

Following Optogenetic Dimerizers and Quantitative Prospects

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ABSTRACT Optogenetics describes the use of genetically encoded photosensitive proteins to direct intended biological processes with light in recombinant and native systems. While most of these light-responsive proteins were originally discovered in photosynthetic organisms, the past few decades have been punctuated by experiments that not only commandeer but also engineer and enhance these natural tools to explore a wide variety of physiological questions. In addition, the ability to tune dynamic range and kinetic rates of optogenetic actuators is a challenging question that is heavily explored with computational methods devised to facilitate optimization of these systems. Here, we explain the basic mechanisms of a few popular photodimerizing optogenetic systems, discuss applications, compare optogenetic tools against more traditional chemical methods, and propose a simple quantitative understanding of how actuators exert their influence on targeted processes.

Optogenetic systems in their broader context

By harnessing the power of genetically encoded light-sensitive proteins, optogenetics seeks to interrogate biological processes via two complementary methods: sensing and actuating (1). Sensors, derived mainly from fluorescent proteins, measure a plethora of outputs. Actuators, composed chiefly of photoreceptor proteins, exert distinctive and diverse cellular functions.

Two major approaches for designing sensors are manipulating fluorescent protein spectral properties and inducing Förster resonance energy transfer efficiency changes under specified conditions (2). While both engineering blueprints translate protein conformational changes into functional measurements of fluorescence, the general principle of design for the first type of sensor relies upon the signal changes of a single fluorescent protein typically arising from β -barrel structural rearrangements when it senses the intended target (3). In contrast, the second type of sensor correlates its conformational changes in the absence or presence of the intended target to an effective measurement of Förster resonance energy transfer efficiency between two fluorescent proteins (4,5). Well-known sensors report changes in intracel-

lular calcium (Ca^{2+}) (6), voltage (7), and secondary messengers (8).

Actuators may also be subdivided into two major groups: microbial opsins (9) and photomultimerizing proteins. From microbial opsins, rhodopsins have come to the forefront of optogenetics with channelrhodopsins (10) and halorhodopsins, which depolarize to activate (11) and hyperpolarize to inhibit action potential firing in the cell, respectively (12). Pioneering work with these *Chlamydomonas reinhardtii* proteins show that they can fine-tune neuronal responses through single-cell excitation (13). Other design tactics with photoswitchable receptors (14) also corroborate the usefulness of optogenetics for many purposes including brain-mapping and studying neurological diseases (15).

While the combinatorial use of sensors and microbial opsins revolutionized many aspects of neuroscience and basic science research, this article will not proceed beyond citing comprehensive reviews for these topics. We will also offer little practical guidance for using optogenetic switches past citing relevant reviews as there are many interesting discussions on how to choose an optogenetic system based upon dynamic range (16), kinetics (17), and experimental requirements (18). Instead, we focus on understanding broader concepts for the various categories of uses for photomultimerizing proteins in probing and engineering biology and medicine today. More specifically, we will discuss the origin and development of optogenetic tools

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and then present a simple quantitative understanding of optogenetic systems derived from these proteins.

Development of optogenetic designs

Since the dawn of evolution, primary producers have been translating light into cellular signals. Accordingly, the more primitive forms of these photomultimerizing actuators originated from scientists studying the regulation of transcription pathways in plants as well as similar mechanisms in other photosynthetic organisms such as algae and cyanobacteria. The mustard plant *Arabidopsis thaliana* is recognized for phytochrome B (PhyB)/phytochrome-interacting factor (PIF) (Fig. 1 A), light-oxygen-voltage (LOV) domain

(Fig. 1 D), and cryptochrome2 (CRY2)/cryptochrome-interacting basic-helix-loop-helix (CIB1)-based (Fig. 1 G) optogenetic systems while isolation of mutant GFP-like proteins from the *Pectiniidae* coral family, also known as chalice coral, brought about Dronpa (Fig. 1 J)-based systems.

The oldest characterized system from the *Arabidopsis* group is the PhyB/PIF system, which may be traced as far back as the 1950s. Lettuce seeds were observed to germinate at differing speeds; red light promoted germination while infrared light repressed germination (19). From these findings, it was assumed that a pigment was chemically changing depending on the wavelength of light. Approximately four decades later, this pigment was identified as PhyB (20). Upon light stimulation, PhyB binds to its partner

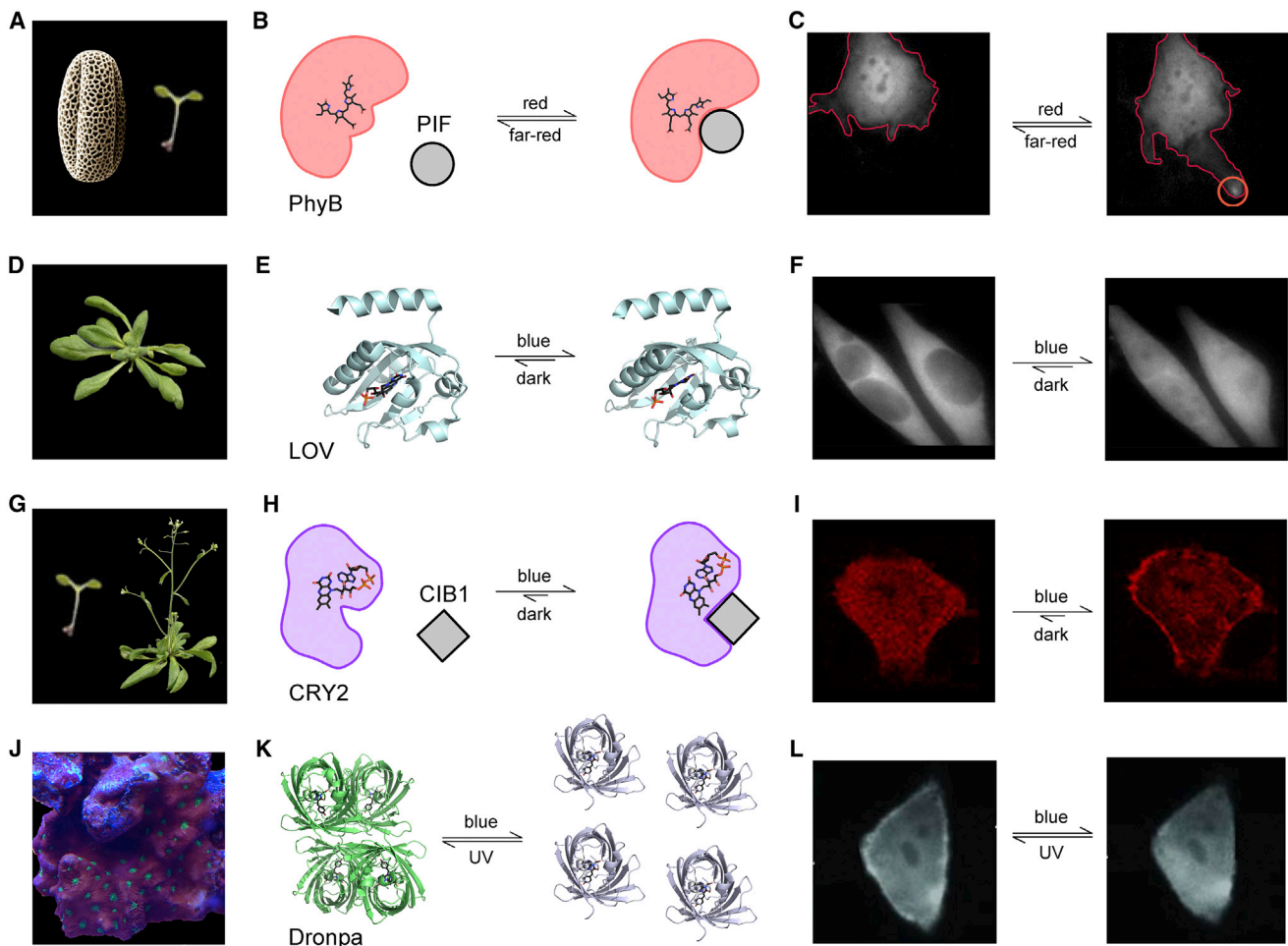


FIGURE 1 Organism of origin and relevant lifecycle is shown for various optogenetic actuators. (A) PhyB is known to be involved in germination and flowering of *Arabidopsis thaliana* (84). (B) Cartoon depiction of PhyB/PIF photoswitching mechanism with chromophore structure (85). (de-etiolation) (C) PhyB/PIF-induced lamellipodia formation (red circle) (86). (D) LOV domain of phototropin is a key component of phototropism and stomatal opening in *Arabidopsis* (84). (E) Atomic structures show two light-dependent configurations of LOV domain isolated from *Avena sativa* (left, Protein Data Bank (PDB): 2V0U; right, PDB: 2V0W) without the α helix (87). An alternate mechanism is LOV photodimerization with an EL222 mutation and VVD. (F) Lov2- $J\alpha$ mediated nuclear localization (88). (G) CRY2 is an important player in flowering and deetiolation of *Arabidopsis* (84). (H) Cartoon rendition of CRY2/CIB1 dimerization with blue light (89). Light-dependent oligomerization of CRY2 is another powerful optogenetic mode of operation. (I) Membrane localization of CRY2-Akt in response to blue light (44). (J) Dronpa is a homolog of GFP found in coral *Pectiniidae*. (K) Crystal structures show reverse dimerization of Dronpa (left, tetramer PDB: 2POX (32); right, monomers PDB: 2IOV (90)). (Green) Fluorescent state; (gray) dark state of Dronpa. (L) Functional exemplar of light-regulated photoswitching of Dronpa (30). For visualization of relocalization, the fluorescence of mNeptune tagged to Dronpa is monitored. Images in (A), (D), and (G) are reproduced from Krämer (91). To see this figure in color, go online.

PIF3, which is a transcription factor with a helix-loop-helix structure, and begins DNA transcription to signal seed germination (Fig. 1 B) (21).

Similarly, the LOV domain was found to be an important photosensing protein when it was discovered that exposure to blue light resulted in heavy phosphorylation of the protein encoded in the NPH1 gene of *Arabidopsis* (22). When LOV is irradiated with blue light, the flavin cofactor forms a covalent bond with a key cysteine residue and changes the conformation of the LOV domain (Fig. 1 E). Many studies focus on optimizing this system as the different types of LOV domains offer a large selection of kinetic rates from highly stable conformations in both the dark state and light state as seen with FKF1 (23) to fast on- and off-rates as with AsLOV2 (24).

CRY2 is a member of the cryptochrome family, and some argue that it takes part in regulating the circadian rhythm like its homolog CRY1 (25). More important for the points discussed here, however, is CRY2's integral role with its partner CIB1 (Fig. 1 H) in modulating transcription leading to *Arabidopsis* flowering (26). Recently, the pair garnered a lot of attention due to their intermediate kinetic rates with an on-rate of milliseconds and an off-rate of minutes (27). The CRY2 mutation E490G also dramatically increases the oligomerization affinity of CRY2 (CRY2olig) and induces clustering at the expense of slower kinetics (28). Nonetheless, CRY2olig provides another powerful flavor of optical signaling through clustering compared to colocalization of CRY2 with its binding partner CIB1.

Lastly, Dronpa was initially found as a homolog of GFP in the coral family *Pectiniidae* (Fig. 1 J) (29). Monomers of Dronpa bind to one another to form dimers, and then these dimers come together to form tetramers. A point mutation, K145N, was introduced to weaken tetramer formation and thereby enhance the probability of finding Dronpa dimers (30). With blue light, Dronpa switches from a fluorescent multimer to dark monomers and reverses with ultraviolet light (Fig. 1 K). Unlike the PhyB/PIF system, Dronpa reverses dimerization, allowing it to photo-uncage enzymes (Fig. 1 L). In addition, the fascinating property of fluorophore switching (31,32) makes Dronpa unique from its fellow optogenetic proteins in it can also serve as a sensor of its own activity (30).

Topics of achievement in discovering and constructing cellular processes

With the elucidation of these systems, many have selected their favorite optogenetic tool to examine their preferred biological problems. Some common themes that emerge through nature and converge with other multimerization systems, such as chemically inducible dimerization (CID) systems (33,34), include manipulating gene expression and toggling molecular concentrations with localization

and clustering-based logic. Classic problems that have been explored include activation of DNA transcription and other well-known molecular pathways, while newer ventures focus on other modes of intracellular signaling such as Ca^{2+} regulation.

There is much success associated with using these tools to control gene expression as many of these systems were derived from light-sensitive transcription pathways. In one case, the EL222 transcription factor, which contains a LOV domain and helix-turn-helix (HTH) motif, was used to upregulate the production of luciferase in HEK293T cells. While LOV binds HTH in the dark, blue light stimulation causes LOV to undock HTH, which leads to HTH dimerization, then HTH dimer-DNA binding, and finally, DNA transcription (35). Another example is using CRY2/CIB1 with epigenetic modifiers such as LITE (36) or permanent genetic modifiers such as TALEN and CRISPR-Cas9 to pattern perpetual transcription profiles in cells spatially (37,38). Similar experiments have also been performed with split-Cas9 and Magnets, a variant of LOV domains, so that upon blue light stimulation, magnets dimerize and produce a functional Cas9 (39). Outside of single-cell applications, these tools have also been implemented in model organisms. The CRY2/CIB1 system has been shown to drive luciferase expression in zebrafish (40) as well as increase GFP levels in *Drosophila* (41) under their respective transcription systems.

More phenotypical assessments of cellular output with optogenetic systems target well-understood molecular pathways such as Rac activation in membrane ruffling and cell motility. A photoactivatable version of Rac1 with LovJ α -directed cell movement with blue light stimulation (42). Comparably, the PhyB/PIF system was also used to show that it is not only possible to direct lamellipodia and filopodia formation (Fig. 1 C) (43) but also possible to generate a train of signals and subsequently evaluate how the functional output of cell survival and cell growth changes with stimulation frequency of the Ras/ERK pathway (18). Moreover, the achievements of optogenetic techniques have not been limited to cell motility. Akt localization to the membrane with CRY2/CIB1 (Fig. 1 I) uncovered the importance of Akt in mechanistic target of the rapamycin growth pathway and the forkhead box O metabolism pathway for the generation of Atrogin1 implicated in muscle atrophy (44).

One powerful notion is using optogenetics to control Ca^{2+} signaling. Ca^{2+} regulation is important for numerous yet dissimilar physiological processes including transcription activation, cell death, and neurological development (45,46). Ca^{2+} release-activated channels (later mapped to the ORAI gene) and stromal interaction molecules (STIM) were first discovered because of their importance in T cell activation. When Ca^{2+} stores are depleted, it is believed that STIM EF-hands undergo a conformational change that allows aggregation of the C-terminal domain of STIM

(CCD) to prompt the opening of ORAI (47). Recently, multiple groups confirmed that it is possible to increase cytosolic Ca^{2+} levels by opening ORAI channels through either clustering CCD with CRY2olig (48) or releasing CCD with the LOV domain (49). Thus, these studies provide additional steps toward examining more complex processes involving Ca^{2+} signaling.

Explicit differences between optogenetics and CID techniques

One established rival technology we would like to consider is CID (34). A few points of contemplation important for understanding the differences between optogenetics and CID include inherent properties of the systems themselves and experimental equipment. Explicitly, these two factors influence the reversibility, spatial specificity, temporal precision, and dynamic range of biological signals generated by these systems during experiments.

Generally, after light-sensitive proteins absorb photons, they enter a high-energy state with a different conformation due to energy transfer. Then, when the light source is removed, they relax and revert to their original conformation. As most CID processes appear irreversible under experimental conditions, optogenetic systems are attractive for their transient responses that enable reversible cycling of signals. One possible resolution for the irreversibility of CID systems, however, is the idea of using a mixture of different CID systems to create antagonistic reversal of the original signal (50). A process that has been highlighted with both optogenetics and CID is KCNQ2/3 channel activity. In 2006, it was proved with CID that PIP_2 levels in the plasma membrane directly modulate KCNQ2/3 so that current decreases upon PIP_2 depletion by an engineered PIP_2 phosphatase (51). Shortly after the popularization of optogenetic techniques, the CRY2/CIB1 system was also applied to the regulation of KCNQ2/3 channels. In addition to replicating the attenuation of current from the original CID experiment, a measurement of the recovery of current and PIP_2 concentrations at the membrane could also be made within 10 min (52).

Another set of experiments for comparison is probing processes that require spatial signaling differences such as cell polarity. Creating molecular concentration gradients is a challenging problem that requires proper equipment for both CID and optogenetic systems. For CID, careful design of microfluidic devices or biomimetic scaffolding is imperative to correctly shape a cell's chemical environment such as with graded rapamycin activation of Rac1 (43). On the other hand, technological advances of microscope setups with optical filters, pinholes (53), digital micromirror devices (54), and even endoscopy tools (55) provide a variety of effective solutions for defining the field of view stimulated with light. Examples include controlling the polarity of yeast for budding with Bem1 (56), using CRY2olig to

cluster receptor tyrosine kinases (57), and using CRY2/CIB1 to manipulate G-protein-coupled receptors that promote lamellipodia and cell migration (58). LOV domains have also been used to optogenetically control protein degradation (59).

Speed of stimulus delivery is an area in which optogenetics exceeds its chemical competitors. Most optogenetic systems produce the desired effect within seconds while CID systems require tens of seconds due to limited delivery of rapamycin. One example of this phenomenon is seen with organelle transport by kinesins and dynein where directed migration of peroxisomes using the LOV domain linked to the respective cytoskeleton motor (60) resulted in an approximately twofold faster recruitment compared to its CID analog (61). Regardless, both systems offer great promise for answering a myriad of biological questions and contributing to the synthetic cell biology toolbox (62), but still require optimization and further specific design to reduce unwanted chemical and phototoxicity effects as well as undesired basal cellular perturbations.

Dynamic range in relation to kinetics

On the topic of dynamic range and speed, it may be useful to contemplate a general scheme for how photomultimerizing proteins behave as optogenetic actuators. Upon photoexcitation, light-sensitive proteins undergo conformational changes (Fig. 2 A) that result in functional outputs. For the photomultimerizing optogenetic tools described above, this conformational change often exposes a hidden surface on the light-sensitive protein that allows it to interact with its target. This bimolecular reaction converts the target into its active form (Fig. 2 B). These actuators may also interact with their target in the dark, albeit with a substantially weaker affinity, leading to basal activity. Thus, the output of target's activity in a cell is directly proportional to the fraction of targets bound to an actuator ($f_{T,b}$). If we assume rapid and complete photoconversion of the optogenetic actuator (free concentration, $[A]$), then $f_{T,b}^{hv}$ after light activation is governed by

$$\frac{df_{T,b}^{hv}}{dt} = k_{on}^{hv} \cdot [A] - \left(k_{on}^{hv} \cdot [A] + k_{off}^{hv} \right) f_{T,b}. \quad (1)$$

Solving Eq. 1 for steady state yields

$$f_{T,b}^{hv}(\infty) = \frac{[A]}{[A] + k_{off}^{hv}/k_{on}^{hv}} = \frac{[A]}{[A] + K_d^{hv}}, \quad (2)$$

where k_{off}^{hv} is off-rate, k_{on}^{hv} is on-rate, and the ratio $K_d^{hv} = k_{off}^{hv}/k_{on}^{hv}$ represents the affinity of the actuator for the target molecule in the presence of light. Because the free concentration of optogenetic actuator in a cell is

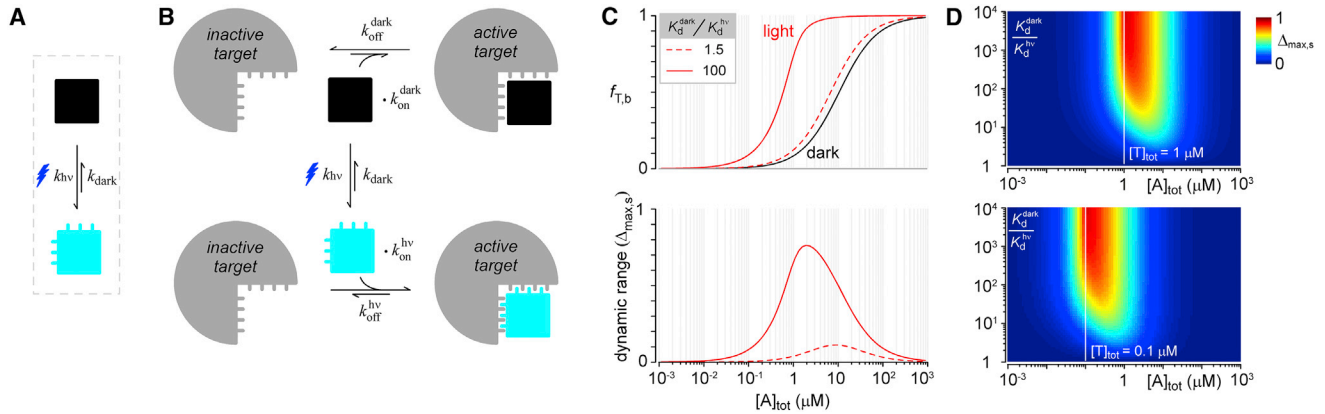


FIGURE 2 Schematic depiction of the behavior of an optogenetic actuator. (A) Light-activation leads to conformational changes in the actuator. (B) Four molecular conformations are possible: unbound dark actuator, bound dark actuator, unbound lit actuator, and bound lit actuator. Physical changes resulting from light allow the protein to interact better with its target under illumination and bias it to reside as a bound lit actuator. (C) The top graph shows a simulation for the changes in fraction bound of actuator-target complexes as a function of actuator concentration due to different fold changes in actuator binding affinity before and after light. Similarly, the bottom graph illustrates the dynamic range of actuator-target complexes for these various conditions. (D) Top graph color maps dynamic range at a total target concentration of $1 \mu\text{M}$ as a function of total actuator concentration and binding affinity fold enhancement for lit versus dark states. An analogous graph is drawn on the bottom for a total target concentration $0.1 \mu\text{M}$. (Open line) Total concentration of actuator and target are equal, which is also the optimal total actuator concentration for maximizing dynamic range. To see this figure in color, go online.

unknown, a more convenient formulation of Eq. 2 assesses $f_{T,b}^{hv}$ in terms of total concentrations of actuator ($[A]_{tot}$) and target ($[T]_{tot}$) molecules:

$$f_{T,b}^{hv}(\infty) = \frac{1}{2} \left(1 + \frac{K_d^{hv}}{[T]_{tot}} + \frac{[A]_{tot}}{[T]_{tot}} \right) - \frac{1}{2} \sqrt{\left(1 + \frac{K_d^{hv}}{[T]_{tot}} + \frac{[A]_{tot}}{[T]_{tot}} \right)^2 - 4 \frac{[A]_{tot}}{[T]_{tot}}} \quad (3)$$

Given that the actuator can also bind to the target in darkness, its dynamics is determined by

$$\frac{df_{T,b}^{dark}}{dt} = k_{on}^{dark} \cdot [A] - (k_{on}^{dark} \cdot [A] + k_{off}^{dark}) f_{T,b}^{dark}, \quad (4)$$

assuming that the on/off-rate constants change instantaneously. Similar to Eq. 2, the steady-state solution of Eq. 4 is

$$f_{T,b}^{dark}(\infty) = \frac{[A]}{[A] + K_d^{dark}}, \quad (5)$$

where the ratio $K_d^{dark} = k_{off}^{dark}/k_{on}^{dark}$ corresponds to affinity of the actuator for the target in the absence of light. Once again, for convenience, $f_{T,b}^{dark}(\infty)$ can be determined in terms of total concentrations of actuator ($[A]_{tot}$) and target ($[T]_{tot}$) molecules,

$$f_{T,b}^{dark}(\infty) = \frac{1}{2} \left(1 + \frac{K_d^{dark}}{[T]_{tot}} + \frac{[A]_{tot}}{[T]_{tot}} \right) - \frac{1}{2} \sqrt{\left(1 + \frac{K_d^{dark}}{[T]_{tot}} + \frac{[A]_{tot}}{[T]_{tot}} \right)^2 - 4 \frac{[A]_{tot}}{[T]_{tot}}} \quad (6)$$

With this framework, the dynamics of the system can be viewed as a simple transition from one steady state to the other in the absence and presence of light ($f_{T,b}^{dark}(\infty), f_{T,b}^{hv}(\infty)$) with the time-course of relaxations determined by the appropriate on-off rate constants. The system's maximal dynamic range ($\Delta_{max,s}$) considering only the signaling aspects of the system is then the difference between the dark and light steady-state solutions,

$$\Delta_{max,s} = f_{T,b}^{hv}(\infty) - f_{T,b}^{dark}(\infty) = \frac{1}{2} \left(\frac{K_d^{hv} - K_d^{dark}}{[T]_{tot}} \right) - \frac{1}{2} \sqrt{\left(1 + \frac{K_d^{hv}}{[T]_{tot}} + \frac{[A]_{tot}}{[T]_{tot}} \right)^2 - 4 \frac{[A]_{tot}}{[T]_{tot}}} + \frac{1}{2} \sqrt{\left(1 + \frac{K_d^{dark}}{[T]_{tot}} + \frac{[A]_{tot}}{[T]_{tot}} \right)^2 - 4 \frac{[A]_{tot}}{[T]_{tot}}} \quad (7)$$

Note that the dynamic range defined here assumes a complete photoconversion of all actuator molecules. In practice, depending on the wavelength of the incident light and the optical fluence of the sample (63,64), expressing the optogenetic dimerizing tool, the actual dynamic range may be substantially lower.

Two key outcomes follow from Eq. 7: (1) The dynamic range of the system relies upon the fold enhancement in the actuator's binding affinity after light activation (Fig. 2 C). For example, if photoexcitation enhances the actuator's binding affinity minimally ($\sim 50\%$), then the fraction of target molecules ($f_{T,b}$) bound to the actuator shifts subtly to the left (compare black curve to red dashed curve, Fig. 2 C, top) resulting in a limited dynamic range of the system ($\Delta_{max,s} < 0.1$). By contrast, a large increase in

affinity ($K_d^{\text{dark}}/K_d^{\text{hv}} > 100$) results in a marked shift in $f_{T,b}$ (compare *black curve* to *red solid curve*, Fig. 2 C, *top*) and a substantial enhancement in the dynamic range ($\Delta_{\text{max},s} < 0.75$). (2) The dynamic range of the system is also sensitive to the total concentration of the actuator in the cell—the “Goldilocks problem”. If the concentration of the actuator is very low, then only a small fraction of target molecules will become bound to an actuator after light stimulation (Fig. 2 C, *bottom*). Similarly, if the actuator is abundant in the cell, then a significant fraction of targets will be prebound to the actuator, resulting in high basal activity and minimal subsequent light-dependent recruitment (Fig. 2 C, *bottom*). By contrast, at intermediate actuator concentrations, there is minimal prebinding in the dark, but light-activation results in substantial translocation of the actuator to its target (Fig. 2 C, *bottom*). Further analysis of the conditions that improve the dynamic range of the optogenetic actuator system can be visualized in Fig. 2 D for a given concentration of target molecules in the cell (*top*, $[T]_{\text{tot}} = 1 \mu\text{M}$; *bottom*, $[T]_{\text{tot}} = 0.1 \mu\text{M}$). In both cases, the dynamic range improves if the fold enhancement in binding affinity upon photoexcitation is increased. In addition, the dynamic range is also improved when the total concentration of actuators in the cell matches the concentration of targets (Fig. 2 D; *top*, *white line* $[A]_{\text{tot}} \sim [T]_{\text{tot}} \sim 1 \mu\text{M}$; *bottom*, *white line*, $[A]_{\text{tot}} \sim [T]_{\text{tot}} \sim 0.1 \mu\text{M}$), akin to impedance matching in electronic circuits.

To optimize the dynamic range of the optogenetic system, one strategy is to maximize the actuator’s affinity for its target in the presence of light, which also broadens the system’s operating range. Strengthening the interactions that form at the interface between the two partners stabilizes the actuator-target bound complex. This manipulation decreases $k_{\text{off}}^{\text{hv}}$ to reduce K_d^{hv} and reflects the enhanced affinity for the actuator-target interaction in the presence of light. An important shortcoming of this approach becomes apparent, however, if we consider the time constant (τ_{hv}) of the optogenetic system in the presence of light,

$$\tau_{\text{hv}} = \frac{1}{k_{\text{on}}^{\text{hv}} \cdot [A] + k_{\text{off}}^{\text{hv}}}, \quad (8)$$

where reducing $k_{\text{off}}^{\text{hv}}$ inevitably increases τ_{hv} , the time required to attain 63% recruitment. Thus, the net improvement in the affinity and the dynamic range comes at the cost of a kinetically slower system. Another complexity for this approach arises from the fact that the actuator binds the target in darkness. To fulfill the conditions of detailed balance in kinetic theory, the strengthened actuator-target interface also enhances the binding of the two proteins in the dark (65), resulting in increased basal activity of the actuator. An alternative strategy to optimize the system is to increase $k_{\text{on}}^{\text{hv}}$ by destabilizing the unbound configuration in the presence of light. This process enhances K_d^{hv} and de-

creases τ_{hv} , resulting in a system with a potentially large dynamic range and fast kinetics.

Although promising, such rational approaches to optimize optogenetic systems remain challenging without extensive structural information. Moreover, the schematic presented here renders the optogenetic process as a single-step reaction with a single time-constant limited with the actuator-target interaction being the limiting step. In most cases, however, the system is more complex with multiple steps that reflect the photophysics of the actuator chromophore and other intermediate relaxation steps. In addition, the dynamics of some optogenetic systems may be limited by the kinetics of downstream signaling by the target. For CRY2, studies argue an intermediate step may be due to stabilization through ATP binding (66). Nevertheless, ongoing studies are actively continuing to address these issues. Examples of specific models that incorporate further details include engineering of light-regulated histidine kinases (67), photocontrolled delivery of toxins to ion channels (68), light-gated transcription factors (35), and analysis of the channelrhodopsin photocycle (10,69). These models have proven insightful for refinement and optimization of various optogenetic tools and may pave the path toward rationally designed synthetic actuators to manipulate biological function.

Particular successes for intentional design of photomultimerizing optogenetic tools include manipulating LOV domains, which have available structures (70). Furthermore, natural processes provide a library of diverse LOV proteins (71,72). A few specific methods that have appeared to tackle this problem are computational modeling and crystal structure analysis with programs such as Rosetta (73,74). By first predicting more stable dark forms of LOV, screening of these mutated proteins revealed a faster system with a larger dynamic range (17,75). These concepts are also generalizable to describe coarse behavior of other systems including CID actuators. Overall, the quest for designing and optimizing optogenetic tools to manipulate various facets of biological function will be greatly facilitated by the increasing number of atomic structures that are available and through in-depth biophysical and kinetic analysis.

Future directions extrapolated from the past and present

Light-sensitive proteins are intriguing and useful for many reasons. Besides their application to basic sciences, medical fields have also employed optogenetics in retinal therapies (76), maintaining blood glucose homeostasis (77), and even adjusting transgene expression with brain activity (78). An idea that has resurfaced on the horizon is directing axonal growth with light. While a study performed more than a decade ago used caged Ca^{2+} to show light directing the growth cone of neurons (79), more recent studies used LOV (60) and CRY2/CIB1 (80) to guide neuronal growth.

Hence, these methods may be very powerful for studying and understanding the formation of synapses and possibly memories.

Nonetheless, one should consider the contrasting advantages between optogenetics and other techniques, as this decision is dependent on a few criteria including equipment, time, space, signal effect, and available wavelengths of light for stimuli and sensor measurements. Merging optogenetics and other orthogonal systems such as CID may also provide extended flexibility for honing cellular control with more complex logics. All in all, these systems provide many tools important for advancing scientific knowledge. As crystal structures emerge in tandem with further understanding of important signaling cascades, the engineer's dream of meticulously designing light-responsive cellular components may be closer than imagined (81–83). Ultimately, this new modality of interacting and manipulating biological systems echoes the brave new world we face.

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