Feature Review

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Natural photoreceptors and their application to synthetic biology

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The ability to perturb living systems is essential to understand how cells sense, integrate, and exchange information, to comprehend how pathologic changes in these processes relate to disease, and to provide insights into therapeutic points of intervention. Several molecular technologies based on natural photoreceptor systems have been pioneered that allow distinct cellular signaling pathways to be modulated with light in a temporally and spatially precise manner. In this review, we describe and discuss the underlying design principles of natural photoreceptors that have emerged as fundamental for the rational design and implementation of synthetic light-controlled signaling systems. Furthermore, we examine the unique challenges that synthetic protein technologies face when applied to the study of neural dynamics at the cellular and network level.

Signaling proteins as parts for synthetic biology

Synthetic biology uses diverse qualitative and quantitative approaches to make biology predictable and 'engineerable'; not only to enable the creation of synthetic systems that serve a useful purpose, but also to understand basic building principles of life. Complex natural systems are decomposed into independent building blocks: for example, protein domains, with well-defined functional attributes and interaction interfaces. In many cases, a small set of parameters can describe the functional characteristics of a protein building block in simplified terms. This grouping principle, called abstraction, enables the reassembly of protein parts and devices that carry out simple functions into synthetic systems that execute more complex ones. Ideally, a synthetic system is entirely predictable from the characteristic of the building blocks that went into its construction.

A class of proteins that has attracted attention as a target for protein engineering is the signaling protein group, which transduces information such as environmental stimuli into an adaptive cellular response. To execute the strategy of abstracting natural systems, we need to distill 'universal' design principles. Design principles are recurring structural and functional motifs that are foundational to the underlying biophysics of signal transduction. Importantly, distilling design principles is amenable to both bottom–up and top–down approaches: We can build and characterize *ad hoc* composed synthetic devices and systems to learn what works and what does not; we can also describe and explain how natural signal transduction pathways are implemented.

Unfortunately, the highly dynamic nature of signaling protein structure and interactions complicates the isolation and study of functionally self-contained protein building blocks. By contrast to engineered genetic elements – where the field has arrived at a framework that can 'describe, explain, predict, and control' [1] parts, devices and systems – fundamental processes such as protein allostery or protein folding we can at best explain, but not reliably predict or control. For this reason, one general approach in protein engineering has been to use the apparent modularity of proteins and isolate smaller, well-defined protein building blocks. For these smaller parts, structure determination by X-ray crystallography and nuclear magnetic resonance (NMR) imaging, or protein modeling can provide a starting point for understanding and experimentation [2–4]. These techniques, however, leave many dynamic parameters such as reaction rates, cell-state dependent modulation, and interaction dynamics ill-defined, requiring us to treat protein building blocks as 'black boxes'. Functional attributes can then be characterized and tuned by controlling a variable signal input while observing the resulting output.

In this review, we focus on light-dependent signaling systems and their application to synthetic biology. We will describe recurring design principles found in natural photoreceptors and review how they have been applied to synthetic signaling devices. Furthermore, we will discuss where these devices fit within the larger fields of synthetic biology, and what challenges their application to a complex multicellular environment, such as the brain, presents.

Natural photoreceptor systems

Several light receptor systems have been described and reviewed in depth elsewhere [5–9]. We will discuss three well-characterized receptor systems in more detail: microbial rhodopsins, receptors that incorporate Light–Oxygen–Voltage (LOV) motifs, and tetrapyrrole-binding phytochromes (PHY).

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Microbial rhodopsins, also categorized as type 1 rhodopsins [10] consist of a seven-transmembrane apo-protein called opsin, and an all-*trans* retinal chromophore covalently linked to a conserved lysine residue forming a protonated Schiff base. The all-*trans* retinal is the most thermodynamically stable isomer of retinal, and it is readily available in a wide range of hosts including mammals, making microbial rhodopsins attractive for synthetic biology applications. Absorption of a photon by the chromophore leads to the isomerization of the retinal chromophore, which initiates a cycle of structural changes called the photocycle. Unlike type 2 rhodopsins in which the 11-*cis* retinal chromophore dissociates after photoactivation, the retinal chromophore remains bound throughout the photocycle in microbial rhodopsins, which allows rapid repetitive photostimulation [11]. Rhodopsins mediate light-driven ion transport across cell membranes, sensory signal transduction in microorganisms, and photo signal transduction in the visual system of animals.

LOV motifs are found in many photoreceptor systems including plant phototropins, the related Zeitlupe/ADO family, the fungal receptor Vivid (VVD), and the bacterial receptor Ytva. As parts of multidomain proteins, they are coupled to diverse effectors: kinases, guanylate cyclases, transcription factors, and ion transporter motifs. LOV domains bind flavin mononucleotide (FMN) which, upon photoexcitation with blue light (440–490 nm), forms a cysteinyl-adduct with the protein backbone. The adduct formation initiates cascades of conformational changes that affect the affinity of the coupled effector domain or influence homo-/heterodimerization equilibria. The LOV photoreceptor recovers to the ground state through thermal relaxation in the dark – over the course of seconds in the case of the plant phototropin LOV2 [12], or hours in the case of the fungal photoreceptor VVD [13].

Phytochromes from plants, photosynthetic eubacteria, or fungi covalently bind tetrapyrrole chromophores such as phytochromobilin, biliverdin, or phycocyanobilin. Depending on the signaling state, phytochromes adopt two states, red-absorbing (Pr) or far-red absorbing (Pfr), that can be readily interconverted with 660 nm and 750 nm light, respectively. Certain algal and cyanobacterial phytochromes can sense blue–green light as well. They do this, not by utilizing an altered chromophore itself, but rather by changing their photochemistry, for example, through forming a second cysteine linkage with the chromophore [14]. The light-induced signaling transduction mechanism is still incompletely understood, but many phytochromes form dimers that adopt different conformations upon illumination. This configuration, in turn, modulates a connected effector domain or induces binding to interacting factors [15,16].

Light is an important environmental cue, and photosensing ensures survival and self-propagation of living systems. Plants and algae respond to light with a series of adaptive changes called photomorphogenic responses – adjusting chloroplast position, avoiding shade, orienting towards the light, etc. – to optimize photosynthesis. Animals sense light to process visual detail of the world around them, and to adjust their internal biological clocks. In protein engineering, by contrast, light has proven to be a very versatile input to control protein devices and systems: it is easily dosed, can be delivered noninvasively, and provides high spatial and temporal control. Fundamentally, all photoreceptor systems have evolved to efficiently couple the absorption of a photon to a downstream signal transduction event. While using different chemistries, folds, and signaling modes, they likely share common features constrained by biophysical parameters, to achieve this goal. What are these 'design principles', and how do we apply them to engineered systems?

Ion transport mechanisms of photoreceptor systems

Microbial rhodopsins that mediate light-driven ion transport fall into two categories: ion pumps and channels. Ion pumps actively transport ions across the membrane independent of an existing gradient. In microbial rhodopsin pumps such as bacteriorhodopsin (BR, an outward proton pump) and halorhodopsin (HR, an inward chloride pump), a full photocycle involves the binding of an ion from one side of the membrane and release to the other side (Figure 1A). In their natural host, the membrane potential created by microbial rhodopsin pumps is used in energy-requiring processes such as synthesizing ATP and maintaining osmotic balance [10,17]. Conversely, ion channels passively allow ion transport down the electrochemical gradient by opening a pore that specific species of ions can traverse (Figure 1B). Microbial rhodopsin ion channels [channelrhodopsins (ChRs)] function as phototaxis receptors in green algae [18] through light-induced conduction of cations [19]. In ChRs, the photocycle is yet to be conclusively determined, but electrophysiological data suggest that there are at least two 'open' states in the photocycle that create a cation selective channel [20,21].

The thermodynamics of signaling

Other receptor systems use allosteric coupling mechanisms instead of ion conduction for signaling (Figure 1C). Allosteric models of light-dependent protein signaling include at least two states of different activity that are occupied according to their free energy difference in the absence of signal (Figure 2A,B) [5]. Small energy differences allow both states to be populated; the protein, therefore, has significant baseline activity (Figure 2C). If the energy difference is larger, one state is sparsely occupied, while the other dominates. When a signal is received (e.g., a photon is absorbed) this change is allosterically coupled to a conformational change in the protein associated with a change in free energy, modulating the occupancies of each state. A signal will exert the largest shift in protein activity when all states in this system had been evenly populated before the signal was received.

In principle, the free energy available for driving dark-to-light state transitions can be as large of the energy of the absorbed photon (e.g., 262 kJ/mol for a 450-nm photon). However, due to thermal and vibrational relaxation, only a fraction of that energy is captured to differentially (de) stabilize conformational states. For the *Avena sativa* phototropin LOV2 (AsLOV2) domains, the total signal-derived change in free energy upon illumination was determined to be 17 kJ/mol [22]. This is the amount of energy available to alter the activity of directly allosterically

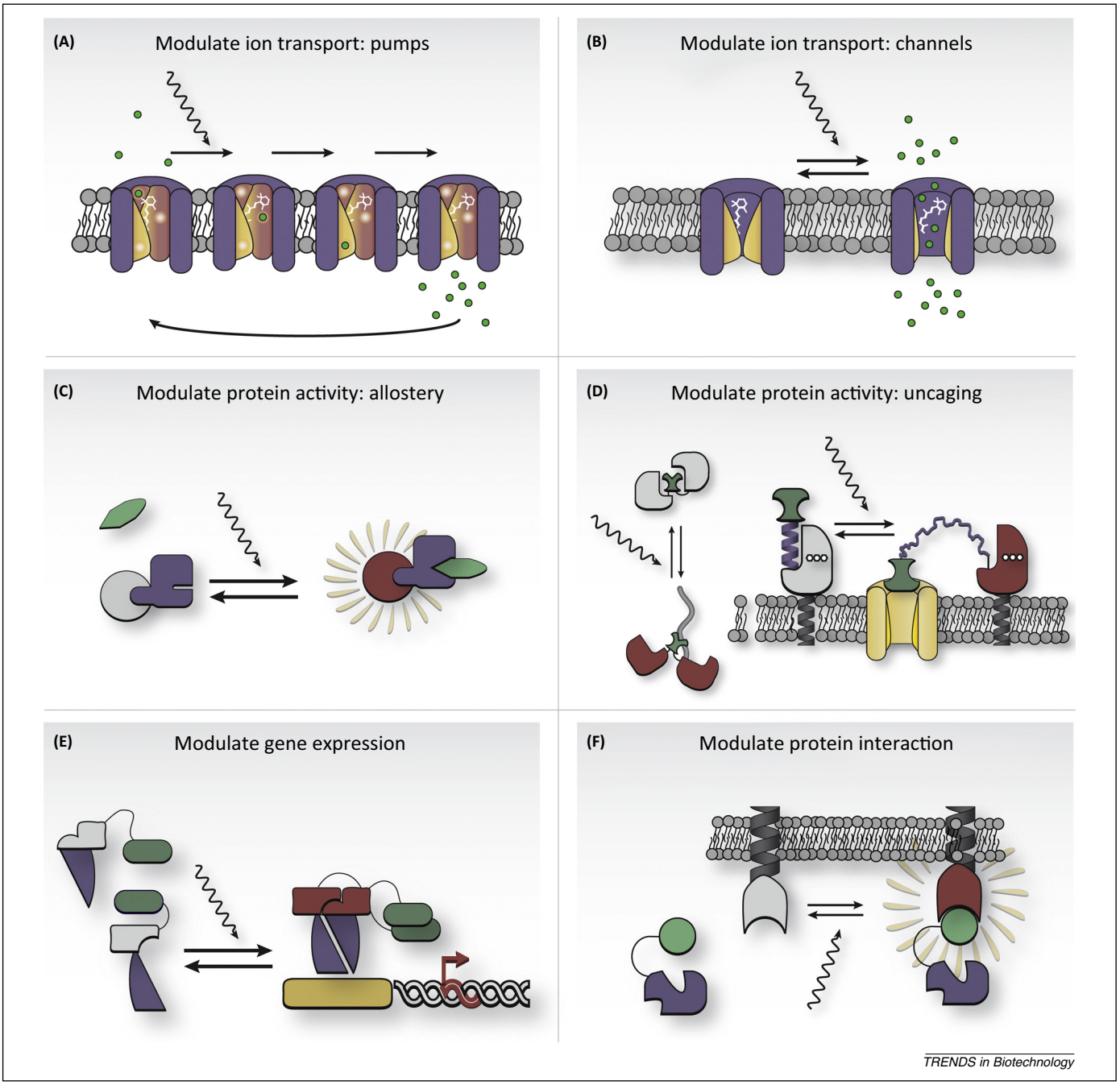

Figure 1. Synthetic signaling devices based on natural photoreceptors. **(A)** Microbial rhodopsin pumps mediate gradient-independent vectorial ion transport. For each completed photocycle, one ion is translocated across the cell membrane. **(B)** Microbial rhodopsin channels mediate ion conduction along electrochemical gradients in response to illumination. **(C)** Photoreceptor domains (grey/red) allosterically control the activity of a fused effector domain (purple) for binding of an interaction partner (green). **(D)** Photoreceptors (grey/red) can control effector domain (green) activity through steric exclusion, that is, by caging them. In response to illumination the effector domain becomes uncaged and gains the ability to bind interaction partners. **(E)** Photoreceptors (grey/red) can control gene expression through controlled assembly or localization of transcription factors (purple) and transactivators (green). **(F)** Photoreceptors (grey/red) can control subcellular localization of signaling proteins (purple) through photo-controlled induction of protein interactions.

coupled effector domains or to cause quaternary structure changes. While this amount represents only 6% of the photon's energy, it is sufficient to bring about large activity changes in a coupled effector domain (Figure 2C).

The modularity of signaling proteins

Nature uses modular building blocks that can be recombined to integrate inputs or to transduce signals in different ways. Across multiple genomes, protein domains correlate strongly with exon boundaries, and thus exon insertions and deletions were proposed to be a major driving force for the evolution of multidomain proteins [23–25]. Exon shuffling can give rise to novel sensor/effector combinations that are subsequently refined by natural selection. Neochromes, for example, contain both phytochrome and LOV domains. Consequently, they sense both red and blue light and transduce a signal that otherwise would have required engaging two separate signaling systems [26].

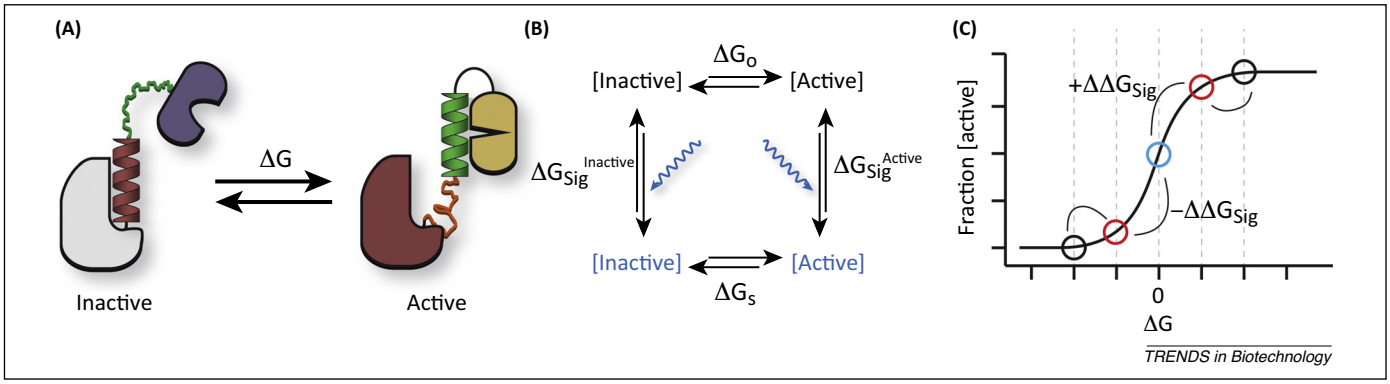

Figure 2. The thermodynamics of signaling. **(A)** An allosteric model of protein signaling that includes two conformational states of different activity, which are occupied according to their free energy difference (ΔG) in the absence of signal. **(B)** The absorption of a photon (blue) is allosterically coupled to a conformational change in the protein associated with a change in free energy (ΔG_{sig}), modulating the occupancies of each state. **(C)** A given total signal-derived change in free energy ($\Delta\Delta G_{sig}$) will exert the largest shift in protein activity when the dark states of the signal protein are evenly populated ($\Delta G_0 = 0$, blue circle). If either the inactive or active state of the signal protein dominates in the dark (red circles), the same change in free energy will yield smaller fractional increases or decreases in protein activity. Adapted from [5].

A challenge in deciphering modular photoreceptor systems is to clearly delineate similarities and differences in coupling and transduction mechanisms. Are there common themes such as the recurring interaction surfaces, or is each solution truly unique? Allosteric coupling of two protein domains creates an ensemble of conformational states, in which a change in either domain influences the conformational distribution of the other. For example, very early on it was noticed that the reaction kinetics of isolated LOV1 and LOV2 domains are markedly different from that of the full length natural photoreceptors [27]. While direct domain interfaces are a feasible way to achieve allosteric coupling, this mechanism would limit the evolvability of modular proteins. The alternative to direct contacts is conformational coupling through an intermediary protein domain. Alpha-helical motifs, called signaling linkers, that are proximate to both sensor and effector domains are examples for natural systems implementing the latter strategy [28]. In transmembrane proteins, alpha-helical elements are known to transduce signals over long distances through rotation, disengagement, pivot, push–pull, and other mechanisms [29,30]. In other multicomponent systems alpha-helical linkers also support localized unfolding and bistability, important for signal transduction through mutually exclusive folding [31,32].

The signal helix

Signaling linkers are 'coupling devices' that communicate changes in conformation of a sensor domain and modify how this signal is transduced to a coupled effector domain. Because they can fulfill specific structural and functional roles without requiring sequence conservation, they are difficult to identify using homology-based methods [33]. Several studies have noted the presence of amphiphatic alpha-helical elements proximate to photoreceptor domains. LOV2 domains, for example, possess both N- and C-terminal extension, A′α and Jα, respectively, that play important roles in signal transduction [34,35]. Similar extensions, can be found in the fungal photosensor VVD [13] and the blue light receptor Aureochrome 1 [36].

The signal transduction mode of linker helices is well understood for the AsLOV2 domain. Crystal structures revealed that the LOV motif core undergoes relatively small conformational changes upon photon absorption [37]. However, the protein scaffold that lines the chromophore binding site on one side also forms an interface with the Jα helix on the other side. Thus, small changes within the flavin binding pocket are allosterically coupled to the Jα helix, which, as a result, is destabilized and unfolds, leading to an increase in the activity of a C-terminal serine/threonine kinase. The extent of unfolding has been debated, but limited proteolysis, NMR, and rational mutagenesis provide strong experimental support for helix propensity being directly correlated with the dynamic range of signal transduction in LOV2 [22,31,38]. Other LOV-domain proteins use the same signaling mode in a different fashion. In the bacterial Ytva LOV domain, no unfolding of the C-terminal linker helix was observed; instead, a rotational or torqueing mechanism for signal transduction was proposed [39]. In the bacterial LOV-containing EL222, the Jα does not unfold but instead unbinds from the protein core and consequently unmasks a dimerization motif for the C-terminal helix–turn–helix motif [40]. This interaction surface facilitates light-induced dimerization and subsequent DNA binding. Conversely, *Rhodobacter spheroides* RsLOV N- and C-terminal extensions form an extensive dimer interface that is disrupted upon illumination [41].

Design principles of natural receptors

By observing natural systems we can recognize specific design principles. For one, the nature of photoreceptor signaling can be described by thermodynamic equilibria of at least two conformational states that respond to a perturbation in the form of photon absorption. These simplified considerations suggest one objective for the engineering of photoreceptors: to maximize their sensitivity and overall dynamic range while minimizing undesired baseline activity in the absence of a signal, we have to maximize the total change in free energy derived from a signal. We can achieve this by maximizing the coupling efficiency between photochemical transitions elicited by the signal and the resulting conformational changes that alter the biological activity of a coupled effector domain.

Second, many photoreceptors are composed of modules with sensor functions and effector functions located on

sequential and structurally separate protein domains. Notable exceptions include rhodopsins, which combine both functions in a single protein fold and support several modes of ion transport. Clearly, another objective in protein engineering is to identify protein domains that encapsulate self-contained functions. However, protein allostery, autoregulation, and quaternary interactions of protein domains might interfere with or alter the intended function and make it difficult to intuit what constitutes a functional module without experimentation. It may also not be obvious where the functional boundaries of protein modules are located. A building block that is completely described by the output it generates in response to an input may encompass more than a single structural motif; it may even encompass the whole multidomain photoreceptor protein.

Third, modular natural photoreceptors often use loosely organized alpha-helical elements packed against the protein core as signal commutators between sensor and effector domains. Conformational changes elicited by photon absorption are transmitted from one side of the chromophore binding pocket to the other against which the various linkers are packed. This shift weakens their binding or folding stability. Furthermore, structural elements packed against the protein core often mask or unmask interaction surfaces in a light-dependent fashion. Ideally, we would use a 'universal' signaling linker, able to connect arbitrary sensor and effector domains, and leverage changes in helix propensity and amphipathic character for the tuning of signal transduction. Since the function of signaling linkers in the context of a multidomain protein depends on the specific nature of the proximally joined protein domains, we need improved predictive models that can take these interdependencies into account.

Applying design principles in engineered signaling devices

The concept of optogenetics [42,43], while originating in neuroscience, now has been applied to biological processes in multiple fields of medicine and biology, for example, to study neural computation, gene expression, and the effects of epigenetic changes. Optogenetics uses light for information transfer and signal modulation in biological systems through control of protein function in targeted cells, with selectable output yield, and with precise spatial and temporal resolution. What all approaches have in common is that the absorption of photons is coupled to a change in functional output. The affected functional output is often a principal cellular messaging system such as membrane voltage, second messenger concentration, or protein localization.

When developing and applying optogenetic technology, the time scale of the perturbed biological process has to be considered; this informs the choice of sensor module. For example, microbial rhodopsins can change membrane voltage within milliseconds, whereas LOV domains actuate downstream effectors on the order of seconds to minutes. The overall size of the optogenetic protein device is another constraint, since the hydrodynamic radius of large sensor domains (e.g., phytochromes) might interfere with the effector's endogenous function. With the notable exception of plant phytochromes, which require phycocyanobilin supplementation, cofactors for widely used receptor systems are readily available in mammals (e.g., flavin, all-*trans* retinal). Delivering light without inducing cellular toxicity is another challenge. For many microbial rhodopsins, light fluences of the order of mW/mm^2 are necessary, whereas intensities several orders of magnitude lower are sufficient for LOV and phytochrome sensors. In addition, the red light that stimulates phytochromes can reach deeper into tissues. If precise spatial resolution is required, scaffolding engineered photoreceptors onto organelles or implementing bidirectional control by patterning deactivating and activating wavelengths can counteract diffusion of light-activated proteins. Lastly, we have to consider what dynamic range is required to achieve a meaningful physiological effect. For example, a 10-fold change in activity is readily achieved with LOV domains and there are instances in which subtle changes can have dramatic effects, especially if they are amplified [44]. In other cases, the light-induced change needs to reach several orders in magnitude [45].

Engineered photoreceptor systems will have a diversity of principal functions, each of which may require different design strategies (Figure 1). Several strategies to design devices that balance specific application requirements were successful: exploitation of conserved signal transduction mechanisms through modular recombination, allosteric control of conformation and activity, and control of function through controlled assembly. These implementations for perturbing living systems can be sorted by how the input (light) affects an output.

Devices that leverage ion transport mechanisms

The distinct ion transport mechanisms of microbial rhodopsin pumps (e.g., HRs and BRs) and channels (ChRs) dictate their application. Microbial rhodopsin pumps provide lower temporal resolution compared to channels because they require a full photocycle (20–30 ms for BR and HR) for ion transport, while ChRs only need to be driven to the open state for ion conduction, which is achieved after approximately 1 ms of light absorption. In addition, speeding up channel kinetics through gene shuffling or mutation allows high frequency control, which otherwise would be limited by the channel closing rate [46,47]. ChRs also support sustained depolarization without the use of continuous illumination. By introducing mutations that can kinetically 'trap' ChRs in its open state, prolonged membrane depolarization is achieved using a short pulse of 470 nm light. The microbial rhodopsin channel can subsequently be closed by a short pulse of 590 nm light [48,49].

Microbial rhodopsin pumps and channels require different strategies for improving ion flux. In microbial rhodopsin pumps, ions are transported one at a time for each completed photocycle (Figure 1A). Therefore, in order to increase the total ion flux, the total number of pumps has to be increased (higher expression), or the photocycle has to be shortened [50]. Conversely, the ion flux generated by ChRs generally depends on the electrochemical gradient and single channel conductance; expression levels are a secondary factor. For ChR2, the single channel conductance

(40–100 fS) is approximately 100 times smaller than that of other high conducting cation channels, suggesting plenty of room for improvement [46,51].

Importantly, microbial rhodopsin pumps and channels leverage distinct mechanisms of action to control membrane voltage. Most known microbial rhodopsin pumps transport ions such that the membrane becomes hyperpolarized via outward transport of protons or inward transport of chloride. The hyperpolarization is effective to inhibit neural activities, but in practice is also self-limiting, since increased hyperpolarization decreases pumping activity [52]. In addition, the active transport of protons and chloride ions can cause cellular responses in the form of bursts of high frequency action potentials after neural silencing using chloride and proton pumps [53–55]. ChRs with altered ion selectivity have the potential to provide an alternative way of inhibiting neural activity by generating hyperpolarizing currents when the membrane becomes depolarized and operating as a shunt. This functionality was recently demonstrated with two ChRs called iC1C2 and ChloC, whose ion selectivity had been shifted towards chloride ions by means of structure-guided mutagenesis [56,57]. When combined with mutations that slow channel kinetics, these ChRs can be used to inhibit action potential formation with a brief pulse of light, instead of the prolonged illumination required for equivalent microbial rhodopsin pumps. Channels with engineered conductance profiles thus provide means to silence neural activities with lower photon fluences and reduce potential concern over light-induced cytotoxicity. Whether these ChRs also cause post-inhibition rebound activity remains to be determined.

Another key property of rhodopsins that dictates their application is spectral sensitivity. Natural rhodopsins cover the entire range of the visible spectrum (400–700 nm) since light absorption in rhodopsins is dependent on the length of the π-conjugated polyene (electron delocalization) in the chromophore and the protonation of the retinal Schiff base. Studies using retinal analogues, site-directed mutagenesis, and metagenomic approaches suggest that rhodopsin spectral sensitivity can be tuned [58–61]. Rhodopsins with high sensitivity in the red (>600 nm) are advantageous for *in vivo* applications, especially when targeting large areas, due to high light scattering in mammalian tissue (particularly in the brain) and hemoglobin absorption (<600 nm). Recently, the development of a red-shifted microbial rhodopsin pump called Jaws enabled the noninvasive silencing of a large brain region through the intact skull [55]. However, it should be noted that rhodopsins sensitive to longer wavelengths require lower activation energy, practically limiting the absorption maximum to approximately 630 nm due to increased thermal noise in their activity [62]. Other applications leveraged the distinct spectral sensitivity of microbial rhodopsins in combination for bi-directional control of membrane potential [63,64] and independent control of multiple cell types [60,61,65]. The recent discovery of a pair of ChRs, named Chronos (high sensitivity and fast kinetics) and Chrimson (maximal spectral sensitivity at 600 nm), and their application to multicolor optical stimulation, identified key parameters and strategies for avoiding cross-activation between colors in such experiments (Box 1) [61].

A novel and useful aspect of rhodopsins is their capability to measure change in membrane potential. Rhodopsins

Box 1. Multicolor activation of ChRs, demonstrated using Chronos and Chrimson

When used in combination, multiple ChRs with distinct spectral sensitivities (Figure IA) are a powerful set of tools that allow independent activation of distinct neural cell types. In multicolor activation experiments, particularly in complex tissues, avoiding cross-activation between the colors can be challenging. This limitation results from any microbial rhodopsins being activated to some degree by blue light, and because the absolute photocurrent generated is a function of cell-variable expression level. Therefore, to avoid crossactivation, blue light powers that ensure separation in sensitivity should be determined across multiple cells with varying expression levels. The blue irradiances are between 0.2 and 0.5 mW/mm^2 for the Chronos–Chrimson pair (Figure IB). Another powerful strategy to avoid cross-activation is to use differences in channel kinetics between ChRs (Figure IC). Since Chronos has an exceptionally fast channel opening rate (Figure IC), using short pulses of blue light (5 ms) further reduces the possibility of cross-activation. Strategies that leverage a separation in spectral sensitivity and kinetics of operation between photoreceptors may provide a generalizable strategy to avoid cross-activation in optogenetic experiments. Figure panels adapted from [61].

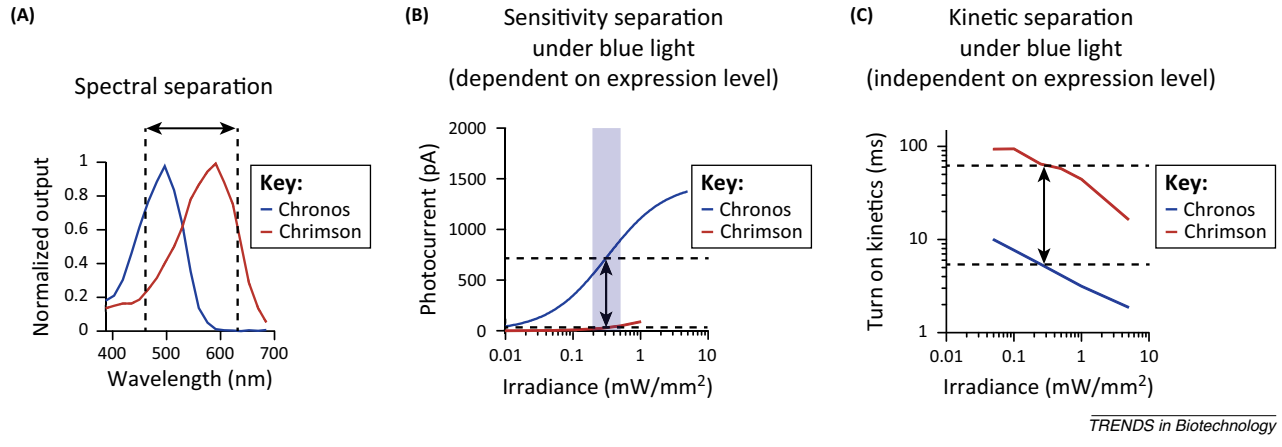

Figure I. Distinct properties of Chronos and Chrimson that enable crosstalk-free multicolor activation.

are fluorescent, but the quantum yield of fluorescence is extremely low ($\Phi \sim 10^{-5}$) [66]. Nevertheless, in certain microbial rhodopsins, such as Archaerhodopsin-3 (Arch), the fluorescence intensity is linearly dependent on voltage and can be used to report membrane potential [67,68]. Arch expresses well in mammalian neurons and has highly voltage-dependent, far-red fluorescence when illuminated with red light (625 nm). Through a random mutagenesis-based screen, the quantum yield of Arch was improved to 8×10^{-3} [69]. The quantum yield is still relatively low, so in order to achieve all-optical electrophysiology (optical excitation using a ChR and membrane potential imaging using Arch), it was critical to reduce the ChR's red-light driven photocurrent. The resulting molecules CheRiff and QuasAr are spectrally orthogonal and were used to probe subtle voltage changes such as back-propagation of action potentials in neurons.

Devices to control protein conformation or activity

Structural homology allowed the generation of devices that resemble natural systems. For example, the light-dependent fusion kinase YF1 is based on the bacterial chemosensor FixL [70]. The FixL PAS motif is replaced with the LOV domain from Ytva, resulting in a dimeric kinase whose activity was initially reduced 1000-fold with blue light. By changing a heptad-motif in the coiled-coil linker, this molecule could be converted into a light-activated kinase. A similar approach was used for the construction of light-activated phosphodiesterase (LAPD) [71]. Structural homology between the GAF domains of human phosphodiesterase 2A and a biliverdin-binding bacteriophytochrome from *Deinoccocus radiourans* permitted swapping them. The resulting devices mediated hydrolysis of the second messengers cAMP and cGMP *in vivo* in response to both red and blue light.

Building on the scheme of allosteric activation, a fusion of AsLOV2 with caspase-7 resulted in the suppression of the caspase's catalytic domain through a pseudoprodomain until autoinhibition was alleviated with blue light [72]. Optimization of the signaling linker was crucial to achieve light-dependent modulation; nevertheless, this system exhibited a high dark state activity, suggesting that further improvements are needed. A light-dependent calcium release system was implemented by inserting a LOV2 domain into positions of the calmodulin–M13 peptide complex that are known to tolerate insertions [73]. Only one of the positions resulted in functional protein, whose affinity for calcium is reduced 230-fold upon blue light illumination. *In vivo*, Ca^{2+} transients act on a millisecond timescale; here, the lack of expression level control and bidirectional switching limits the temporal resolution by which Ca^{2+} can be modulated. Nevertheless, this synthetic protein demonstrates that domain insertions – not commonly found in natural photoreceptors – can be used for engineering light-modulated devices.

In addition to tandem fusions and domain insertions, another strategy is to use photoreceptors for modulating protein activity through photocaging (Figure 1D). Structural information in combination with computational approaches such as rigid-body docking and loop modeling enabled the development of a photoactivated GTPase Rac1 [44]. The fusion of Rac1 with an AsLOV2 domain enabled the affinity to the effector PAK to be increased from 2 μM in the dark – here Rac1 interactions surfaces are sterically blocked by the LOV2 domain – to 0.2 μM with blue light illumination. Since this shift is within the same affinity regime as the endogenous GTPase, a 10-fold change is sufficient to have a physiologically relevant effect. Thus, PA-Rac1 demonstrates that large dynamic ranges are not necessary when the downstream effector is amplified by additional systems such as phosphorylation cascades. Interestingly, an important aspect of the structural basis for Rac1 light-control turned out to be an extensive non-evolved interface between the LOV2 domain and Rac1, mediated by a hydrophobic cluster and several buried waters. While the generation of a photoactivated cell division control protein 42 (Cdc-42) seemed to suggest that creating corresponding interfaces in LOV2 fusions with other GTPases would be a way to generalize this strategy, it has not found widespread adoption.

Another example of photocaging involves lumitoxins, which combine a membrane anchor, AsLOV2, and an ion channel-blocking peptide toxin to disinhibit specific endogenous ion channels with blue light [74]. The signal magnitude mediated by lumitoxins is expected to be a function of difference in tether length and rotational freedom in dark and light states, in addition to the ligand's affinity. Similarly, fluorescent proteins themselves can be used as photoactuators. In the photochromic fluorescent protein DRONPA, the reversible unfolding of a beta-barrel sheet can be induced with green light. This unfolding leads to the monomerization of DRONPA. By fusing DRONPA monomers to the N and C termini of a given protein, its activity could be caged, requiring light for activation [75]. Since DRONPA-based protein cages do not require optimized linking regions, as in the case of lumitoxins, this methodology promises great potential for generalization.

Devices to alter gene expression

One of the earliest engineered protein devices to control gene expression with light (Figure 1E) was based on a VP16 transactivator fused to a flavin-binding FKF1 that dimerizes with a GIGANTEA protein-fused Gal4 binding domain under blue light illumination [76]. Cryptochrome 2 (CRY2)/cryptochrome-interacting basic helix-loop-helix 1 (CIB1)-based systems further expanded the optogenetic tool box with devices that provided more fine-grained temporal control and reduced baseline activity in the dark. These new tools make it possible, for example, to recruit a split Cre recombinase to loxP loci [77] or target transcription activator-like effector nucleases (TALENs) and histone modifying proteins [78]. Utilizing different regions of the light spectrum, UV resistance locus 8 (UVR8)- and PHY-based systems provide multiplexing capabilities for the control of several gene products, each of which can be driven independently [79]. Even though the action spectra of UVR8, CRY2, and PHY overlap, with careful choice of pulsed illumination, one can selectively activate faster switching (PHY) distinctly from slower-switching (UVR8) components. Devices that employ light-controlled homodimerization (e.g., LightOn, LOV-TAP, EL222) feature differing levels of baseline activity, kinetics, dynamic

ranges, and response curves of varying linearity and providing further tool diversity [31,45,80].

In many instances, the bistability and helix propensity of the coupling linker needed to be adjusted by rational mutation for optimal device function and to compensate changes in conformational equilibria that resulted from tandem fusion of protein domains. For example, when designing the light-dependent gene expression system LOV-TAP, the output domain (TrpR) and LOV2 where fused using a shared α-helix with steric overlap that could interact with either sensor or effector, but not both [31]. This bistability ensured a low energy barrier for conformation state transitions and thus sufficiently large rates for photoconversion. However, in this scheme, repressing baseline activity and retaining input sensitivity depend oppositely on the affinity of the bistable signaling linker in the low-energy state. The higher the affinity for docking to the photosensor core in the dark, the lower the baseline activity of a coupled effector, but also the lower the input sensitivity to illumination. In the prototype LOV-TAP, the K_d for binding to DNA increased six-fold with illumination. A high baseline activity indicated that the affinity of the shared helix for the LOV2 domain was too low in the dark state. Adding the output domain TrpR had shifted the LOV2/Jα docking equilibrium too far to the undocked, and thus active, side. As predicted, mutations that increased the helix propensity could rescue the affinity for the dark state LOV2 and increase the dynamic range to 70-fold [32].

Devices such as EL222 that exhibit non-linear photoswitching can incorporate the same signal linker design to achieve high dynamic ranges [45]. A large dynamic range (ultra sensitivity) is a desirable feature of switches, and can be achieved through cooperative effects when there are more than one sensor (input) domain [81]. EL222 demonstrates that light-dependent dimerization driving a downstream effect is an effective way of implementing cooperativity with simple sensor–linker–effector fusions.

Devices to change protein localization and interaction

Localizing proteins to specific intracellular compartments is a common theme in intracellular signal transduction. Two component systems consisting of *Arabidopsis thaliana* phytochrome B (PHYB) and its binding partner phytochrome interacting factor (PIF) or a membrane-tethered LOV2, for example, were used to recruit photo-uncaged peptide epitopes, guanine nucleotide exchange factors (GEFs), kinases, and phosphatases to the cell membrane, where they activated downstream signaling partners involved in cell morphology and cytoskeleton remodeling [77,82,83] (Figure 1F). Eventually, the PIF/PhyB system was combined with live-cell signaling readouts (e.g., translocation of transcription factors to the nucleus) to map connectivity, strength and timescales of intracellular signaling pathways [84,85]. In yeast, the localization of a variety of proteins could also be controlled with light by fusing PHYB to proteins that endogenously target different sub-cellular compartments [86]. It is worth noting, however, that the addition of a large PHYB domain altered the localization of the resulting fusion protein.

Another line of engineering efforts has used photoreceptors to mask and unmask protein interaction surfaces in a light-dependent fashion either to control membrane recruitment [83,87], target proteins for the ubiquitin-independent degradation pathway [88,89], expose nuclear localization signals [90], or to recruit activators of store-operated calcium channels [91].

When adding peptide epitopes as C-terminal extensions to the Jα helix, one can optimize helix packing using theoretical frameworks and *in silico* analysis combined with site-specific mutations. In several cases, the dynamic ranges of such devices could be optimized; however, background activity remains an issue that has to be addressed on a case-by-case basis. Our understanding of the biophysics of light-dependent protein switches continues to improve, but we have not yet succeeded in developing 'universal' coupling linkers whose structure and function are independent of the identity of proximal protein domains. For example, when implementing the caged version of the small flexible peptides ipaA and SsrA, the light-switched affinity to their respective binding partners was enhanced 19-fold for ipaA and 8-fold for SsrA [83]. Surprisingly, mutations that affected linker helix propensity and increased the dynamic range in LOV-TAP 70-fold decreased it to five-fold for the caged ipaA peptide. The authors hypothesized that this behavior was due to charge repulsion in this specific device and demonstrate that further optimization could improve the switching magnitude to 49-fold. Another example for this context-dependent performance are tunable, light-controlled interacting proteins (TULIPs) [87]. TULIPs activate proteins by recruiting them to the membrane. A specific TULIP called LOVpep contains a short peptide that binds to engineered ePDZ protein interaction domains with tunable affinity. Unlike what was observed with LOV-TAP, mutations that increased helix docking in the dark to the LOV core decrease the dynamic range of mitogen activated protein kinase (MAPK) activation, and vice versa. Interestingly, the degree of caging required for successful activation upon illumination also changed depending on the substrate recruited to the membrane. For one recruited signaling protein (Ste5), the variant that had the highest dynamic range (1.6-fold) but lowest dark state caging gave the largest overall physiological response, whereas for another (Ste11), insufficiently caged variants resulted in constitutive activity. Strongly caged variants were required to bring Ste11 within a light-sensitive range.

Devices to change protein complexes and protein clusters

Engineered photoreceptors have also proven useful for controlling the oligmerization state of proteins, either to induce the protein's forward trafficking, or to control their activity. Clustering of small GTPases via CRY2-mediated multimerization, for example, could trigger the activation of coupled intracellular signaling pathways complete with downstream events such as cytoskeletal remodeling [92]. Proteins fused to UVR8 aggregated intracellularly in the dark, but could enter the secretion pathway upon illumination with a short pulse of UV [93]. Another technology platform combines an intrinsically multimeric

protein with CRY2/CIBN to multimerize, and thus inactivate, proteins with blue light [94]. By using single chain antibodies, this technique promises to be extendable so that the activity of arbitrary proteins can be controlled without requiring their genetic modification.

Applying synthetic biology to complex systems – lessons from neuroscience

Optogenetics has gathered significant attention in neurobiology, where it is applied to the grand challenge of neuroscience: to explain and control circuit function, behavior, and cognition through the systematic perturbation and recording from individual neurons [95,96]. Similar to other synthetic protein systems, optogenetic devices will face challenges when applied in the brain. Any of the design choices that we can make – spectral band, transduction mechanism, light sensitivity – is, in practice, limited by the complexity of this tissue and by cell-specific parameters.

For example, only a fraction of microbial rhodopsins originating in bacteria, archaea, and algae were functional when heterologously expressed in mammalian neurons [60,61]. The generation of well-expressing chimeric rhodopsins and the use of trafficking signal sequences ultimately allowed researchers to develop strategies to improve membrane targeting, and in some cases, to target expressed proteins to specific sub-cellular regions, including the axon initial segment [97,98], but this approach is not generalizable [68]. It is likely that many of these microbial proteins misfolded, misassembled, or exhibited instabilities because they are maladapted to the subtly different protein translation and trafficking machinery of neurons. In some cases, a cell's inability to handle certain heterologous proteins may be due to rate limiting steps during protein elongation or post-translational modification that lead to the accumulation of folding intermediates, which have a tendency to aggregate. Folding pathways and conformational stability are optimized in many natural proteins, making the accumulation of folding intermediates unlikely. Because we lack good models of protein folding that can assess engineered protein systems for stable folding intermediates, some groups have instead taken up the strategy of increasing the expression system's folding capacity and resilience by engineering these cells with specific chaperones and foldases [99].

Another strategy to overcome protein folding and trafficking limitations has been to codon-optimize genes, since codon usage affects translation efficiency and accuracy, as well as protein folding [100]. Furthermore, there is evidence for tissue-specific tRNA expression levels and codon usage in human genes [101–103]. Whether or not the differential codon usage is a form of translational control is debated, but theoretical models suggest that selection pressure against toxic and misfolded proteins is sufficient to explain covariation between codon usage and mRNA levels in several taxa, including humans [104]. Among metazoan tissues, covariation is the strongest in neuronal tissue, perhaps reflecting this postmitotic tissue's sensitivity to cytotoxicity from misfolded protein. Furthermore, experimental evidence shows that synonymous mutations affect the mRNA stability and synthesis of two neuronally expressed proteins, catechol-O-transferase (COMT) and the dopamine receptor D2 (DRD2) [105,106]. Tissue-specific codon usage thus appears to be an important aspect of optimizing heterologous protein expression in the brain, yet most synthetic protein systems are only optimized to adhere to the target organism's overall codon usage. This simplification, aside from disregarding tissue-specific effects, implicitly discounts any position-dependence of codon adaptation that can optimize protein synthesis initiation and elongation rates. More experiments are needed to determine the effect of various optimization schemes.

Achieving precise control over the expression levels of synthetic protein systems has also proven to be important. For example, under certain conditions, long-term high-level expression of ChR2 may cause formation of abnormal neuronal morphology and connectivity [107]. While promoters of different strengths can address this need to some extent, cell-to-cell variability is still a major source of functional heterogeneity. Recently, a feedback mechanism was introduced that allows activity levels of targeted proteins to be 'clamped' at a desired concentration using light [84], but it has not yet been applied to complex tissue.

These challenges suggest that we should work 'backwards from the goal' and define a figure of merit by which we can assess the function of synthetic protein devices in living systems. Ideally, functional assessment is implemented as an activity-based assay that closely matches the intended application space. Prototyping and iterating the development of synthetic protein devices and systems *in vivo* is possible with recent advances in 3D printed tissues [108], organoid systems from induced pluripotent stem cells [109–111], or using lower organism systems to model human disease and pharmacology [112–114]. While uncertainty remains, for example, regarding which mechanochemical signaling pathways support controlled maturation and self-organization of printed tissue and human pluripotent stem cells (hPSC) cells into the desired cytoarchitecture, one of the biggest challenges now is to define a precise set of human diseases that can be accurately recapitulated in these systems.

The methodologies we can use to implement our engineering choices are constantly improving. The growing collection of sequenced genomes continues to supply us with a broad diversity of natural photoreceptors identified by homology. Mining these databases can teaches us which of the many structural features of component proteins are important for implementing a specific set of functions in known transduction systems. However, exploiting these databases also allows us to discover new transduction mechanisms when we encounter previously unknown sensor and effector domain combinations. This insight, in turn, improves information-intensive approaches to rational protein design based on structural information, homology structure predictions, expression analysis, and structure-guided mutagenesis. For example, computer-aided design of *de novo* functional proteins has made great progress in recent years and was successfully applied to the rational design of new enzymes, grafting protein domains onto new proteins scaffolds, and optimizing protein folding based on molecular dynamics and free energy perturbation [3,115,116]. However, predictions and optimizations are

far easier to make than to experimentally verify, which is why some studies took advantage of the powerful framework of directed evolution to improve protein devices with suboptimal performance [117–119]. Directed evolution is an approach that requires a good starting point – a sufficiently functional protein – and a way to apply selection pressure. The latter often requires the use of very simple cell models such as bacteria and cell lines, but this approach carries the risk of selecting for protein variants that work well in these simple systems but not in neuronal tissue [120]. Recent studies have, therefore, used primary neuron cultures for medium-throughput approaches [78,121].

In addition to the design process of a tool, we need to pay attention to the practical aspects of their application in the living brain. Ideally one would want to deliver genetically encoded devices through the blood stream in a noninvasive fashion, but this strategy is impeded by the blood–brain barrier. Creating transgenic animals that express exogenous genes in specific cell types is time consuming; therefore, the most widely used method for gene delivery is the injection of viral vectors near the targeted brain region [122,123]. While effective, viral delivery methods are limited in amount of genetic payload [5–8 kB for most commonly used adeno-associated viruses and lenti viruses]. Other methods such as *in utero* electroporation in rodent model systems enable the delivery of larger sets of genes and are compatible with cell-type specific promoters or small molecule-regulated expression systems. However, these methods lack translational potential and require extensive training in animal surgery, and necessitate precise positioning of the electroporation electrodes for regionalized transfection of different regions of the brain [124].

Finally, the complexity of the brain, which arises from high density connections involving many cell types, necessitates cell-type specific expression systems. High-content image analysis and translational profiling approaches have been successful in systematically characterizing expression patterns of genes, and discovering novel cell-type, region-specific, or activity-specific promoters [98,125–128]. However, the correlation between neuronal gene expression patterns, morphology, and electrophysiology can be weak and variable over time, for example, as result of learning, disease, or development. Accordingly, there is great need for light-controlled ternary expression systems (akin to split Gal4 [129]) that take advantage of the modular nature of transcription factors and allow the intersectional expression of DNA binding and activation domains from two promoters, which would increase cell-type specificity.

Concluding remarks and future perspectives

How can synthetic photoreceptor devices contribute further to our understanding of cell signaling and multicellular networks? To identify technologies and approaches that address future needs, we should consider what signaling methods specific cell types employ, and then find precise ways to perturb them. For example, since a large number of ion channels and receptors engage intracellular signaling pathways through a limited number of signaling nodes, the amplitude and frequency of signal patterns originating from these channels and receptors must matter. Any signaling node will have multiple varying inputs, and it is this dynamic signaling that encodes information and ultimately elicits a cellular response. Precision technologies, such as optogenetics, may allow us to isolate pathways so that we can distinguish between competing modes of signaling and off-target effects. Importantly, when designing tools to perturb and interrogate these systems, we are presented with specifications restricted to the timescales and biophysics of cell signaling. We need technologies that function well within these constraints to probe the relation of a pathway's inputs and outputs in the context of intact cells and tissues, and to deduce what processes contribute to the phenotype and its diseased state. Ultimately, we might find specific circuit elements or signaling patterns that can be repurposed in therapeutic or prosthetic networks.

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