mRNA shuttling motifs. However, local rhodopsin translation in the axon has not been
376 successfully applied, potentially due to a lack of transmembrane protein synthesis in the vertebrate
377 axon¹⁰⁶.

378

379 **[H2] Light delivery techniques**

380 Although the vast majority of advanced light targeting approaches have been developed with the
381 specific applications of neuronal and cardiac optogenetics in mind, these methods are generalizable
382 and are beginning to be applied to other systems¹⁰⁷. Optogenetics is readily applicable to light-
383 accessible preparations such as cultured cells, tissue slices, transparent organisms such as zebrafish
384 larvae or to the cortical surface of the mammalian brain, allowing for extensive flexibility in light
385 delivery. For whole circuit or brain region optogenetics, light needs to reach the target with sufficient
386 irradiance to induce opsin activation. Ideally, light should be guided into the target structure with
387 minimum damage to the tissue. In behaving animals, stimulation should also be conducted with
388 minimal disruption to the measured behavior, limiting implantable weight and tether stiffness. Whole
389 circuit/region optogenetic stimulation is typically carried out using a multimode optical fiber, guiding
390 the light from the source to the target (Figure 4a-b). Optical fibers targeting a deep brain region can
391 be permanently implanted by attaching a fiberoptic implant to the skull using dental cement. The
392 dimensions of the fiber and its optical properties strongly influence the spatial profile of light reaching
393 the brain. Most commonly, flat-cleaved optical fibers are used. However, the high radiant flux density
394 necessary at the fiber tip to achieve a sufficient irradiance within the targeted volume, can lead to
395 heat-induced changes in neuronal activity and behaviour^{108,109}. It is therefore advisable to consider
396 tissue heating when planning the experiment and to use opsin-free light stimulated controls. One
397 approach to minimizing the irradiance required in optogenetic experiments is to maximize the
398 operational light sensitivity of the opsin used (Supplementary Figure 3). Another factor is wavelength,
399 as absorption is higher for shorter wavelengths and therefore the peak temperature increase is lower

400 for longer wavelengths at the same radiant flux density. Increased optical fiber diameter also reduces
401 the peak light power density. However, wider fibers also cause more tissue damage and have a higher
402 chance of illuminating blood vessels, which strongly absorb visible light and thus increase potential
403 heating-related artifacts. This tradeoff can be at least partially mitigated by the use of tapered optical
404 fibers (Figure 4b), which can be used to flexibly illuminate a large brain volume¹¹⁰.

405 In these conventional optogenetic experiments, visible light is mostly delivered non-specifically to
406 large tissue regions and genetic targeting strategies are used to express the optogenetic actuator in
407 specific cell types. This approach has enabled tissue function to be mapped with unprecedented
408 anatomical and cell-type specificity. However, widefield illumination synchronously activates or
409 silences entire populations of all opsin-expressing cells, which does not replicate the physiological
410 case: adjacent cells belonging to genetically defined classes have been observed to exhibit divergent
411 activity patterns. To investigate complex population activity patterns, whole-region optogenetics is
412 insufficient. DMD coupled to single-photon excitation have enabled single and multi-target excitation
413 in head-restrained and freely-moving animals and found in situ applications in control of excitation
414 waves underlying cardiac arrhythmias^{111,112,113,114}. However, the use of visible light has limited these
415 approaches to superficial brain layers or low scattering samples. Recent developments in opsin
416 engineering, optical microscopy and multiphoton laser source development have given rise to circuit
417 optogenetics¹¹⁵, which allows modulation of neuronal activity deep in scattering tissue with single-
418 spike precision and single-cell resolution (Figure 4c-e). Specifically, combining variants with
419 enhanced kinetics^{30,116-118}, higher conductance^{116,118,119} or shifted absorption peaks^{30,119,120} with
420 optimized targeting and expression strategies¹⁰¹⁻¹⁰⁴, enable neuronal control with single-cell, single-
421 spike precision at millisecond temporal resolution and the generation of action potential (Ap) trains
422 with high (50–100 Hz) spiking rates^{121,122}. In parallel, advanced optical techniques, based on two-
423 photon (2P) excitation (Box 2) have been developed to precisely guide light through tissue. The small
424 single-channel conductance of commonly used optogenetic actuators such as ChR2 (40–90 fS)¹²³, and
425 the limited number of channels or pumps recruited within a conventional 2P focal volume, mean that
426 it is generally necessary to use spiral scanning or parallel light shaping using computer generated
427 holography or the generalized phase contrast method (Supplementary Figure 5) combined with
428 temporal focusing (Supplementary Figure 6)¹⁴ to increase the portion of excited membrane^{124,125} and
429 to sufficiently depolarize a neuron to firing threshold or effectively silence it. Holographic light
430 multiplexing with spiral scanning¹²⁶ or ad hoc spatiotemporal shaping approaches (Supplementary

431 Figure 7) have been used to generate patterned illumination at multiple axially distinct planes
432 ^{116,127,128}. Multiplexing divides the available laser power between targets and thus requires powerful
433 lasers. Due to the higher peak photon density, amplified low-repetition rate (200 kHz – 10 MHz) fiber
434 lasers enable higher rates of 2P absorption compared to titanium:sapphire oscillators (at the same
435 average power) and can therefore be used to reduce the necessary power to generate physiological
436 signals ¹²⁹. Additionally, these sources deliver tens of Watts of power, facilitating the simultaneous
437 photostimulation of hundreds of cells throughout mm³ volumes. The combination of these
438 technologies has recently led to the first demonstrations of multi-target neural circuit manipulation
439 ^{104,118,130}.

440 The ability to control neuronal activity with single-cell precision and millisecond temporal
441 resolution allows to functionally probe neuronal networks beyond the resolution of synchronous
442 modulation of entire networks or genetically defined network components. For instance, using
443 temporally precise single-cell excitation in visual cortex and olfactory bulb, the minimal number of
444 co-activated cortical neurons necessary for visual perception ¹³¹ and the dependence of olfactory
445 perceptual detection on both the number of activated neurons and their relative spiking latency was
446 characterized ¹³². The requirement of high numerical aperture objectives has limited 2P-optogenetics
447 to circuits in superficial ($\leq 500 \mu\text{m}$) cortical areas of mouse brain, transparent zebrafish larvae ¹³³ or
448 in-vitro applications. Micro-endoscopes are small optical probes that can be inserted into living
449 tissues, and represent a promising solution to extend optical brain manipulation to deeper brain
450 structures both in combination with holographic spiral scanning ¹³⁴ or multi-temporally focused light
451 shaping approaches ¹³⁵. Three-photon (3P) optogenetics, which relies on longer wavelengths and
452 exhibits a cubic dependence of excitation efficiency on excitation power, could potentially be used to
453 stimulate neuronal circuits in deeper brain regions (600 μm – 1 mm) with single-cell resolution.
454 However, to date, 3P photostimulation has only been demonstrated in vitro ¹³⁶.

455

456

457 **[H1] Results**

458

459 **[H2] Output analysis**

460 When designing optogenetic experiments, care should be taken to verify the impact of the optogenetic
461 manipulation on the targeted cells. This can be achieved in a number of ways, including

462 electrophysiological recordings in vitro or in vivo, optical recordings with genetically-encoded
463 sensors, immediate early gene labeling and noninvasive imaging modalities. Below we outline the
464 major techniques used in such experiments, and the considerations that should be taken into account
465 when designing and performing such experiments.

466

467 **[H3] Electrophysiological recordings**

468 To interpret the results of optogenetic manipulations, it is often necessary to determine the extent of
469 optogenetic tool expression and its physiological effects on the targeted neurons. In the case of light
470 gated ion channels or pumps, recording the electrophysiological changes induced by the optogenetic
471 manipulation is the most direct way to characterize light-mediated effects (Figure 5a-c). As these
472 effects can vary greatly between cell types, brain regions and even viral serotypes¹³⁷, it is crucial to
473 validate the optogenetic effector in every new experimental system before proceeding to behavioral
474 or other functional readouts. To describe effects on the level of spike rates and timing, whole-cell
475 recordings are often not necessary. Instead, extracellular recordings are often used (Figure 5d-f), given
476 their higher throughput and minimal crosstalk with light delivery (see¹³⁸ for discussion of light-
477 induced electrical artifacts). However, higher frequency spiking activity does not necessarily indicate
478 increased synaptic transmission from the stimulated neurons¹³⁷. Synaptic depression and depletion of
479 neurotransmitter release can lead to erroneous interpretation and should be taken into account when
480 performing optogenetic excitation experiments, particularly with neuromodulatory and neuropeptide-
481 releasing neuronal populations.

482

483

484 **[H3] Optical recordings**

485 Fluorescent reporters are another common method for monitoring the effects of optogenetic
486 manipulations. These techniques enable recording from the same cells over several recording sessions
487 and the concurrent recording of high numbers of cells. However, given that optogenetics itself relies
488 on light delivery, fluorescent reporters can be efficiently integrated only if a spectral or light power
489 separation can be achieved to minimize the crosstalk between the recording and manipulation
490 modalities (Supplementary Figure 2). Additionally, when combining red-shifted Ca²⁺ indicators with

491 optogenetic actuators, extra care must be taken, as these can show blue-light-activated photoswitching
492 behavior that can resemble Ca^{2+} activity in their amplitude and kinetics ¹³⁹.

493 While genetically encoded calcium sensors continue to be the state of the art in terms of optical
494 activity readout, voltage indicators are gradually reaching a level of maturity that could allow for
495 wider adoption by the field ¹⁴⁰. Novel fluorescent sensors for neurotransmitters, neuromodulators and
496 other small molecules are continuously developed¹⁴¹⁻¹⁴³. Another approach to read out gross neuronal
497 firing rate changes is to characterize the expression of **immediate early genes [G]**, for instance via
498 immunohistochemistry on the protein level ¹⁴⁴ or on the mRNA level using quantitative PCR, in-situ
499 hybridization or single cell RNA sequencing ¹⁴⁵. Immediate early gene expression can be used to
500 determine the relationship between the modulation of specific neuronal populations and global brain
501 activity ¹⁴⁶. However, the temporal precision of this approach is limited to the average neuronal
502 activity over minutes to hours and, unless combined with targeted recombination approaches ¹⁴⁷, only
503 a single manipulation can be characterized per animal.

504 Although channelrhodopsin variants with peak single-photon (1P) excitation wavelengths spanning
505 the visible region of the electromagnetic spectrum have been engineered ²⁶, performing crosstalk-free,
506 multi-colour 2P experiments is not trivial. Ideally, spectrally orthogonal channelrhodopsins and
507 activity reporters would be chosen, but, unfortunately, the 2P action spectra of commonly used opsins
508 are extremely broad (Supplementary Figure 8) ²⁶. As previously introduced, opsins with red-shifted
509 action spectra exhibit persistent activation in the blue range, which coincide with wavelengths used
510 for 2P imaging of commonly used activity reporters (920 – 980 nm). One approach to reduce crosstalk
511 is to use opsins with fast kinetics. Although this approach does not eliminate sub-threshold network
512 perturbation, the (relatively) fast repolarization of neurons expressing channelrhodopsins with fast
513 off-kinetics means they are unlikely to fire action potentials due to excitation by the imaging laser
514 during scanning. Successful employment of this method requires careful titration of imaging
515 parameters, including imaging power, frame rate and field of view. This is an interim approach until
516 high efficiency blue-shifted opsins, red-shifted activity indicators and amplified lasers in the
517 appropriate spectral range become more widely available.

518

519 **[H3] Alternative recording modalities**

520 Electrophysiological and optical recording modalities both suffer from potential interactions with the
521 light required to excite optogenetic actuators. The **hemodynamic response [G]** is an alternative
522 physiological response to neural activity which can be exploited to report the impact of optogenetic
523 modulation. For superficial brain areas such as the cortex, the hemodynamic response can be
524 measured via intrinsic imaging^{148,149}, while functional magnetic resonance imaging¹⁵⁰ can be utilized
525 to record brain-wide hemodynamics. Although the noninvasive nature and the ability to measure the
526 hemodynamic response throughout the entire brain are major advantages, the main drawbacks are that
527 the temporal resolution of this approach is fundamentally limited by the specificity and kinetics of the
528 hemodynamic response itself and the limited spatial resolution of neurovascular coupling¹⁵¹. Heating
529 should also be taken into account here as it can directly impact the hemodynamic response¹⁵².
530 Functional ultrasound imaging is a rapidly developing technology which could be used to perform
531 brain-wide detection of neural activity triggered by localized optogenetic stimulation. Although this
532 method still relies on changes in neurovascular blood volume changes, it can be performed at a
533 fraction of the cost of functional MRI recordings and is rapidly advancing to allow better spatio-
534 temporal resolution and portability¹⁵³.

535

536 **[H2] Linking neural to behavioral readouts**

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

543 Choosing the locus of intervention may be instructed by previous literature, lesion experiments
544 and behavioral pharmacology. For example, we know that silencing the motor cortex with compounds
545 like muscimol or baclofen causes motor impairment while optogenetic stimulation elicits muscle
546 contraction¹⁵⁴. While gain of function experiments may be a starting point, cell type-specific
547 optogenetic inhibition of genetically-defined neurons in the motor cortex would provide a more
548 complex picture, better dissociating physiological motor response from an artificial perturbation¹⁵⁵.
549 Another way to determine the brain region and cell types of interest is the use of activity markers such
550 as the immediate early genes *c-Fos* or *Arc*. Finally, technological advances in wide-field optical

551 monitoring of intracellular calcium may allow to visualize the activity of large cortical areas^{151,156} and
552 selectively silence defined cortical regions transcranially¹⁵⁷. Alternatively, high density electrical
553 recordings^{158,159} can elucidate the activity of many neurons in deeper structures. This allows the
554 experimenter to identify circuits with activity patterns that may be relevant to the behavior to be
555 studied.

556 Observational experiments should be implemented to characterize the functional properties of
557 the cell population to be modulated (Figure 6). This may be achieved using electrophysiology in vivo
558 — for example, by tetrode recordings of photo-tagged neurons¹⁶⁰ or genetically encoded calcium
559 sensor imaging¹⁶¹. The choice of the optogenetic intervention should ideally be instructed by these
560 observational investigations and match the dynamic range of the activity observed. Additional
561 selectivity can be achieved by aiming at axon terminals rather than cell bodies. Effectors aiming at
562 hyperpolarizing terminals or creating shunting inhibition may not always be efficient or at times even
563 perturb para-membranous ion concentrations such that the effect is difficult to predict³⁹. With the
564 advent of $G_{i/o}$ -coupled effectors^{53,54}, presynaptic inhibition is more straightforward, but it remains
565 good practice to validate the efficacy of inhibition, as well as its spatial selectivity, particularly with
566 the highly light-sensitive effectors. It is particularly important to take into account the firing frequency
567 of the cells under investigation as presynaptic inhibition is potentially less efficacious at higher firing
568 rates.

569 There are two distinct approaches for optogenetic manipulations, one with an acute effect, the
570 other with long-lasting effects. Acute manipulations require behavioral observations in real time.
571 Ideally, a small set of optogenetic trials should be randomly interleaved with control trials. This allows
572 us to assess not only the acute effects on optogenetic trials, but also to determine if there are longer-
573 lasting changes to the subsequent control trials. Such laser on-off protocols can be used to control for
574 adaptive behavioral changes throughout a given session. However, often the particular structure of
575 the behavioral paradigm does not allow for hundreds of trials. The timing of the optogenetic
576 stimulation or inhibition should therefore occur in a behaviorally defined window, and be only as long
577 as is strictly necessary.

578 Long-term observation is appropriate when optogenetic interventions exploit synaptic
579 plasticity mechanisms. For example, synaptic potentiation typically is achieved by high stimulation
580 frequency, while depression requires sustained low frequency stimulation. Optogenetic synaptic

581 plasticity protocols are particularly suited to study learned and adaptive behavior. The goal of long-
582 term observation experiments is to induce synaptic plasticity at identified synapses and observe the
583 effect on behavior at a later time point when optogenetic stimulation is no longer active. For example,
584 low-frequency optogenetic stimulation can restore baseline transmission in cortico-accumbal
585 synapses that have been potentiated by cocaine exposure ¹⁶². Similarly, daily optogenetic stimulation
586 of orbitofrontal to dorsal striatum axons for 10 minutes triggered long-term changes in synaptic
587 strength and inhibited compulsion ¹⁶³.

588

589 **[H1] Applications**

590 The vast majority of applications of optogenetics have involved neuroscience and brain research.
591 Many of the general principles and approaches of optogenetics can be extended to other organs,
592 particularly to those with excitable cells — like skeletal muscle, heart, retina and gut — as well as to
593 microorganisms and plants. These newer applications often present unique challenges and
594 opportunities. Below, we illustrate some of these aspects with three select examples from visual,
595 cardiac and plant applications.

596 **[H2] Vision restoration**

597 Retinal degenerative diseases, such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD),
598 result in the loss of rod and cone photoreceptor cells, leading to partial or complete blindness ^{164,165}. Rendering
599 inner retinal neurons responsive to light is one of the most obvious medical applications for optogenetics
600 (Figure 7A). The first proof-of-concept study involved the ubiquitous expression of ChR2 in retinal
601 ganglion cells in retinal degenerated mice¹⁶⁶. The approach since then has been reported by numerous
602 studies using different optogenetic tools, retinal cell targeting strategies and animal models
603 (Supplementary Table 1).

604 Multiple clinical trials using ChRs for treating RP-related blindness have been initiated since 2015, with
605 encouraging results (Supplementary Table 2). Recently, the first published case study reported the
606 partial restoration of vision (in the form of perceiving, locating and counting objects) in a blind patient
607 with RP ¹⁶⁷. Positive preliminary results have also been reported in other clinical trials (Supplementary
608 Table 2). However, further efforts will be required to improve the outcome of optogenetic vision

609 restoration, including the development of effective optogenetic tools and treatment strategies, and the
610 improvement of gene delivery efficiency.

611 [H3] Optogenetic tools

612 ChRs have been the more commonly used optogenetic tools for vision restoration in animal models
613 and the ones used so far in clinical trials. Two main issues should be considered when choosing an
614 optogenetic tool for vision restoration. The first is the tool's expression efficiency and long-term
615 safety in mammalian neurons; problems with the expression of an optogenetic tool are difficult to
616 correct and usually result in cell toxicity in the long-term. The second issue is the low operational
617 light-sensitivity of ChR-expressing retinal neurons in general caused by the small unitary conductance
618 and substantial inactivation. The requirement of high light-intensity to activate the ChR-expressing
619 retinal neurons constrains this application and also raises concerns regarding tissue photochemical
620 damage, especially for short-wavelength sensitive ChRs. One solution to mitigate the potential
621 photochemical damage is to use red-shifted ChRs, such as Chrimson, since the threshold of light
622 intensity causing tissue photochemical damage is shifted to higher light-intensities for longer
623 wavelengths¹⁶⁸⁻¹⁷⁰. Another solution is to improve the light sensitivity of a ChR expressing cell by
624 slowing its closing kinetics or off-rate with molecular engineering¹⁷¹ (Supplementary Figures 1 and 2)
625 combined with genome mining for more potent ChRs³⁰. This strategy has been recently used to further
626 optimize the more effective ChR variant CoChR. Functional vision is restored with improved CoChR
627 mutants under ambient light conditions in a blind mouse model¹⁷². A third solution is to use GPCRs,
628 including animal opsins (for example, rhodopsin and cone opsins)¹⁷³⁻¹⁷⁵ or engineering of optoGPCR
629 chimeras¹⁷⁶, taking advantage of their high light sensitivity due to intracellular signal amplification.
630 Further studies will need to evaluate the most effective optogenetic tools or develop better ones for
631 this application.

632 [H3] Gene delivery

633 AAV vectors are the current choice for transgene delivery in the retina both in animal studies and in clinical
634 trials¹⁷⁷. Intravitreal injection is a preferred route of viral vector administration due to its safe
635 operation and ability to achieve widespread delivery to the retina. However, in non-human primates
636 and in humans, virus transduction was mainly conferred to a narrow region surrounding the fovea or
637 parafoveal region^{178,179}, due to the barrier of a thick limiting membrane in the retinal surface of

638 primates ²², which is one of the major factors limiting the outcome of AAV-mediated optogenetic
639 therapy. Further development of more efficient gene delivery vehicles or techniques is required.

640 [H3] Retinal cell targeting

641 Most animal studies and clinical trials have employed ubiquitous promoters to express depolarizing ChRs in
642 retinal ganglion cells. However, unlike the normal visual processing features in the retina including
643 the segregation of ON and OFF signal pathways and the presence of antagonistic center-surround
644 receptive fields (Figure 7Aa), this treatment strategy converts all retinal ganglion cells to ON cells
645 (Figure. 7Ab). Although useful vision could still be generated as demonstrated in animal studies and
646 reported from clinical trials, it is commonly believed that restoration of vision to mimic the intrinsic
647 visual processing features in the retina would result in a better outcome. To this end, one strategy is
648 to target an optogenetic tool to distal retinal neurons. Targeting a depolarizing ChR to ON bipolar
649 cells using the mGluR6 promoter has been the most commonly employed strategy (Figure 7Ac). Due
650 to the unique rod pathway in the mammalian retina, this could lead to ON and OFF responses at the
651 level of retinal ganglion cells ¹⁸⁰⁻¹⁸³, and possibly center-surround receptive fields. Targeting surviving cone
652 photoreceptors with a hyperpolarizing optogenetic tool, such as eNpHR, has also been reported ¹⁸⁴. As a
653 limitation for this strategy, the distal retinal neurons are more susceptible to severe retinal
654 deterioration or remodeling than retinal ganglion cells after the death of photoreceptors¹⁸⁵. Multiple
655 treatment strategies will need to be developed for treating patients with different retinal degenerative
656 conditions.

657 **[H2] Cardiac research**

658 The key benefits for clinical translation are sought in more versatile optogenetic pacing or suppression
659 of wave propagation during arrhythmias, compared to currently used cardiac devices like pacemakers
660 and cardioverter/defibrillators (Figure 7B) ¹⁸⁶⁻¹⁹³. Strategies for rhythm control enabled by
661 optogenetic actuators aim to lower the energy needed to power cardiac devices and extend battery life
662 by delivering longer lower-energy light pulses — electrical pulse duration is limited due to
663 electrochemical toxicity via Faraday effects. Optogenetic actuators also eliminate discomfort and pain
664 during classic cardioversion/defibrillation for better quality of life by using cell-specific genetic
665 targeting to engage the fast conduction system ^{190,194} or to specifically target myocytes and avoid
666 unintended contractions of thoracic skeletal muscle, diaphragm and vocal cords like pain-inducing
667 electrical defibrillation ¹⁹⁵. Computational modeling of the action of optogenetic tools in the heart

668 helps to explore strategies for control of arrhythmias, both with excitatory/depolarizing opsins and
669 with inhibitory/hyperpolarizing opsins^{113,189,190,196,197}. Longer-term in vivo clinical applications face
670 the challenges of genetic modification of the hard-to-access cardiac muscle, potential immune
671 responses and realizing embedded miniaturized light control devices that are reliable and safe¹⁹³.
672 Light penetration in the haemoglobin-rich heart muscle requires operation in the near-infrared and
673 opsins excitable within that range, along with stabilization techniques to counter mechanical
674 contractions. The atria are thinner (human atria are < 5mm) and present an easier target, along with
675 more accessible autonomic nerves, such as the vagus nerve¹⁹⁸.

676 AAV9 is the most efficient AAV serotype for targeting the ventricular myocytes in vivo when
677 using an ubiquitous or a specific promoter, such as Myh6¹⁹⁹. The heart atria can be targeted
678 optogenetically using the NPPA promoter and local viral gene delivery¹⁹². Cre-Lox transgenic mouse
679 models with suitable promoters have been used to transform the fast conduction system cells
680 (Cx40)¹⁹⁴, sympathetic neurons (tyrosine hydroxylase, TH)²⁰⁰, and parasympathetic neurons (choline
681 acetyltransferase, ChAT)^{198,201} (Figure 7Ba). To translate the approaches from rodents to larger
682 animals, more work is needed in finding minimally-invasive ways of transgene delivery to the heart
683 and in minimizing immune responses. Previous clinical trials on gene therapy for cardiac disorders
684 found that a large portion of the patients had antibodies against the viral vectors used, thus reducing
685 the efficacy of the therapy²⁰². Most of the published studies have used ChR2-H134R as excitatory
686 opsin. In general, more efficient and fast inhibitory opsins are desirable for arrhythmia control
687 applications. There may also be a niche for **step-function-like depolarizing [G]** opsins that have fast
688 recovery from inactivation as clamping tools in arrhythmia management. Bidirectional closed-loop
689 control could make an all-optical approach, named **optical clamp [G]** at the whole organ level a reality.
690 However, this will require spectral compatibility to accommodate not only for an excitatory and an
691 inhibitory opsin, but also for the optical readout of a voltage indicator.

692 Overall, clinical applications of optogenetics in the heart face many challenges compared to
693 the more accessible, immune-privileged applications to the eye that have seen translational advances.
694 Considering the potential impact for control of arrhythmias, efforts should continue to improve the
695 genetic targeting by more specific promoters, safer viral vectors, longer-wavelength opsins for better
696 penetration and miniaturized distributed light sources. Basic science experiments with optogenetic
697 tools provide invaluable insights for improvement of current cardiac devices and may yield new

698 strategies for arrhythmia control ^{112,114,193,203,204}. These new strategies take advantage of the ability to
699 produce complex space-time control patterns by light (unlike discrete signals from electrode arrays)
700 to steer waves of excitation towards non-arrhythmic behavior at very low energy. Optogenetics-
701 empowered high-throughput systems can more immediately improve cardiotoxicity testing and drug
702 development. All-optical cardiac electrophysiology, which combines optogenetic actuators and
703 optical/optogenetic sensors ²⁰⁵⁻²⁰⁷, offers immediate adoption and translation (Figure 7Bc).
704 Cardiotoxicity testing is crucial in the development of any new pharmaceutical, and high-throughput
705 optogenetic methods with patient-derived cells represent impactful technology for personalized
706 medicine ^{208,209}. Optogenetic techniques using hyperpolarizing opsins like ArchT have been used to
707 dynamically alter the action potential characteristics of induced pluripotent stem-cell-derived
708 cardiomyocytes (iPSC-CMs) towards a more mature phenotype to better predict drug responses ²¹⁰.
709 The maturity of tissue-engineered constructs of such patient derived iPSC-CMs can be improved
710 through chronic optogenetic pacing ²⁰⁹ towards new regenerative solutions for the heart
711 (Supplementary Figure 9).

712

713 [H2] Plants

714 A large set of photoreceptors that control phototropism, diurnal rhythms and photomorphogenesis
715 play fundamental roles in plant growth and development. Blue-light absorbing phototropins and
716 cryptochromes or red/far-red light absorbing phytochromes are found in almost all plant tissues
717 (Figure 7Ca). Therefore, when using optogenetics tools in plants, the **light regime [G]** used needs to
718 be considered. The light required for plant growth will activate optogenetic tools when light of the
719 entire visible spectrum is used; this can be avoided by combining a blue light-regulated transcriptional
720 repressor with a red light-triggered switch ²¹¹, allowing plants to grow at ambient white light. The use
721 of flavoprotein-based optogenetic tools in plants has been described in detail recently ^{212,213}. Based on
722 the light-oxygen-voltage (LOV) domain, a synthetic light-gated K⁺ channel with considerable dark
723 activity, called BLINK1, was recently expressed in Arabidopsis guard cells for control of stomatal
724 behavior ²¹⁴. The mechanism of BLINK1 light activation that clamps the membrane potential to E_K
725 and facilitates stomatal opening and closing in the same way, remains to be clarified. A rather simple
726 but valuable technique to avoid non-specific activation of rhodopsin-based optogenetic tools is to
727 grow plants exclusively in red light ^{98,215}. Both chlorophyll a and chlorophyll b absorb red light (Figure

728 7Ca), and tobacco plants exclusively grown in red-light are hardly distinguishable from those grown
729 in white light⁹⁸. Green light is the least absorbed wavelength by endogenous plant photoreceptors,
730 therefore, green light allows for optogenetic manipulation with only minimal crosstalk⁹⁸ (Figure
731 7Ca), especially with GtACR1.

732 Rhodopsin-based plant optogenetic approaches are still in their infancy compared to their
733 long-standing use in animals. The combination of ubiquitous rhodopsin expression with global or
734 local light-emitting diodes (LED) or laser light applications have been used in plants^{98,215-217}.
735 However, cell type-specific expression with global green light exposure certainly bears great
736 potential, when combined with red light growth conditions. Use of the LeLAT52 pollen-specific
737 promoter²¹⁸ allows plants to be grown in white light under greenhouse conditions for optogenetics-
738 inspired research on pollen tubes⁹⁸. For local rhodopsin stimulation at the single-cell level, fiber-
739 optics or laser light pulses have been successfully applied^{98,217}. The Fluorescence Recovery After
740 Photobleaching (FRAP) module of conventional laser scanning microscopes allows local optogenetic
741 stimulation of plant cells when using rhodopsins, like GtACR1, with activation kinetics in the lower
742 ms range⁹⁸.

743 To perform plant optogenetics with rhodopsins, retinal can be added externally²¹⁵
744 (Supplementary Figure 10 a-c), or plants can be empowered to produce retinal by expressing a β -
745 dioxygenase from a marine bacterium targeted to the chloroplasts to synthesize retinal from
746 carotenoids efficiently⁹⁸ (Supplementary Figure 10 d-f). In contrast to animal cells, the plant cell
747 extracellular medium is low in ions and mostly moderately acidic, which may result in different
748 electrical responses in plant and animal cells using the same rhodopsin (Supplementary Figure 11).
749 Activation of ACRs in the soma of neurons leads to membrane hyperpolarization²¹⁹, while
750 depolarization occurs in plant cells⁹⁸ due to the outward-directed anion gradient. When expressed in
751 leaves or pollen tubes, activation of GtACR1 by green light (530 nm) resulted in membrane
752 depolarization by about 60–100 mV within milliseconds⁹⁸. Local GtACR1 activation on one side of
753 the dome of apically growing pollen tubes has been used to demonstrate the involvement of an anion
754 efflux in polar growth⁹⁸ (Supplementary Fig. 10 e, f), supporting earlier studies on the role of anion
755 transport in polar growth^{220,221}. In guard cells, native anion channel activity can be mimicked when
756 GtACR1 is triggered by a series of light pulses (Figure 7Cc-f), demonstrating that anion channel
757 driven depolarization is sufficient to close stomata²¹⁷. Although plants do not have neuronal-like
758 networks, voltage changes in the form of depolarization waves are transmitted between leaves or even

759 between different organs²²²⁻²²⁴. The role of these long-range electrical signals can now be investigated
760 with the help of GtACR1. Through GtACR1 induced anion efflux, depolarizations of any shape and
761 intensity can be optogenetically generated to mimic the voltage changes observed in plants such as
762 variation potentials, system potentials or action potentials²²⁵⁻²²⁷.

763 A wide-range of processes in plants are induced by changes in cytoplasmic Ca^{2+} and H^+ levels
764 ^{245,246}. For both ions, there is a strong inward gradient, in contrast to animal cells, where there are
765 minimal differences in intracellular and extracellular pH (Supplementary Figure 11). The slow cycling
766 ChR2 variant XXL with high proton conductance²²⁸ is excellent to impose light-induced pH changes,
767 and has already been used to feed the P-type plasma membrane H^+ pump with substrate and study its
768 voltage dependence²¹⁵(Supplementary Figure 10 b, c). The resting potential of plants is negative with
769 respect to E_K (-120 to -180 mV) due to the voltage dependent activity of P-type plasma membrane H^+
770 pumps. The latter hyperpolarize the membrane and acidify the cell wall space²²⁹. This voltage
771 deflection is used by the plant to open hyperpolarization active Shaker type K^+ channels²³⁰ and
772 electrophoretically move K^+ ions into the cell²³¹. The combined driving proton-motive-force (PMF)
773 of the electrical gradient and that of the H^+ is used by solute transporters using protons as co-substrate.
774 The plant optogenetics toolbox therefore needs to be complemented by light-driven H^+ pumps such
775 as Arch3. Great potential for the study of Ca^{2+} signaling is the ChR2 variant XXM with increased
776 Ca^{2+} conductivity and medium open state lifetime²³². Combined with electrophysiology and Ca^{2+}
777 imaging, the molecular mechanisms for long-distance Ca^{2+} signaling could be resolved. Ca^{2+}
778 signatures can represent either single events or rhythmically recurring signals. Whether and how
779 different Ca^{2+} signatures control specific processes in plants is still largely unexplored. In the future,
780 Ca^{2+} -permeable ChRs could be used to elicit defined Ca^{2+} signatures.

781

782 **[H1] Reproducibility and data deposition**

783 **[H2] Reproducibility of optogenetic tools**

784 Reproducibility in optogenetics experiments depends on the consistency of the tools used, the
785 organism/cell type, genetic transformation procedures and light delivery. Adherence to minimum
786 reporting standards for all relevant parameters of an experiment can help to increase reproducibility.

787 Optogenetic actuators are used in a diverse range of organisms, tissues and cell types. Because
788 of differences in codon usage between the original host and the organisms in which the tools are
789 applied, it is common to codon-optimize the coding sequence to facilitate translation in these
790 heterologous systems. New codon-optimized sequences should be tested for expression, membrane
791 targeting and function before applying these novel constructs in optogenetic experiments. The
792 sequences of codon-optimized constructs should be appropriately reported in publications to allow
793 reproduction of findings in other laboratories. However, even with codon-optimization or adding
794 traffic motifs, the intracellular aggregation of many optogenetic actuators can still pose a problem for
795 their applicability, particularly for translational applications. A thorough evaluation in targeted
796 organism/cell type is needed because the intracellular aggregation not only reduces expression
797 efficacy but also affects cell health or causes cell death.

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

806 **[H2] Reproducibility of opsin expression**

807 Evaluation of the viral titer is needed to optimize viral vector spread and expression level, and to
808 minimize overexpression-mediated off-target effects. Many opsin viral vectors were designed to co-
809 express a fluorophore. Standard histological methods can be used to visualize the strength and spatial
810 extent of viral vector expression. Characterizing viral expression for every experimental animal can
811 increase interpretability by correlating the variability in behavioral effect to the variability in
812 expression area and, for instance, optical fiber placement. Even when an experiment is planned based
813 on published work, the experimental design should be validated in each new experiment due to the
814 potential variability of viral vector batches, optical hardware and mouse strain. When presenting
815 results obtained using viral vectors, the source of the viral vector, its purification and titration
816 methods, and the duration of expression should be reported.

817 Viral vector expression can impact cell health or change the electrophysiological properties of
818 the targeted neurons. It is therefore necessary to include a control group injected with a titer-matched
819 virus that expresses a control transgene. Researchers often use a virus encoding the same fluorophore
820 that is co-expressed with the opsin. This control group can be used to evaluate direct effects of the
821 virus injection surgery and potential phototoxic or heating effects due to the light delivery paradigm.
822 Strong opsin expression has been reported to affect cell physiology²³³. It is therefore advisable to
823 include an opsin expressing group where no light is applied. Where the experiment allows for multiple
824 repeats of the same manipulation, light and no light conditions can be tested in the same group, which
825 presents a within-animal control.

826 Transgenic animals for optogenetics research should be genotyped continuously to confirm
827 suitability for the experiments. For in vivo optogenetics with viral delivery, even when using the same
828 tools in the same organism type, variations in responses may be due to variations in the immune
829 response of the subjects (animals or humans) to the viral capsid, or the cargo (opsin and/or fluorescent
830 reporter). To obtain reproducible data with viral delivery, testing for neutralizing antibodies can be
831 implemented²³⁴. Appropriate control groups, immunohistochemistry and histology should be done
832 routinely in animal experiments to demonstrate consistency of the optogenetic transformation.

833 **[H2] Reproducibility of light delivery**

834 Activation of optogenetic tools depends on the photon irradiance or photon exposure in case of short
835 flashes and the spectral profile of the delivered light. The spectral profile should be reported by listing
836 the light source, all filters and optical components used in the experiments. Insufficient irradiance
837 may lead to failure to engage the optogenetic tools and therefore failure to reproduce the phenotypic
838 changes; excessive irradiance may lead to adverse thermal effects and photoreceptor bleaching that
839 also affect reproducibility. For single-photon excitation, the spatial pattern of the delivered light is
840 variable and highly depends on the positioning of the light source and the tissue properties. While
841 total power is trivial to report, the normalized values of irradiance are influenced by the uncertainties
842 of area estimation and the non-uniform spatial profile of light delivery. At a minimum, effort should
843 be made to measure and report irradiance at the tissue point-of-entry. Whenever possible, light-tissue
844 interactions can be simulated²³⁵ to yield relevant estimates of irradiance at points of interest.

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

854 **[H2] Data and metadata sharing**

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

862

863 **[H1] Limitations and optimizations**

864 **[H2] Tissue heating and photodamage**

865 **[H3] Single-photon optogenetics**

866 Optogenetic experiments based on illumination with visible light excitation (450–630 nm) typically
867 use optical fibers coupled to lasers or high-power LED for large ($\sim\text{mm}^3$) field illumination, relatively
868 long (0.5 – 60 s) exposure times and excitation powers on the order of milliwatts (0.5–20 mW). Under
869 these conditions, the main cause for concern with respect to photodamage is heating due to light
870 absorption. This has been investigated both theoretically, using Monte Carlo with finite-difference
871 time-domain simulations¹⁰⁸ or the finite element method²³⁶, and experimentally using
872 thermocouples^{108,236}, infrared cameras²³⁷ or electrophysiological recordings²³⁸. Depending on the
873 precise stimulation protocol used, these experimental and theoretical studies report a wavelength and

874 power density dependent temperature increase between 0.3–6 K throughout the volume of illuminated
875 tissue^{237, 108}. Temperature variations on the order of only 2 K can affect ion channel kinetics and
876 conductance²³⁹, synaptic transmission²⁴⁰ and neuronal firing rate¹⁰⁸, and lead to a bias in turning
877 behavior across various brain regions²³⁸. Importantly, changes in temperature can induce
878 physiological changes in the absence of detectable changes in behavior²⁴¹. It is extremely important
879 to carefully design optogenetic experiments to minimize photon exposure and absorption, for instance
880 by using short illumination duty cycles²³⁷ and opsins with long open state lifetimes and red-shifted
881 absorption peaks^{30,104,242}. Simulations^{108,237,243,244} can be used to guide experimental design, but,
882 since the effects of heating vary between cell types and brain regions, opsin-negative controls should
883 always be performed.

884

885 **[H3] Multiphoton optogenetics**

886 Generating sufficient rates of multi-photon excitation requires the use of pulsed lasers with high peak
887 energies, but since typical optogenetic stimulation protocols irradiate cells on millisecond timescales,
888 the temperature rises induced by single-cell multi-photon photostimulation are of the order of 10^{-1} K
889²⁴⁴. Much larger temperature rises are induced during multi-target excitation due to the diffusion of
890 heat from each target into the surrounding tissue. The resultant temperature increase occurs over
891 hundreds of milliseconds and can approach or even exceed the 2 K threshold for thermal damage²⁴⁴.
892 This effect can be mitigated by ensuring that the separation between adjacent targets is larger than the
893 thermal diffusion length.

894 The risk of non-linear photodamage increases with peak fluence and could be a dominant
895 source of photodamage in the case of spiral scanning which typically requires higher photon density
896 than parallel illumination. Non-linear photodamage can be reduced by increasing the repetition rate
897 of the pulsed laser source although this will increase photo induced temperature rises²⁴⁴. In all-optical
898 experiments which combine 2P optogenetics with 2P imaging, the possibility of thermal or nonlinear
899 damage induced by the imaging laser should also be considered²⁴³.

900

901

902 **[H2] Interpreting optogenetic experiments**

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

915 Non-physiological activity patterns can occur at the single-cell level as well as at the broader
916 circuit scale ^{245,246}. On the single-cell level, ion pump-mediated hyperpolarization for instance can
917 lead to rebound excitation upon inhibition release ²⁴⁷ or to supra-physiological ion concentrations ^{42,94}.
918 High-frequency light pulse trains or constant illumination of an excitatory pyramidal neuron
919 expressing a CCR can, for instance, lead to depolarization block, effectively reducing rather than
920 increasing its firing rate ²⁴⁸. Whether such rebound excitation or depolarization block occurs and to
921 what extent is hard to predict, as it depends on many experiment-specific parameters which can greatly
922 vary between laboratories. While axonal stimulation can be used to effectively isolate the activity of
923 an anatomically-defined projection pathway, optogenetic stimulation of axons can cause **antidromic**
924 **activation [G]** of both neuronal cell bodies as well as collaterals to other brain regions, leading to
925 reduced specificity which should be taken into account.

926 At the circuit level, particularly when a large portion of cells expresses ion translocation-based
927 optogenetic tools such as ion-channels or ion-pumps, the simultaneous activation of these tools can
928 lead to transient but significant changes in the ion composition of the local extracellular space, thereby
929 indirectly affecting nearby non-opsin expressing neurons ²⁴⁹. Electrophysiological characterization of
930 the optogenetic manipulation can be performed to quantify the extent of such unintended effects,
931 allowing the optimization of light power and illumination paradigms. Optogenetic tools that modulate
932 biochemical activity within the cells or ones that act on slower timescales, or only induce subthreshold

933 depolarization, are less prone to the caveats imposed by highly synchronous neuronal activation²⁵⁰.
934 Finally, optogenetic firing rate modulation experiments are mostly designed to acutely alter the firing
935 rates of targeted cells, which can have different effects than chronic manipulations. Brain circuits
936 regulate their overall activity to achieve a homeostatic equilibrium, such that when the firing rate of
937 a circuit is transiently increased or decreased, it can acutely affect the independent functions of
938 downstream circuits and lead to markedly different results compared with chronic manipulations²⁵⁰.
939 Acute effects are normally more severe, and could lead to overestimation of the roles of targeted
940 regions in a given behavior. While chronic manipulations such as lesions do not suffer from this
941 limitation, plastic changes during lesion recovery can also lead to an underestimation of the necessity
942 of a given input to a local circuit. In summary, a sound experimental strategy should balance the use
943 of acute and powerful optogenetic approaches with chronic experiments, pharmacological
944 manipulations or lesions, and use caution in claims of causality based purely on manipulations that
945 might suffer from any of the above-mentioned artifacts.

946

947 **[H1] Outlook**

948 **[H2] Refinement of the optogenetic toolbox**

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

955

956 **[H2] Enhancement of basic research**

957 Optogenetics will further advance the investigation of neural circuits. This will not only establish
958 links of causality between neural activity and behavior but eventually generate sufficient knowledge
959 for a theory of the brain to emerge. Empirically observed neural activity in optogenetic experiments
960 taking into account the activity of individual neurons may eventually allow deriving the neural code,
961 which, when integrated into a solid theoretical framework, will bring the neurosciences at par with
962 other fields of natural sciences.

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

970

971 **[H2] Open routes of translation**

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

981 With the recent proof-of-principle of optogenetic vision restoration in humans with RP,
982 longer-term gene therapy options remain open for optogenetics, though several challenges need to be
983 addressed. Optimization of gene delivery vectors that are safe and produce long-lasting expression
984 and optimization of light delivery to the desired organ is essential. Light delivery deep into the tissue,
985 beyond the limited optical depth penetration minimizing the use of optical fibers is also needed. One
986 non-conventional solution is to introduce in situ sources of biological light, such as luminopsins.
987 Triggered by a chemical process like simple substrate delivery, these luminopsins do not require
988 device implantation and can be tuned to control inhibitory or excitatory actuators. Further remote
989 trigger methods involve energy-conversion schemes via mechanoluminescent nanoparticles. The
990 energy could be provided by intermittent focused ultrasound, thus recharging light emitting materials
991 that can deliver short opsin-engaging pulses. Mechanosensitive TRAAK K⁺ channel for example
992 could be specifically activated by ultrasound with submillisecond kinetics ²⁵¹ providing a new,
993 orthogonal dimension for external non-invasive manipulation of neural circuits.

994 Clinical applications of optogenetics to the heart face many challenges compared to the more
995 accessible, immune-privileged applications to the eye that have seen translational advances.
996 Considering the potential impact for control of arrhythmias, efforts should continue to improve the
997 genetic targeting by more specific promoters, safer viral vectors, longer-wavelength opsins for better
998 tissue penetration and miniaturized distributed light sources. Basic science experiments with
999 optogenetic tools provide invaluable insights for improvement of current cardiac devices and may
1000 yield new strategies for arrhythmia control ^{112,193}. In the meantime, optogenetics-empowered high-
1001 throughput systems can more immediately improve cardiotoxicity testing and drug development.
1002 Similarly, in vitro assays for drug development and personalized medicine may use humanized
1003 optogenetic tools, patient-derived cells and engineered tissues coupled with highly parallel all-optical
1004 electrophysiology techniques to yield a low-cost, faster and more efficient drug-development pipeline.

1005 Likewise, scalable optogenetic control of living plants, as discussed here, or microbes can be
1006 leveraged to address problems related to energy, food, biotechnology and climate challenges. As these
1007 do not involve deployment in the human body, they can be implemented on a shorter time scale, with
1008 less technical and regulatory obstacles.

1009

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1036

1037 **Competing interests**

1038 Z.-H.P. is a co-inventor on patents related to optogenetic vision restoration and is also a co-founder
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1044
1045

1046 **Glossary terms:**

1047

1048 **Microelectrode:** an electrode with micrometer-sized tip used to record single neuron activity.

1049 **Optrode:** an electrode coupled to an optical fiber used to record and manipulate neural activity in
1050 cells expressing an optogenetic actuator.

1051 **Optogenetic actuator:** a light sensitive protein that transiently modifies cellular properties during
1052 illumination.

1053 **Bidirectional voltage modulation:** Changing the voltage in the depolarizing (excitatory) or
1054 hyperpolarizing (inhibitory) directions.

1055 **Immediate early genes:** genes that are rapidly induced by elevated neural activity such as *c-Fos*.

1056 **Optical clamp:** Using light and real-time feedback to keep membrane electrical parameters, such as
1057 voltage or action potential shape, at set desired value.

1058 **Antidromic activation:** Retrograde propagation of an action potential from the axon to the neuronal
1059 soma.

1060

1061

1062

1063

1064 **Figure legends**

1065 **Figure 1. Principles of Optogenetics.** DNA encoding a sensory photoreceptor derived from a
1066 microorganism, plant or animal (orange) is cloned under the regulation of control elements that allow
1067 the targeting of specific host cells (blue), packed into a vector such as a viral vector or bacteria and
1068 injected into the tissue, organ or organism of interest. The targeted cell (orange) now expresses the
1069 light-sensitive protein and can be controlled with light in a variety of ways, depending on the specific
1070 photoreceptor expressed.

1071

1072 **Figure 2. The optogenetic actuator toolbox. a)** Key advances in the development of optogenetic tools. Not all
1073 available tools are highlighted here. Major developments are shown above the arrow, and first applications of
1074 channelrhodopsins to model organisms including humans are shown below the arrow. **B)** Tools for optogenetic
1075 manipulation of membrane voltage and local ion concentrations (top), second-messenger, G-protein-signaling and
1076 kinase-signaling (middle) and the light-controlled interaction of photoreceptors with tethered partner proteins for
1077 subcellular application (bottom). LOV-domain based dimerizers expose a “aged” signaling peptide after light-
1078 triggered unfolding of the Ja-Helix²⁵². Cryptochrome 2 and Phytochrome B interact with CiBN or PIF-domains after
1079 blue or red light absorption respectively^{253,254} **c)** Commonly-used optogenetic tools for excitation or inhibition of
1080 neuronal activity including cation conducting ChRs *eT3ChR*²⁵⁵, *Cheriff*²⁰⁵, *CoChR*³⁰, *CrChR2_{TC}*²⁵⁶, *ChroME*¹⁰⁴ and
1081 derivatives, *SSFO/Soul*^{120,257}, *ChRmine*²⁵⁸, *bReaChES*²⁵⁹ and *f-Chrimson*¹¹⁷, chloride and potassium conducting
1082 ChRs (e.g. *GtACR1*, *GtACR2*²¹⁹ and *HcKCR1*²²), inward directed proton pumps (e.g. *NsXer*²⁶⁰) and outward
1083 directed proton, sodium and chloride pumps (e.g. *Arch3.0*¹⁷¹, *eKR2*²⁶¹, *eNpHR3.0*²⁶²), all plotted according to their
1084 peak excitation wavelength and temporal kinetics. **D)** The soluble enzyme *bPAC*⁶² and the rhodopsin-guanyl-cyclase
1085 *CaRhGC*⁶⁸ produce cAMP and cGMP following illumination whereas the non-bleaching opsins *mOPN4*⁴⁸,
1086 *eOPN3*⁵⁴, *PPO*⁵³ and *JellyOP*⁴⁷ activate different G-protein pathways. **e:** Genetically encoded sensors with diverse
1087 excitation spectra (depicted on the x axis) can be used to monitor changes in Ca²⁺ voltage, and pH, such as *GCaMP*
1088 and *R-CaMP*¹⁵⁶ and *FRCaMP*²⁶³ for Ca²⁺, *ASAP3*²⁶⁴, *Voltron*²⁶⁵, *VARNAM*²⁶⁶, *Quasar*²⁰⁵ and *Archon*²⁶⁷ for voltage,

1089 and pHluorin²⁶⁸ for pH. In experiments combining sensors and actuators, both need to be chosen carefully to
1090 minimize optical crosstalk.

1091

1092 **Figure 3. Cell-type specific targeting of optogenetic tools.** **A)** Transgenic mice constitutively expressing an opsin gene
1093 from their genome allow simple experiments that only require the addition of a light-delivery apparatus. Promoter “A”
1094 activity (indicated by A) will lead to transgene expression (indicated in green). **B)** A transgenic animal expressing a
1095 recombinase such as Cre under the control of a cell-type specific promoter is crossed with a second line carrying a
1096 conditional expression cassette encoding the desired opsin. Dual transgenic offspring will then show organism-wide
1097 expression of the opsin in all cells that underwent promoter activation at any stage of development (indicated in green).
1098 Cre expression (indicated by A) is unnecessary once the conditional expression cassette was activated. **C)** Where a short
1099 minimal promoter sequence is available, targeted viral vector injections can be used to restrict expression spatially as well
1100 as by the gene expression profile. A viral vector containing the specific minimal promoter sequence upstream of the opsin
1101 gene will lead to expression in specific cells expressing the promoter (indicated by A), only in the region targeted with
1102 the injection (indicated by the blue box). **D)** Approaches a and b can be combined to achieve both spatial and gene
1103 expression specificity in cases where short specific promoters are not available, or where promoter activity is not specific
1104 during development. **e:** Projection neurons can be addressed by injection of an axon terminal-transducing, retrograde
1105 traveling viral vector encoding for the opsin or a recombinase into the target region. The recombinase encoding viral
1106 vector is injected in a projection target (area B, indicated by the red box) and travels retrogradely. A second viral injection
1107 of conditional expression cassette encoding the desired opsin into an upstream region (area A, indicated by the blue box)
1108 will then lead to opsin expression only in neurons within area A that project to area B **f:** AAV capsids engineered for
1109 improved blood-brain barrier penetration allow brain-wide (mostly sparse) expression of an opsin through intravenous
1110 injection of the viral vector.

1111 **Figure 4. Optical approaches for optogenetic stimulation.** **a-c)** Single-photon wide-field illumination
1112 (indicated in blue) of all genetically targeted opsin-expressing neurons using excitation through optical fibers.
1113 **a)** Illumination using a flat cleaved optical fiber causes high peak light power density at the fiber-tissue
1114 interface. **b)** A tapered fiber increases the optical fiber-tissue interface resulting in a reduced peak light power
1115 density. **c)** Single-photon multi-target patterned illumination by spatially shaping the intensity of the excitation
1116 beam by means of a digital micromirror device (DMD), placed in a plane conjugated to the sample plane. The
1117 light distribution at the DMD plane and at the sample plane only differ by a spatial scaling factor corresponding
1118 to the magnification of the optical system. The axial resolution is proportional to the square of the lateral spot
1119 dimensions. **d-e)** Two-photon multi-target illumination by holographic light shaping: **d)** a spatial light
1120 modulator (SLM) placed at a plane conjugated with the objective back aperture, generates a 3D distribution of
1121 holographic spots which are scanned with a spiral trajectory to cover the cell surface. The axial extension of
1122 the generated spot is optimized to illuminate the upper and lower cell membranes. **e)** An SLM is used to
1123 generate multiple extended spots with a size large enough to cover the whole cell soma. Temporal focusing is

1124 used to maintain micrometer axial resolution independently of the lateral spot size. . **f)** A timeline indicating
1125 critical optical developments which have enabled new optogenetic experiments throughout the past fifteen
1126 years. Single- photon and two-photon milestones are colored in blue and red respectively. Holographic light
1127 shaping for neuronal activation was developed simultaneously for single- and two- photon activation as
1128 indicated by the red-blue gradient for the milestone in **f)**.

1129 **Figure 5: Expected results in optogenetic experiments.** **A)** Expression of optogenetic actuators such as ChR2 or NpHR
1130 in neurons leads to the emergence of light-driven photocurrents, which can be recorded using the whole-cell patch clamp
1131 technique (left). Cells expressing the chloride-conducting NpHR will show an outward current (top right, voltage clamp
1132 recording with cell resting at -70 mV) while cells expressing the cation-conducting ChR2 will show an inward
1133 photocurrent (bottom right, voltage clamp recording with cell resting at -70 mV). **B)** Whole-cell current-clamp recordings
1134 in a neuron expressing the excitatory ChR2, showing action potentials evoked by brief light pulses (blue bars). **C)**
1135 Hyperpolarization and silencing of spontaneously-occurring action potentials in a neuron expressing eNpHR3.0. **d)**
1136 Extracellular recordings, coupled with local light delivery, used to reveal the activity of neurons in vivo, using the awake
1137 behaving optrode configuration²⁶⁹ **e)** Raster plot showing action potentials (represented as black dots) occurring rapidly
1138 after a 5-ms blue light pulse delivered into the target brain region. **F)** Raster plot showing the activity of neurons expressing
1139 the inhibitory anion-conducting GtACR2, showing increased inhibition of action potential firing with increasing light
1140 intensity. Part F is reprinted from ref X, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

1141 **Figure 6. Establishing links of causality with optogenetics.** Experimental road map based on identifying the neural
1142 correlate of behavioral sensitization to cocaine²⁷⁰. **A)** When injected, cocaine elicits a locomotor response quantified in a
1143 cyclotron. The response is enhanced upon a second injection of the same dose. **B)** *c-Fos* is an immediate early gene
1144 highlighting the neurons particularly active, which provided the entry point to identifying the mPFC to NAc projection as
1145 the behavioral relevant circuit. **C)** Slice electrophysiology enables observation of the selective potentiation of glutamate
1146 transmission onto D1R-MSNs²⁷¹. **D)** A depotentiation protocol (LTD at 12Hz) validated in slices restores standard
1147 transmission. **E)** In vivo validation involves opto-tagging, where spontaneously occurring spikes (grey, dashed trace) are
1148 compared to optogenetically-evoked spikes (blue trace). Waveform and latency are important parameters. **F)** The LTD
1149 protocol is eventually applied in vivo to reverse sensitization. Part B adapted with permission from ref X, PUBLISHER.
1150 Part D adapted with permission from ref X, PUBLISHER.

1151 **Figure 7. Optogenetic application for vision restoration, cardiac research, plant modification.** **A)** Strategies for
1152 optogenetic restoration of vision following photoreceptor degeneration. (Aa) Visual processing pathways in the normal
1153 retina, illustrating the rod/cone, ON/OFF pathways and the antagonistic center-surround receptive fields of retinal
1154 ganglion cells. ON cells, including rod bipolar cells and AII amacrine cells, are shown in gray tones, and OFF cells are
1155 shown in black. The ON and OFF regions of the receptive field of retinal ganglion cells are indicated by + and -,
1156 respectively. (Ab) Ubiquitous expression of a depolarizing optogenetic tool (green) in all retinal ganglion cells to convert
1157 them to ON cells. (Ac) Targeting a depolarizing optogenetic tool in ON bipolar cells to produce ON and OFF response in
1158 retinal ganglion cells and possibly the center-surround receptive fields. Abbreviations: AII, AII amacrine cells; BC,

1159 bipolar cells; RBC, rod bipolar cells. (Modified from ²⁷²). **B**) Optogenetics in cardiac research. Ba) Cell-specific targeting
1160 is used for the sympathetic (red) and parasympathetic (blue) nervous control of the heart using TH and CHAT promoters;
1161 cardiomyocytes (CM) from the upper or lower chambers of the heart (atria, A, or ventricles, V) can be selectively light-
1162 sensitized; specific targeting of the fast conduction system (CS), cardiac fibroblasts (FB), vascular cells (VC) or
1163 macrophages (M) is also of interest. (Bb). Rhythm control can include optical pacemaking by short pulses (top trace),
1164 heart rate modulation by low-level constant (middle trace) or pulsed light by activating the sympathetic nervous system
1165 (increase) or the parasympathetic nervous system (decrease), and arrhythmias can be terminated to restore normal rhythm
1166 through a single long pulse (bottom trace), series of pulses and/or spatially-patterned light. Bc) Cardiotoxicity testing, a
1167 required component in drug development, is enabled by high-throughput optogenetic platforms, which can integrate
1168 patient-derived iPSC-cardiomyocytes for personalized therapy.. **C**) Optogenetic approaches in plants. Ca) This scheme
1169 displays carbon dioxide entering through the stomata with loss of water and oxygen. Cb-Cg: Expression of rhodopsins to
1170 control plant cell behavior. Cb) Absorbance spectra of anion channelrhodopsins GtACR1 (black) in relation to endogenous
1171 relevant plant photoreceptors. Cc) Optical fiber illumination of a leaf from an Arabidopsis plant mounted in a microscope
1172 setup for Cd) simultaneous optical stimulation and electric recordings of guard cells embedded in the leaf epidermis. Ce)
1173 A representative membrane voltage recording from wild-type tobacco (red) and tobacco with stable GtACR1 expressing
1174 guard cell (black) in response to a 525 nm light pulse (10s) of 0.57 mW/mm² in the presence of background red-light (630
1175 nm, 0.018 mW/mm²) to elicit stomatal opening. Cf and Cg). Closure of the stomatal aperture is only induced in GtACR1-
1176 expressing cells in the presence of green light, indicated by the green bar in Cf and the green light spot in Cg. Part A
1177 adapted with permission from ref X, PUBLISHER. Part Cc, image courtesy of COPYRIGHT HOLDER. Parts Cd, Cf and Cg
1178 adapted with permission from ref X, PUBLISHER.

1179

1180

1181 **Box 1: Compartment-specific functions of microbial rhodopsins.**

1182 ACRs (such as GtACR2, iC⁺⁺ and iChloc) can be used to control the chloride conductance in a light dependent
1183 manner. However, the effect on neuronal excitability depends on the chloride reversal potential, which can
1184 differ between subcellular compartments, as well as the membrane potential. The left schematic shows a neuron
1185 colored according to the typical chloride reversal potential. In the somatic compartment (top inset) shunting
1186 inhibition occurs, if the reversal potential of a channel is close to the resting membrane potential of
1187 the cell. In this case, its activation does not lead to a substantial current, but rather to a conductance
1188 that will oppose any change of the membrane potential from the channel reversal potential, referred
1189 to as shunting conductance. The input resistance of the neuron is reduced, causing a smaller amplitude
1190 of subsequent excitatory potentials. If the channel reversal potential lies between the resting potential
1191 and the action potential threshold, however, the effects of shunting inhibition are more complex. In
1192 this case, ACR activation will lead to a depolarization, but excitatory potential amplitudes will still

1193 be reduced. Consequently, ACR activation will still lead to a reduced spike rate. Upon channel closing
1194 the input resistance then increases while the membrane potential is still depolarized, leading to a
1195 transiently increased excitability. Furthermore, if ACRs are activated over extended periods of
1196 excitatory drive, Cl⁻ can accumulate in the cell, and the depolarizing phase of shunting inhibition will
1197 become more accentuated, leading to activity-dependent effects of shunting inhibition. At the
1198 presynaptic terminal (bottom) ACR activation leads to depolarization, and potentially even action potential
1199 initiation, especially at light onset when the pool of activatable voltage gated sodium channels is large. ACR
1200 based optogenetic manipulations should thus take the unique features of compartment-specific physiology
1201 into account.

1202

1203 **Box 2: Single-photon vs two-photon excitation, mechanism and focal volume**

1204 In single-photon (1P) excitation the absorption of a photon by a chromophore induces a molecular transition
1205 from the ground state (S₀) to the excited electronic state (S₁), while in two-photon (2P) excitation, the same
1206 transition can be induced by the quasi-simultaneous absorption of two photons. Since 2P cross-sections are
1207 typically much smaller than those for 1P, significantly higher photon fluxes are generally required to generate
1208 similar excitation rates, requiring more complex and expensive components such as ultrafast lasers. There are
1209 two main implications of two photon absorption in microscopy. First, since the probability of excitation is a
1210 quadratic function of the instantaneous photon density, targets at the focal plane are much more likely excited
1211 than out of focus targets, whereas in 1P excitation all targets throughout the light path can be excited. Second,
1212 the use of photons of lower energy and therefore of longer wavelengths (deep red and IR) can penetrate more
1213 deeply (~700 μm) in scattering tissue.

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 1773 and applications in medicine, cardiovascular science and plants, among many other uses.

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