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Perspective

Optimizing optogenetic constructs for control over signaling and cell behaviors

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Abstract

Optogenetic tools have recently been developed that enable dynamic control over the activities of select signaling proteins. They provide the unique ability to rapidly turn signaling events on or off with subcellular control in living cells and organisms. This capability is leading to new insights into how the spatial and temporal coordination of signaling events governs dynamic cell behaviors such as migration and neurite outgrowth. These tools can also be used to dissect a protein's signaling functions at different organelles. Here we review the properties of photoreceptors from diverse organisms that have been leveraged to control signaling in mammalian cells. We emphasize recent engineering approaches that have been used to create optogenetic constructs with optimized spectral, kinetic, and signaling properties for controlling cell behaviors.

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Introduction

Photoreceptor proteins mediate light-dependent responses in organisms ranging from bacteria and algae to plants and mammals. Two features of this diverse class of proteins have made it possible to engineer novel genetically encoded constructs to control a wide range of biochemical processes in intact cells using light. First, photoreceptors are often found to retain their light-dependent signaling capabilities when expressed in different organisms and cell types. Second, light-responsive domains often act in a modular fashion, which has enabled their integration into engineered constructs that retain light sensitivity and incorporate novel signaling outputs.

Recent reviews have focused on optogenetic tools based on photoreceptors such as opsins, phytochromes, cryptochromes, and light-oxgen-voltage sensing (LOV) domains used to control signaling activities with light and the biological insights that these tools can provide [1-3]. Here, we focus on how optical modulators of cell signaling have been designed by selecting light sensitive proteins and recruiting their unique properties such as activation spectra, light-sensitivity, kinetics and signaling characteristics to control cellular responses.

Naturally occurring light activated signaling proteins

Some signaling functions can be controlled using naturally occurring light-sensitive versions of enzymes or transmembrane receptors. In this section we focus on light activated adenylyl cyclases and G protein coupled opsins. Both offer examples of light-sensitive proteins that can be used in a variety of organisms and cell types to achieve subcellular control over signaling events. They also illustrate how light-sensing and signaling domains can be combined in new ways to achieve desired spectral, kinetic and signaling properties.

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Light-activated adenylyl cyclases

Photoactivated adenylyl cyclases from the flagellate *Euglena gracilis* (euPAC), and the soil bacterium *Beggiatoa sp.* (bPAC) have been shown to be capable of generating cAMP in mammalian cells and model organisms in response to blue light [4-9]. bPAC is advantageous because of its smaller size (350 amino acids), lower dark activity and larger light-induced increase in activity [7]. Its cyclase activity increases >100-fold in response to blue light and decays within 20s upon remove of the optical stimulus [7]. bPAC encodes an N-terminal BLUF (blue light receptor using flavin adenine dinucleotide) domain, and a C-terminal adenylyl cyclase and functions as a homodimer. Understanding the mechanism of photoactivation remains an active field of study [10, 11]. An LOV regulated adenylyl cyclase was recently discovered from the cyanobacterium *Microcoleus chthonoplastes* PCC 7420 (mPAC) [12]. Compared to bPAC, mPAC exhibited higher constitutive activity as well as higher light induced activity [12].

Subcellular compartmentalization and temporal control of second messengers such as cAMP are thought to be central to how this ubiquitous second messenger achieves diverse cellular responses that are specific to different signaling inputs. For example, some G protein coupled receptors (GPCRs) are now thought to be capable of generating cAMP both at the plasma membrane and at endosomes [13]. Light activated adenylyl cyclases are enabling new insights into this regulation. By fusing bPac with domains that targeted it to either the plasma membrane or endosomes, cAMP production was optically stimluated at these sites, showing distinct patterns of changes in gene expression [14].

G protein coupled opsins

G protein coupled receptors (GPCRs) regulate diverse cellular behaviors and physiological responses. GPCRs are therefore the most important target for therapeutic drugs. Methods that allow optical control over these pathways can be valuable both for probing the mechanistic basis of these pathways and identifying their roles in regulating cell physiology.

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Optical control can be especially useful if it allows spatially restricted regions of a single cell to be activated. This can help create asymmetric signaling activity and direct polarized cell behavior such as migration, asymmetric cell division and neurite outgrowth. Since light can be switched on and off almost instantaneously, temporal control over signaling is also potentially possible with appropriate optical triggers.

G protein coupled opsins possess unique properties that make them excellent candidates for acting as such optical triggers [2, 15, 16]. They are spectrally distinct and coupled to different G protein subunits with contrasting effects on second messengers. They can be used in the native form without the introduction of a light sensing domain. Their wavelength selectivity allows intracellular signaling activity to be imaged using fluorescent proteins of spectrally distinct excitability. Opsins sense the extracellular signal first, so it is possible to measure the activity of all signaling molecules downstream. Responses to opsin activation are mediated by endogenous molecules thus maintaining the molecular integrity of the cell. Importantly, color opsin deactivation and recovery occur rapidly in contrast to rhodopsin [17] and since they diffuse relatively slowly along the plasma membrane activated areas are restricted and temporal control is acute.

Since chimeric forms of different GPCRs in which the extracellular and intracellular loops have been swapped are functional, it is possible to obtain opsins with residues and domains swapped to create novel combinations of spectral sensitivity and second messenger effects. Distinct opsins of this nature can be used to control multiple signaling pathways with different wavelengths of light. For example, jellyfish opsin normally optimally senses green light but a chimera has been developed in which the extracellular loops have been substituted with human blue opsin so that cAMP increase is induced on sensing blue light [15]. A Gs coupled blue sensing opsin can be used with fluorescent proteins spanning a larger part of the visual spectrum.

Opsins require 11-cis-retinal as a cofactor for light activation. Rhodopsin appears to be capable of functioning in cell lines and in the whole brain without the need for exogenous 11-cis-

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retinal [18, 19]. In contrast, cone opsins require 11-cis-retinal addition to function in cell lines [15] but are able to show activity in the brain with endogenous retinal[19]. When using opsins to regulate signaling and cell behavior they are repeatedly exposed to pulses of light. Rhodopsin rapidly loses the capability to respond to subsequent light pulses [20], and this process is found to be slower in the case of color opsins [15]. A valuable property of some opsins -- bistability [21] -- can be recruited to overcome this deficit. For example, OPN3 is a bistable opsin that is capable of being activated and deactivated with two distinctly different wavelengths of light without progressive inactivation [22]. This opsin can be activated repeatedly without the requirement of endogenous or exogenous 11-cis retinal which can be valuable for extended periods of optical control. Alternatively, the light activated photoisomerase RGR converts all-trans-retinal to 11-cis-retinal and might be used to replenish 11-cis-retinal levels in experiments requiring prolonged opsin activation [23].

Achieving optical control of a desired signaling output

For most signaling proteins, naturally occurring light-sensitive versions are unavailable. However, optical control over several such proteins has now been achieved using genetically encoded constructs that combine a light-sensitive input domain with a signaling output domain from the protein of interest. Light-induced conformational changes in the input domain can be relayed through various mechanisms to achieve control over the signaling domain as outlined below. An emerging theme has been the versatility of different light-sensing domains. A given type of photosensory module, whether it be a cryptochrome, phytochrome, or LOV domains can often be utilized in multiple different approaches to control signaling.

Light induced heterodimerization – controlling a protein's proximity to its effectors

Light-induced heterodimerization has been leveraged to control protein activity through two main approaches. In the first approach, each partner in the dimerization pair is fused to a

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protein of interest. Optically induced dimerization brings them together to facilitate their interaction. In the second approach, one partner of the dimerization module is fused to a subcellular targeting sequence. The second partner is fused to the protein of interest, so that upon optical activation, it is recruited to the targeted subcellular region. Light induced recruitment of various signaling protein domains to the plasma membrane has been used to control cellular responses such as those regulated by small G proteins [24, 25], lipid kinases and phosphatases [26], and heterotrimeric G protein subunits [27]. Light induced recruitment to other subcellular locations such as the nucleus, cytoskeleton or organelle membranes has also been used to control a variety of processes [28-30].

Several light-inducible dimerization pairs have been applied in optogenetics, but two naturally occurring dimerization pairs, both from *Arabidopsis*, have been the most widely used. The first is the red light induced interaction between the phytochrome phyB and its binding partners PIF3 [31] or PIF6 [24]. The second is the blue light induced interaction between the cryptochrome CRY2 and its binding partner CIB1 [32]. Each scheme has unique properties that can be advantageous depending on the application.

Like other plant phytochromes, phyB contains a photosensory core module (PCM) that binds a phycocyanobilin (PCB) cofactor. An advantage of the phyB/PIF scheme is that it is activated by non-toxic red light, and can be rapidly reversed using infrared light [24]. A disadvantage is that mammalian cells do not contain endogenous PCB, so the cofactor must be added. To overcome this constraint, cells can be genetically engineered to synthesize their own PCB [33]. Bacterial phytochromes use a biliverdin chromophore that is present in mammalian cells [34]. This can form the basis for an engineered inducible dimerization tool that is sensitive to red light and does not require exogenous cofactor.

In contrast, CRY2 binds to the flavin cofactor FAD which is ubiquitous in mammalian cells. Its activation by blue light is convenient for live cell imaging because it allows imaging of longer wavelength fluorescent proteins to be performed without causing photoactivation of

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CRY2. Both full length CRY2 and a truncated version consisting of only the photolyase homology region (CRY2PHR) have been used, and the optimal choice was reported to be context dependent [35].

It is also important to consider the design of the signaling domain and how it is fused to the light-sensing domain. For example, optical control over the actin-remodeling protein cofilin used a partially impaired mutant version in order to minimize background activity in the dark prior to light induced recruitment to the cytoskeleton [36]. Likewise, light-induced activation of Wiskott-Aldrich Syndrome Protein (WASP) involved heterodimerization with a GDP bound version of Cdc42 that would normally have minimal capability to activate WASP [31]. Although the GDP bound states of small G proteins such as Cdc42 are often considered “off states”, there was sufficient activity that when combined with a large increase in effective concentration it could activate WASP.

Light dependent unmasking of proteins and peptides

Optical control over protein activity can also be achieved by masking its interaction surfaces in a light-dependent manner, as exemplified by work using a light-oxygen-voltage sensing domain from *Avena sativa* (oat) phototropin 1 (AsLOV2). Like other LOV domains, AsLOV2 contains a flavin-based blue light sensing chromophore. In the dark state, the LOV domain interacts with a C-terminal helix termed the Jα helix. Light exposure causes unwinding of the Jα helix, and this change has been used to achieve unmasking of a protein fused to the C-terminus. The approach has been used to obtain optical control over the interaction between a constitutively active Rac1 mutant and its effectors [37]. A similar principle has been used to optically control access of peptides that activate or inhibit specific proteins inside the cell [38, 39]. It has also been used for optical control over the concentration of peptide ligands on the outer surface of the plasma membrane [40].

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Optogenetic constructs based on light induced unmasking generally require more careful engineering than those based on light induced heterodimers, because of the need for effective masking in the dark state. However, this approach offers the advantage of only requiring a single genetically encoded construct. Also, it can be used to control proteins that may not be effectively regulated by subcellular targeting alone. In addition to the unmasking scheme described above, a diverse array of optogenetic tools have been engineered using LOV domains, as described in the sections below. Together, this collection of tools shows the remarkable versatility of light-sensitive domains for controlling protein functions.

Optically controlled association and dissociation of protein clusters

Optical control of protein clustering has been used for both light induced activation [41] and inactivation. Oligomerization induces activation of proteins such as the small G protein Ras, and several types of receptor tyrosine kinases (RTKs). Blue light activation of CRY2 not only induced binding to CIB1, but can also generate CRY2 oligomers in the absence of CIB1. This property has been exploited to activate Ras or various RTKs [41-43]. A modified version called CRY2olig was identified in a yeast two hybrid screen that exhibits dramatically enhanced oligomerization [44]. An alternative approach for inducible clustering, also demonstrated to provide optical activation of RTK signaling, used a bacterial LOV domain capable of forming light induced homodimers [45].

Whereas clustering leads to increased activity of certain proteins, in other cases it can lead to an inhibition of activity by sequestering the protein away from its effectors. This property has been leveraged for light-induced inhibition by cluster formation using a CRY/CIBN based scheme that incorporated a multivalent protein to enhance light-induced clustering [41]. Conversely, an alternative approach used a mutant of the fluorescent protein Dronpa that oligomerizes in the dark and dissociates under cyan light to enable light-induced protein activation [46].

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Optimization of optogenetic constructs

Optimizing optogenetic constructs for control of protein activity and cellular responses involves several considerations such as spectral characteristics, light-sensitivity, binding affinities, and kinetic properties. Variations in these properties can often be found across different members of families of naturally occurring light sensitive proteins. Additionally, mutagenesis can often uncover variants not found in nature that have properties better suited for engineering optogenetic tools. Information from structures and computational modeling has helped identify residues of interest for directed mutagenesis. High throughput methods involving imaging [47] or phage display [48] technologies have allowed optimized properties to be selected from large numbers of photoreceptor mutants. Below we describe some of the advances that these approaches have enabled toward generating optimized optogenetic tools.

Tuning the masking characteristics of LOV domains

Most of the photoreceptors used for engineering optogenetic tools respond very rapidly upon absorption of a photon, and this step does not typically limit kinetic control of cell signaling. In contrast, the rate at which a light-activated optogenetic construct reverts back to its “inactive” dark state upon removal of the light source varies widely among photoreceptors. Mutagenesis studies of the LOV domains from a variety of proteins have demonstrated the ability to drastically alter the off-rate kinetics by controlling the stability of a conserved cysteinyl-flavin adduct associated with photoactivation. For example, mutations in AsLOV2 designed to alter side chain interactions with the flavin cofactor and the surrounding water molecules were identified that alter photocycle times from 2s to over 2000s [49]. For the LOV protein YtvA from *Bacillus subtilis*, mutagenesis identified a variant that reverts to the dark state 85 times faster than wild-type following photoactivation. Mutations of the slow-cycling fungal LOV photoreceptor Vivid (VVD) altered the off-rate kinetics by over for order of magnitude [50].

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Ideal optogenetic tools have a large photoswitching dynamic range with minimal signaling in the dark state and a significant increase in signaling upon optical activation. Guided by an analytical model of photoswitching, mutational stabilization of the dark state interaction between the Jα helix and core domain of AsLOV2 helped increase the dynamic range of an LOV based optogenetic tool from 5-fold to 70-fold [51].

Engineering improved light-induced heterodimerization schemes

Two recently engineered light-induced dimerization schemes, iLIDs and Magnets [48, 52], may prove advantageous over the commonly used CRY2/CIBN and PhyB/PIF schemes for certain applications. Both schemes leverage LOV domains as optical switches, but they do so in distinct ways as described below (Fig. 1). These are not the first LOV based dimerization schemes [53, 54], but they are the most extensively optimized and represent the cutting edge for optical control over heterodimer formation. They offer small size and different ranges of affinities and kinetics that will allow them to be tailored to control of different protein activities.

The iLID scheme is based on the known interaction of the bacterial SsrA peptide with its binding partner SspB. Optical control over the interaction was achieved by embedding SsrA in the C-terminal helix of asLOV2 [38]. A multifaceted optimization procedure using computation, phage display and high-throughput binding assays improved the light-induced change in binding affinity from twofold to over 50-fold [48]. Two iLID pairs were developed: iLID nano switches from 4.7μM to 130 nM affinity upon blue light exposure, whereas iLID micro switches from 800 nM to 4μM. As noted by the authors, the availability of inducible dimerizers with different ranges of affinities will be useful because the concentration threshold required for activity varies for different signaling proteins.

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The Magnets heterodimerization pair was generated through mutagenesis of Vivid (VVD), a fungal photoreceptor derived from *Neurospora crassa* that is almost entirely composed of an LOV domain and can switch from a monomer to a homodimer in response to blue light [55-57]. Mutations were introduced at the N terminal cap of VVD to alter the electrostatic properties of the homodimer interface and generate two VVD mutants, pMag and nMag, that selectively form heterodimers when exposed to blue light [52]. Additional mutations in the Per-Arnt-Sim (PAS) core of VVD resulted in modified pMag/nMag interactions with dissociation rates in the dark ranging from 25 sec to 4.7 h [52]. The ability of the fastest dissociating pMag/nMag pair, as well as both iLID pairs, to dissociate in less than a minute will enable better dynamic control over protein activity within a cell compared to the CRY2/CIBN scheme. Alternatively, the slowest dissociating pMag/nMag pair will be useful for controlling slow processes where minimizing repeated exposure to blue light could be important to avoid phototoxicity.

Spectral tuning

The magnitude of signaling output produced by an optogenetic construct depends on the intensity and wavelength of light used for photoactivation. Tuning the activation spectrum can generate new capabilities (Fig. 2). For example, spectral tuning can enable independent optical control of two different processes in the same cell using different wavelengths of light. Alternatively, it can allow live cell imaging of fluorescence proteins to be performed orthogonally to photoactivation. It can also enable the signaling response to be activated by far-red or near infrared wavelengths of light that are advantageous for *in vivo* applications due to their deeper tissue penetration.

The activation spectrum is determined largely by the specific chromophore and the neighboring residues within the protein. For example, 11-cis-retinal in solution has peak absorption at 380nm but by interacting with different opsins exhibits peaks varying from 360 to 560 nm (17). This natural variation in spectral characteristics can be leveraged for spectrally

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selective control over two different opsins. For example, mouse blue SWS1 opsin and human red opsin have been shown to both control GIRK currents with wavelength specificity [19]. It will be highly desirable to extend this approach to control the activation of two different families of G proteins in the same cell.

A further red shifted opsin would also be useful for dual control in cells coexpressing CRY2 or LOV based constructs. Screening of naturally occurring opsins could uncover such a red shifted opsin. Alternatively, a mutagenesis approach will be aided by the extensive characterization of the residues involved in spectral tuning of opsins [17, 58-61]. Spectral tuning can be altered by substituting the specific retinal bound to the opsin with an alternative form. For example, when 11-cis-retinal in a goldfish opsin is replaced with 11-cis-3,4-dehydroretinal, the λmax is red shifted more than 25nm [61]. Synthetic retinal analogues can be used for this purpose. Such analogues have been shown to modify spectral and kinetic characteristics of microbial opsins [62]. Regardless of the method used to red shift opsins, receptors activated at lower energy infrared wavelengths are likely to suffer from increased background noise due to thermal activation [63].

The flavin cofactors used by BLUF, LOV, and CRY modules are far less amenable to spectral tuning than the retinal and tetrapyrrole cofactors for opsins and phytochromes [10]. For example, mutagenesis of bPAC would be unlikely to achieve a red or infrared sensitive adenenyly cyclase. However, a near infrared activated cyclase was engineered by an alternative approach using a bacteriophytochrome. In the native protein, the light sensing domain is linked to activation of a histidine kinase. By replacing the histidine kinase with a structurally homologous adenylyl cyclase, infrared light activated cyclase activity was achieved [34]. A similar method was utilized to engineer a red light activated phosphodiesterase [64].

Conclusions

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The ability to recruit the unique properties of a variety of photosensitive proteins has helped design optical tools to modulate signaling activity in a single cell with spatial and temporal precision. The challenges ahead are in overcoming some of the limitations of existent approaches. This includes the ability to quantitatively image the molecular and cellular responses to stimulation by optogenetic tools which at present is constrained by spectral overlap. A potential avenue towards surmounting this is to utilize the properties of additional light sensitive proteins and by developing more innovative strategies for optical control. This can be achieved by identifying novel light sensitive proteins with useful properties such as a collection of algal phytochromes that sense light optimally at wavelengths that span the entire visual spectrum [65]. It can also be done by finding appropriate applications for existing protein systems in which conformational changes and protein-protein interactions have been shown to be light driven (e.g., [66-68]).

Among the most important challenges is to apply these optogenetic tools to whole animals to address long standing basic questions with regard to development, differentiation and tissue physiology. The ability to shine light on a single cell and achieve subcellular control can be translated to individual cells within a population and can be useful for studying cell-cell communication. For example, activating ERK in single cells lead to propagation of ERK activity pulses in neighboring cells [69]. Optical control can be used to direct cell migration during morphogenesis or extend neurites in predetermined directions. The long term goal should be to apply these tools towards light driven therapeutic intervention.

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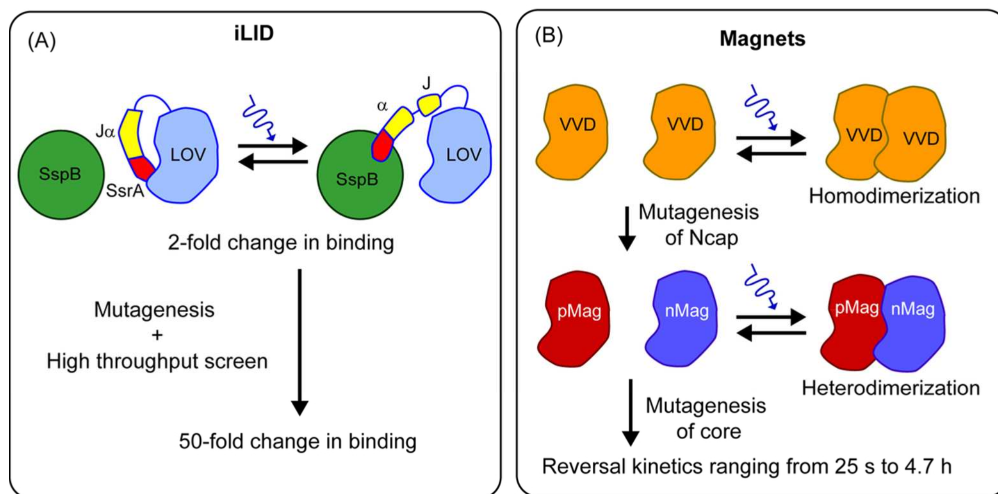
Figure Legends

Fig. 1. Optimization of light induced heterodimerizers. The two schemes are based on different LOV domains. (A) The iLID scheme uses the AsLOV2 domain to optically control the binding of the bacterial SsrA peptide to SspB. As initially constructed, this pair exhibited only a two-fold increase in binding affinity upon photoactivation. A high throughput screen identified mutations in the LOV domain that generate a 50-fold increase in binding upon photoactivation. (B) The Magnets scheme uses the LOV domain containing protein Vivid (VVD) that homodimerizes upon photoactivation. Mutagenesis of the Ncap region generated two variants, pMag and nMag, that selectively form heterodimers upon photoactivation. Subsequent mutations to the core domain generated widely varying unbinding kinetics upon removal of the blue light.

Fig. 2. Optimization of spectral characteristics. (A) Selective optical control of two responses. These could for example be opsins coupled to different G protein types. Initially, overlapping activation spectra prevents selective activation (top). Spectral tuning allows each response to be controlled independently with a different wavelength of light. (B) Spectrally independent optical activation and fluorescence imaging. Initially, the activation spectrum overlaps with the laser lines used to excite fluorescence proteins (FPs) such as Venus and mCherry. Using opsins with altered spectral tuning allows both FPs to be imaged without photoactivating the optogenetic construct. Photoactivation can then be performed independently with a shorter wavelength of light.



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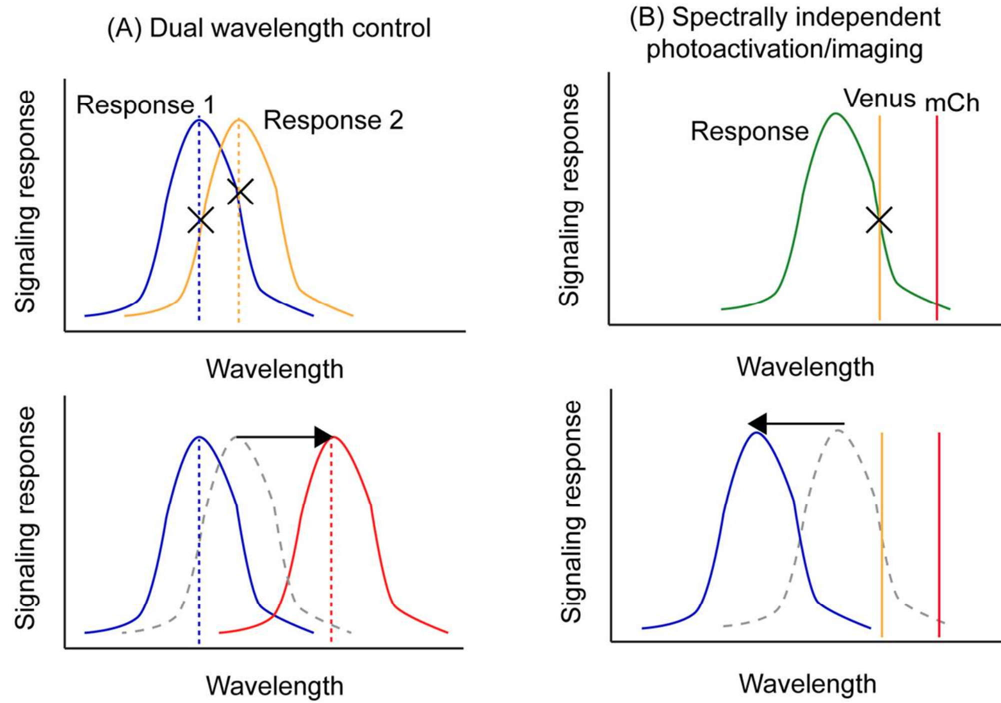

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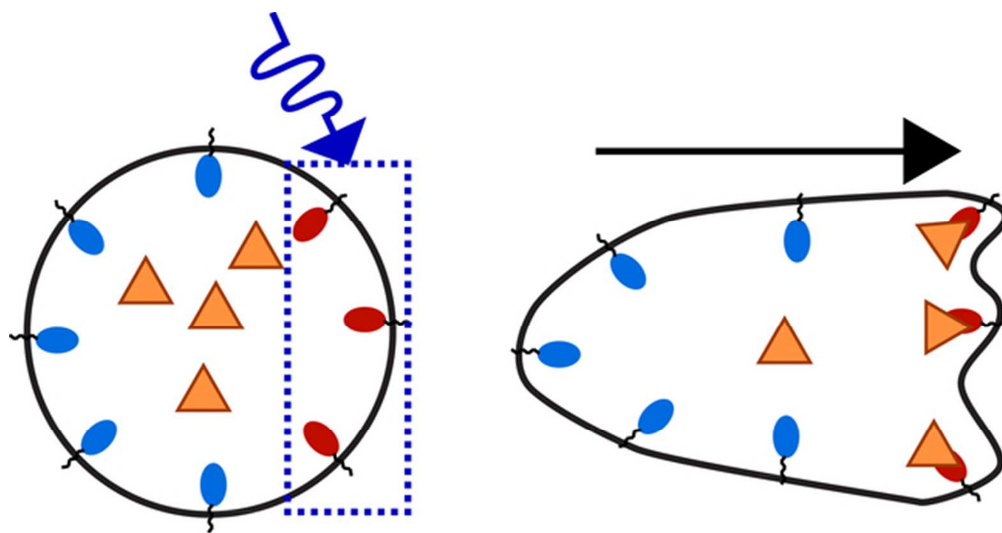

This perspective highlights recent advances in the design of optogenetic tools that provide dynamic subcellular control over signaling and cell behavior.
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