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Optically inducible membrane recruitment and signaling systems

Pimkhuan Hannanta-Anan[1], Spencer T Glantz and Brian Y Chow

Optical induction of intracellular signaling by membrane-associated and integral membrane proteins allows spatiotemporally precise control over second messenger signaling and cytoskeletal rearrangements that are important to cell migration, development, and proliferation. Optogenetic membrane recruitment of a protein-of-interest to control its signaling by altering subcellular localization is a versatile means to these ends. Here, we summarize the signaling characteristics and underlying structure-function of RGS-LOV photoreceptors as single-component membrane recruitment tools that rapidly, reversibly, and efficiently carry protein cargo from the cytoplasm to the plasma membrane by a light-regulated electrostatic interaction with the membrane itself. We place the technology-relevant features of these recently described natural photosensory proteins in context of summarized protein engineering and design strategies for optically controlling membrane protein signaling.

Address
Department of Bioengineering, University of Pennsylvania, 210 South 33rd Street, Philadelphia, PA 19104, USA

Corresponding author: Chow, Brian Y (bchow@seas.upenn.edu)
[1] Current location: Department of Food Engineering, Faculty of Engineering, King Mongkut's Institute of Technology Ladkrabang, Chalongkrung Road, Ladkrabang, Bangkok 10520, Thailand.

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Introduction

Over the nearly past two decades, optogenetics [1,2] and optochemical [3,4] approaches to controlling the excitability and signaling of genetically targeted cells and cell-like systems [5,6] have transitioned from specialized upstart technologies to core techniques in cell biology. This prominence has driven the discovery of natural proteins that push the boundaries of known photosensory signaling mechanisms [7••,8•,9,10], the creation of innovative photoreceptor engineering strategies [11,12•,13,14••], and the solving of high-resolution structures of natural and engineered light-activated proteins [15••,16,17] – all of which efforts have been aimed at informing how to construct photoinducible signaling tools from natural proteins.

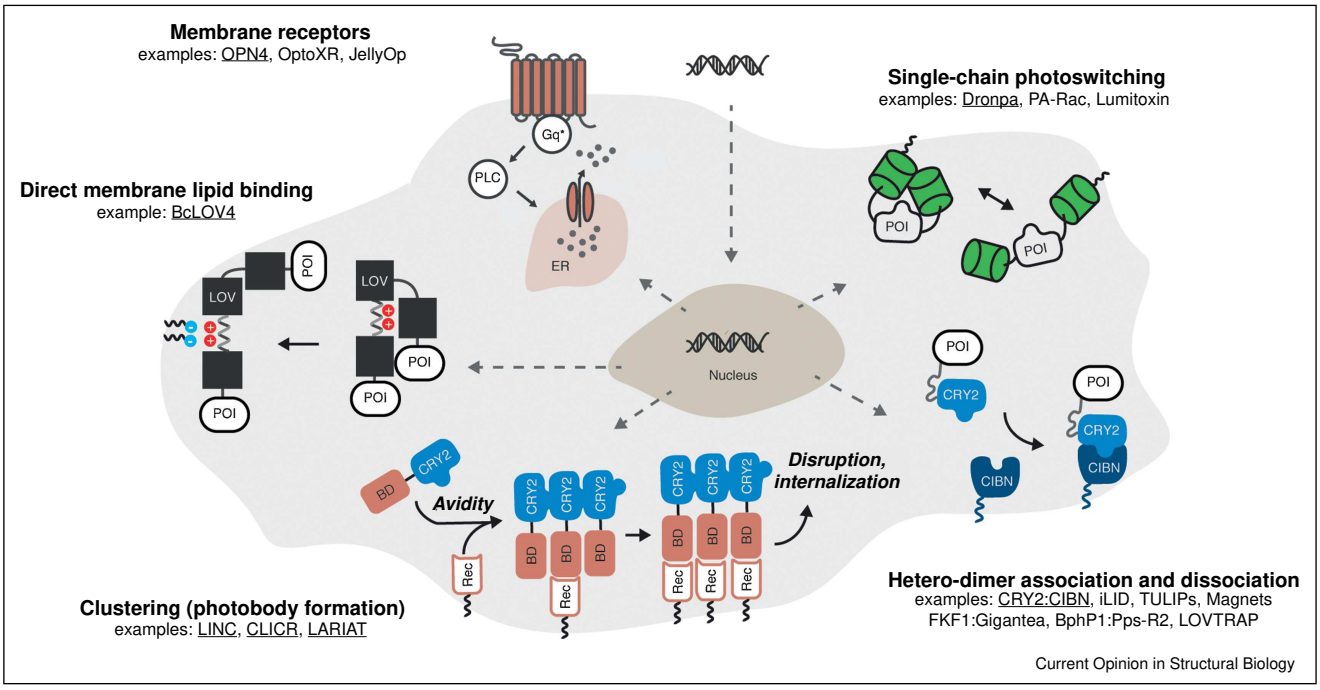
Outside of neuroscience and muscle biology where electrogenic control over excitable cell spiking dominates, technology development and application have extensively focused on mammalian transcriptional activation (as well-summarized by others [18]) or of primary focus here, intracellular signaling by membrane receptors [19–22] and membrane-associated proteins [23,24•,25–28,29•,30] for controlling the effector functions of kinases, G-protein-coupled receptors (GPCRs), and small GTPases that are involved with second messenger signaling, environmental sensing, and cytoskeletal rearrangements in cell migration, development, and proliferation. The signaling of these effectors and the phenotypic cellular behaviors they regulate occur on the timescale of $\sim 10^0$–10^3 s, which is similar to the typical photocycle of a non-electrogenic photosensory protein. Thus, the spatiotemporal dynamics of signaling by such effectors, and their roles in information encoding/decoding schemes that govern cellular dynamics [22,23,24•,25] are well-suited for optogenetic analyses.

Here, we will first briefly describe approaches for optical control over intracellular signaling by membrane proteins, with an emphasis on optogenetic approaches with fully genetically encoded tools in mammalian cells that alter subcellular localization of membrane-associated proteins (Figure 1). This emphasis reflects the current prevalence in reports, not an implied importance of these model/expression systems or signaling pathways/applications over others. The contextual summary highlights general signal induction strategies at a high-level, as opposed to detailing the myriad specific tools reported to date – for which we point to these recent and exhaustive reviews by others [1,2,18]. We will also summarize the signaling structure-function of very recently reported light-oxygen-voltage (LOV) photoreceptors that directly bind the plasma membrane by a light-regulated protein-lipid electrostatic interaction [7••,8•], in context of guiding the design and engineering of single-component optogenetic membrane recruitment tools.

Allosteric switching of effector biochemical function

There are numerous ways to classify optogenetic tools: by photoreceptor from which they are derived, by induction wavelength, and by application, to name a few. For the sake of simplicity, we organize the approaches for optical

Figure 1



Fully genetically encoded strategies for optically inducible intracellular signaling by integral membrane and membrane-associated proteins in mammalian cells. Examples schematized are underlined. Abbreviations: POI, protein of interest; BD, binding domain; CIBN, cryptochrome interacting binding partner; CRY2, cryptochrome; ER, endoplasmic reticulum; Gq*, activated $G\alpha_q$; IP_3, inositol triphosphate; IP_3R, IP_3 receptor; LOV, light-oxygen-voltage; PLC, phospholipase C; Rec, membrane receptor.

induction of membrane protein intracellular signaling into two effector-centric modes: (i) photo-switching of effector biochemical activity (described in this section), and (ii) photocontrol over subcellular localization of a constitutively active effector to regulate signaling (as described in the next section). Here, photo-switching implies that the protein-level enzymatic/binding efficiency of the signaling effector domain/segment is altered between the photoactivated and dark-adapted states of the photosensory domain. These photo-switching tools are typically single-chain proteins since they do not rely on engineered binding partners.

Co-opting the natural signaling function of photosensory membrane receptors is conceptually straightforward. For example, human melanopsin (OPN4), a light-activated $G\alpha_q$-coupled GPCR normally found in non-vision-forming retinal ganglion cells, initiates calcium release from the endo/sarcoplasmic reticulum and downstream calcium-dependent transcription and excitability [22,31]. Invertebrate opsins from diverse organisms also engage mammalian signaling pathways [21,32], such as a $G\alpha_s$-coupled opsin from jellyfish (JellyOp). Chimeric opsins created by substituting the signaling-responsible cytoplasmic loops with those of ligand-responsive GPCRs (often termed 'OptoXRs') have been derived from both mammalian opsins [20] and more recently, microbial opsins [33].

The most common component for constructing single-chain, chimeric photo-switches is the light-oxygen-voltage (LOV) sensor domain from Phototropin 1 of *Avena sativa* (AsLOV2), a non-dimerizing monomer with existing high-resolution structures of its active signaling state by NMR [34••] and X-ray crystallography [35] (PDB code 2v1a). LOV signal transmission is mediated by flavin photocycling-initiated protein conformational changes that disrupt a β-sheet interaction with a C-terminal Jα-helix [34••], to which effector proteins-of-interest (POI) can be fused such as small GTPases as in photoactivatable Rac (PA-Rac) [27], (extracellular) surface-displayed peptide toxins (Lumitoxins) to antagonize endogenous channels [36], and small peptide tags [12•,37,38•,39,40]. The signaling function of the POI is presumably diminished in the dark by steric hindrance or occlusion of its binding site by the LOV domain fused to the terminus, although the structured molecular contacts between these domains are seldom reported (assuming they exist). A recent report describes how effector loop regions can be computationally designed to interact with the AsLOV2 flavin binding pocket, such that light-induced

conformational changes introduce 'extrinsic disorder' to these loop regions that disrupts effector signaling in a structurally principled manner [15••]; importantly, theses mechanistic assertions were confirmed by structure determination and correlated signaling assays [15••].

Monomeric photoswitches can also be constructed by engineering single-chain proteins from light-activated homodimers, such as the β-barrel green fluorescent protein-based Dronpa [28]. Dronpa dimerization is bi-directional or photo-switchable with two different colors of light (ultraviolet and blue), a beneficial feature because its shutoff can be independent of its thermal reversion between states. Beyond allosteric switching, homodimer dissociation can be effective in disrupting oligomerization-dependent signaling activity of membrane-associated proteins, as demonstrated with receptor tyrosine kinases (RTKs) fused to bacterial cobalamin binding domains [19]. Although this latter system requires cofactor supplementation in mammalian systems, natural LOV that photo-dissociate (PDB code 4hj6) [10] exist that bind mammalian-endogenous flavins, and have been used to engineer chimeric Cas9 DNA-binding domains in bacteria [41].

Oligomerization systems to alter subcellular localization of effector cargo

Whereas allosteric systems switch the effector protein-level activity, optically induced signaling by a constitutively active POI merely requires a change in the subcellular localization of the POI that consequently changes its local concentration or availability to its partner. These signaling systems are ostensibly easier to design and engineer than allosteric photoswitches because effector signaling is largely decoupled in structure-function from the conformational changes of the photosensory domain, thus requiring only a modest amount of linker engineering (in length and rigidity) between the photosensor and its passive cargo, the POI, to work in principle.

Nature has already 'engineered' several photosensory heterodimerization pairs suitable for membrane signaling in mammalian cells (using endogenous cofactors) when stimulated by blue light (e.g. cryptochrome CRY2 with CIBN, and the LOV domain FKF1 with Gigantea) or near-infrared light (e.g. bacteriophytochrome BphP1 with Pps-R2) [29•,42•,43]. In the most common design configuration, an interaction domain is non-specifically localized to the plasma membrane by prenylation at a C-terminal 'CAAX' motif, and the dark-adapted photosensor initially sequesters a fused POI in the cytoplasm, unavailable to signal at the membrane; the POI is recruited to the membrane to increase signaling upon light-activated heterodimerization.

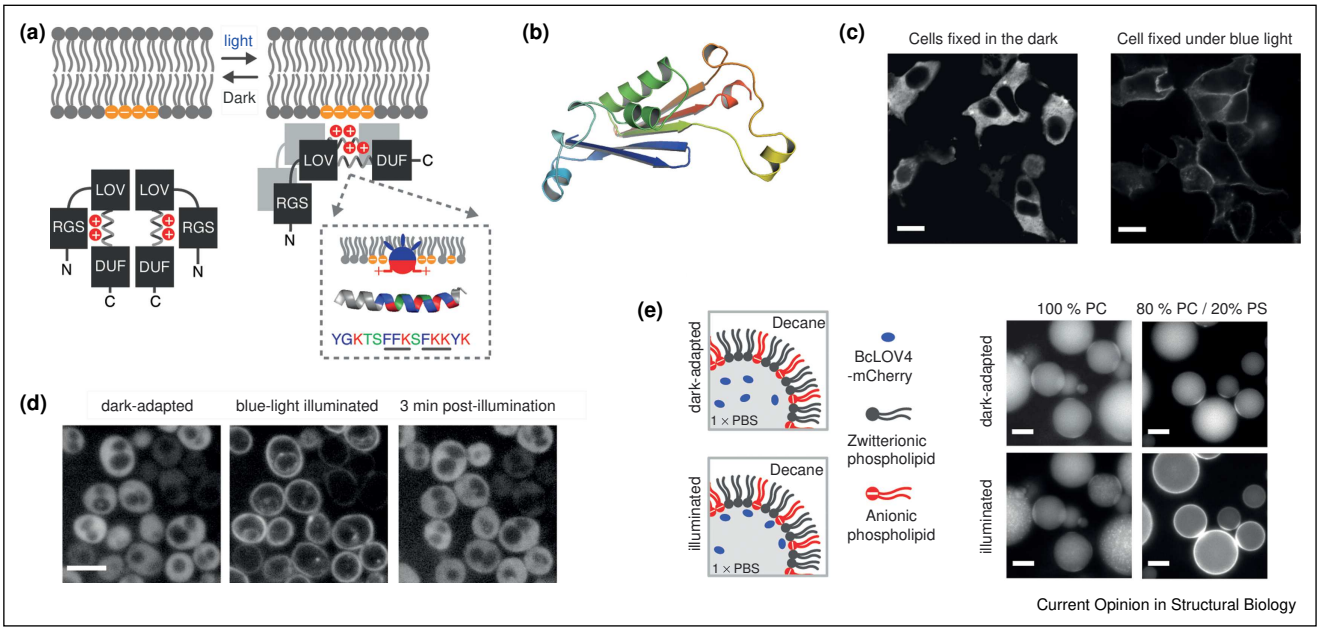
Despite the ready availability of natural heterodimer pairs, many engineered heterodimer pairs have also been reported. These artificial heterodimerization pairs can be derived from natural homodimerizers by creating a highly charged and electrostatically asymmetric dimerization interface that promotes electrostatic stabilization between heterodimers and repulsion between homodimers, as done with the Magnet [13] system derived from the LOV protein Vivid (VVD, PDB code 3rh8). Several reported AsLOV2-based photoswitches expose the binding regions of short peptides [12•,37,38•] with known binding partners to drive heterodimer association for the downstream purpose of altering the subcellular localization of another effector, as is the case with iLID (SsrA peptide binding to SsrB) (PDB code 4wf0) and TULIPs (epitope binding to a PDZ domain). Thus, it is important to note that the signaling mode classification used here is organizational, not fundamental in nature or mutually exclusive.

Optically induced dissociation of a thermally stable heterodimer is rarer. When the naturally fused regulatory and effector domains of cyanobacterial orange carotenoid protein (OCP) are split, the resulting engineered domains form a stable heterodimer in the dark that is disrupted by blue light stimulation ([44], see also the contribution of Kerfeld to this issue). While the ketocarotenoid cofactor bound by OCP is not endogenously biosynthesized in mammalian cells, carotenoids are often bioavailable by supplementation. To the best of our knowledge though, Nature has not provided such a heterodimerization pair involving a photoreceptor that binds an endogenous mammalian cofactor; even though bacteriophytochromes (and phytochromes) optically dissociate from their interaction partners from the photoactivated state, the dark-adapted photoreceptor is unbound. However, an artificial pair has been cleverly created in the LOVTRAP system [14••] (PDB code 5efw), in which the binding of Zdark (an engineered Z subunit of Protein A) to the critical Jα-helix of dark-adapted AsLOV2 is disrupted when the latter photocycles.

While these modular systems are fairly 'plug-and-play,' beyond linker engineering they do require protein expression level tuning, in relative expression level between heterodimerization partners and total expression level of the POI, for suitable dynamic range to ensure a strong signaling change upon illumination without permanent association in the dark-adapted state. These expression level setpoints can be reasonably locked in with proper clonal selection of stably transducing cell lines [23,24•], but heterogenous expression across a cell population can lead to inconsistent function for applications in primary cells and transiently transfected cell lines.

Beyond dimers, oligomerization state can be grossly controlled to alter subcellular localization using cryptochromes, which are known to cluster into large ($\sim 10^{-7}$ m) internal bodies [45] of unknown colloidal structure (as do phytochromes [46] that bind

Figure 2



BcLOV4, a membrane-interacting photoreceptor and single-component system for dynamic membrane recruitment across diverse model systems. **(a)** Schematized signal-transmission mode of membrane recruitment by a directly light-regulated and high-affinity electrostatic interaction with anionic phospholipids, which are largely enriched in the plasma membrane in mammalian cells. The interaction is inhibited by an N-terminal RGS (Regulator of G-protein signaling). A critical membrane binding site exists in a polybasic amphipathic helix in the linker region between the LOV sensor and a C-terminal DUF (domain of unidentified function). *Inset:* Schematized helix-membrane interaction and amphipathic helix sequence from BcLOV4, with known membrane binding motifs underlined. Blue = hydrophobic. Red = Basic. Green = Polar. **(b)** Pymol rendered model of predicted DUF structure by *de novo* energy minimization modeling in Rosetta. The DUF has a PAS-like mixed α-helix/anti-parallel β-sheet topology, suggesting that the LOV-DUF interaction is an evolutionary conserved PAS-PAS interaction. **(c–e)** Fluorescence micrographs of light-activated membrane recruitment in diverse contexts: (c) Expressed as FLAG epitope-tagged protein in mammalian HEK cells, fixed in the dark/light, and visualized by immunocytochemistry with Alexa488-conjugated anti-FLAG antibody. Scale = 10 μm. (d) Expressed in fungal *S. cerevisiae* cells and visualized by mCherry-fusion tag. Scale = 5 μm. (e) *In vitro* as purified recombinant mCherry-tagged protein that binds lipid-stabilized water-in-oil emulsion interfaces. BcLOV4 does not bind purely zwitterionic interfaces of phosphatidylcholine (PC), but binds anionic interfaces with 20% phosphatidylserine (PS) that emulates mammalian plasma membrane inner leaflets. Scale = 25 μm. Images modified from Ref. [7••]. Copyright 2018 National Academy of Sciences.

mammalian-exogenous phycocyanobilin). Homo-oligomeric clustering at the membrane can disrupt membrane protein signaling by internalization as reported with LINC [47] and the related LARIAT (hetero-oligomers of multimeric proteins) [48] systems. Conversely, photobody formation can activate signaling by enhancing the overall avidity of the fused POI to an endogenous membrane receptor (CLICR, [49]). This latter approach is promising because it requires a single transgene. However, the aggregates are large and the indirect membrane recruitment is a multi-step process.

A more straightforward single-component approach to membrane recruitment, in which a single-transgene tool carries a POI cargo directly to the membrane, without aggregation and a multi-step binding process, would be highly useful. Next, we will discuss a recent advance to this end: the discovery of natural LOV photoreceptors that are directly recruited by the plasma membrane itself in a blue light-dependent manner.

Single-component membrane recruitment by BcLOV4

Recently, we and colleagues reported the identification and characterization of a class of LOV photoreceptors that directly associate with the plasma membrane inner leaflet by a light-switched and high-affinity electrostatic interaction with anionic phospholipids [7••,8•] (Figure 2). Their photocycle-coupled signal transmission is mediated by an unmasking of a membrane-interacting polybasic amphipathic helical linker that couples the LOV sensor Jα-helix to a highly structured C-terminal DUF domain (domain of unidentified function) (Figure 2a–b); an N-terminal RGS domain (regulator of G-protein signaling) inhibits the electrostatic interaction in the dark-adapted state, but has no detectable interaction with mammalian proteins. Of relevance here, these RGS-LOV (RGS-associated LOV) proteins function as single-component optogenetic tools for dynamic membrane recruitment from the cytoplasm by binding the plasma membrane itself (Figure 2c–d). To date, we have most thoroughly characterized BcLOV4 from *Botrytis cinerea*,

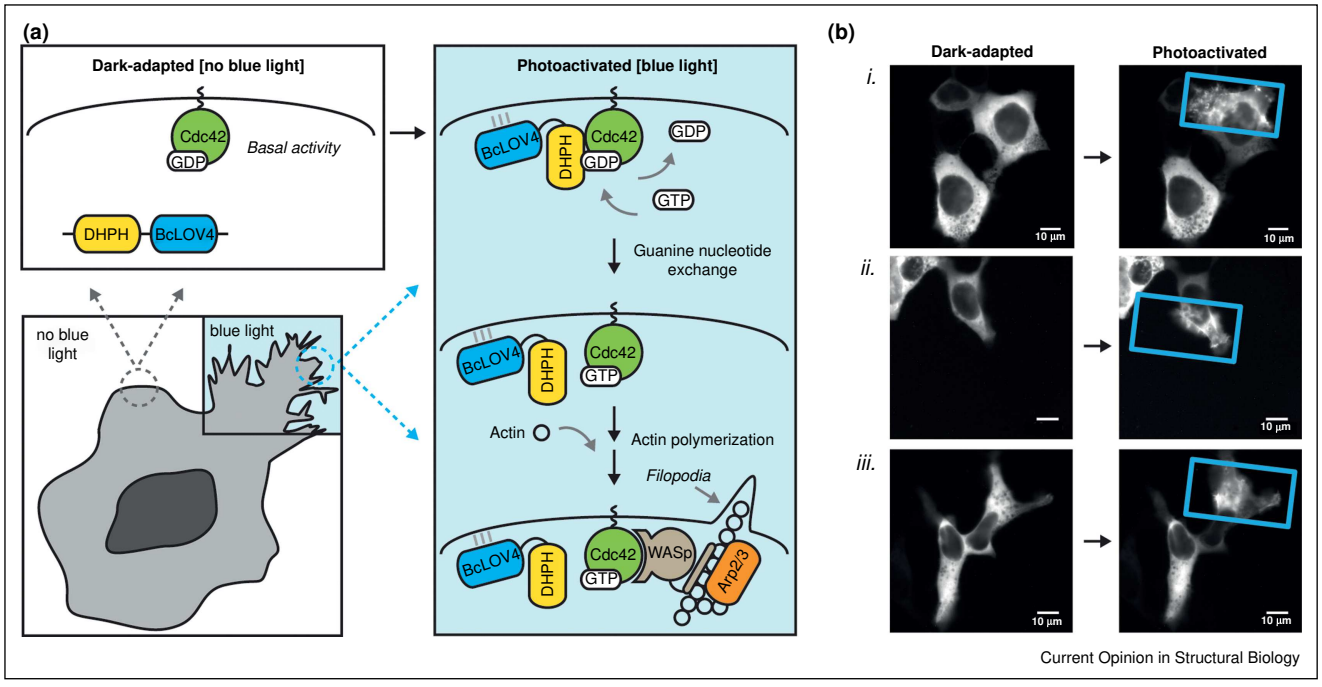
but the dynamic membrane association phenomenon is general (see Figure S5 of Ref. [7••]) to its fungal RGS-LOV homologs [7••,8•]. Here, we will expand upon the discussion of BcLOV4 as a single-component optogenetic tool with a contextual emphasis on mammalian signaling.

BcLOV4 membrane recruitment is fast ($\tau_{on} \sim 1$ s) because it has a high affinity for interfaces of mammalian plasma membrane-like composition ($K_D \sim 130$ nM for 80% phosphatidylcholine (PC)/20% phosphatidylserine (PS)). This timescale is near diffusion-limited, such that it associates with the plasma membrane near instantaneously upon diffusing to it. It also undocks quickly from the membrane in the dark ($\tau_{off} \sim 1$–1.5 min), and mutants with much longer residence times of ~10 min have been engineered by lengthening the photocycle (see Figure S8 of Ref. [7••]). These undocking timescales are approximately one minute longer than the respective photocycles, and thus, the photocycle is partially rate-limiting in the overall dissociation kinetics of the system, with other rate-determining factors still to be elucidated (as discussed further below).

While its high-affinity lipid interaction in the photoactivated state ensures robust membrane recruitment, its low affinity in the dark-adapted state ($K_D \sim$ low micromolar) keeps it well-sequestered in the cytoplasm even when over expressed in eukaryotic cells. Because its mammalian binding 'target' is the inner leaflet itself, which is a giant endogenous sink for active-state protein, the relative stoichiometric tuning of interaction partners is unnecessary to achieve a high on:off ratio for the interaction in the light versus dark. This feature distinguishes it from heterodimerization systems that may require the selection of clonal cell lines with optimized expression levels to overcome the inherent expression level heterogeneity of transiently transfected cells in order to ensure robustness [23]. Accordingly, its single-component operation simplifies transgene delivery and cell line development, and also frees optical bandwidth by eliminating a second fluorescent protein tag needed in heterodimerization systems to visualize individual components in live cells.

BcLOV4 is versatile. Beyond robust performance in mammalian cells (Figure 2c), it also functions when expressed in yeast (Figure 2d) and *in vitro* as purified recombinant protein in lipid-stabilized water-in-oil emulsions (Figure 2e), the latter related to optochemical control in droplet-based *in vitro*-compartmentalized

Figure 3



Spatially localized and optically induced cytoskeletal rearrangements by BcLOV4-derived opto-DHPH. **(a)** Schematic overview of spatially precise opto-DHPH membrane recruitment and consequent activation of Cdc42 by the DHPH domain of the Intersectin GEF, which drives downstream actin polymerization. Cdc42 = Cell division control protein 42. DHPH, Diffuse B-cell lymphoma homology, Pleckstrin homology domain; GEF, Guanine exchange factor; Arp2/3, Actin-related protein-2/3; WASp, Wiskott-Aldrich Syndrome protein. **(b)** Optically induced filopodia formation in HEK cells visualized by fluorescence imaging of a C-terminal mCherry tag. Only the blue light-illuminated (rectangle) regions show pronounced protusions, which are induced with very little stimulation (duty cycle = 0.8% = 0.5 s per minute, $\lambda = 450$ nm, 15 mW/cm^2; spatially patterned by a digital micromirror device). Post-illumination times (i) 300 s, (ii, iii) 500 s Supplementary Video 1 corresponds to cell (i).

signaling systems [5]. Because its primary membrane interaction site is internal (in linear polypeptide space), it tolerates protein fusions to its N-terminus and/or C-terminus when engineering chimeras. As stated, optogenetic chimeras for membrane recruitment are commonly employed in mammalian cytoskeletal biology applications, and indeed, BcLOV4 can be effectively applied to that end as demonstrated here (Figure 3).

Opto-DHPH is a chimera of BcLOV4 (mammalian codon-optimized and with C-terminal mCherry visualization tag) with an N-terminal DHPH (Dbl-homology, Pleckstrin-homology) domain of the Intersectin1 (ITSN) guanine exchange factor (GEF) that activates the Cdc42 small GTPase and downstream actin polymerization (Figure 3a). This particular Cdc42-GEF signaling pathway has been manipulated by numerous optogenetic heterodimerization systems based on iLID, CRY2, and BphP1 [26,29•,50]. Spatially patterned illumination (using a digital micromirror device [51]) of opto-DHPH in transfected HEK293 cells causes pronounced filopodia formation that is restricted to the blue light-illumination field (Figure 3b and Supplementary Video 1). Optogenetic induction of the cytoskeletal change is very efficient; cells robustly respond to very sparse optical stimulation (<1% duty cycle or 0.5 s per minute) at experimental light levels (photocycling $ED_{50} = 15\ mW/cm^2$ at $\lambda = 450\ nm$). Thus, BcLOV4 is clearly promising as a single-component tool for optogenetic membrane recruitment and signaling of its fusion partner as cargo.

Optogenetic implications of RGS-LOV signaling structure-function

The RGS-LOV signal transmission mode is distinct from known PAS-superfamily (Per-Arnt-Sim [52], to which LOV belong) lipid-binding proteins, which are integral membrane proteins (e.g. PhoQ: PDB code 3bq8, LuxQ: PDB code 2hje), and we are unaware of other photosensory proteins that are directly recruited to the membrane in response to light. However, despite the novelty of the photosensory signal transmission mode, *de novo* structural predictions by energy minimization modeling in Rosetta [53•] suggest that the highly structured DUF domain is PAS-like [7••] (Figure 2b), which would bring RGS-LOV in line with evolutionarily conserved tandem PAS proteins [52].

Importantly, RGS-LOV signaling follows known determinants of canonical LOV protein signaling. Optical membrane recruitment can be largely abolished by a cysteine-to-alanine mutation that prevents canonical photoadduct formation, and conversely, BcLOV4 can be made constitutively active or permanently membrane localized in the dark by mutating a conserved glutamine at the Jα helix terminus to structurally mimic an active 'lit' state [54]. These mutants are useful as experimental controls for implicating photocycle involvement in signaling and accounting for the non-LOV role of blue-light alone in assays [7••,27]. Likewise, a kinetic mutant that approaches functional bi-stability (membrane undocking timescale of ~10 min) has been rationally engineered by lengthening the photocycle [7••].

The membrane-interacting amphipathic helix, which was initially identified through bioinformatics based on sequence conservation across 66 RGS-LOV homologs and secondary structure predictions [7••], contains known plasma membrane interaction motifs rich in phenylalanine and lysine ('FFK' and 'FKK'), which are found in other membrane-associated proteins (e.g. BAD: PDB code 1g5m, M2 proton channel of Influenza A: PDB code 2rlf). Its hydrophobic residues embed into the phospholipid bilayer while the surrounding cationic residues bind to the anionic membrane phospholipid headgroups such that the helix sits on top (not across) of the inner leaflet (Figure 2a *inset*). This non-stereospecific lipid-interaction motif is thus non-selective among lipid headgroups beyond their anionic charge density [7••]. Thus, why do RGS-LOV preferentially bind the plasma membrane? The simplest explanation is that the mammalian plasma membrane is the most-enriched subcellular structure for anionic phospholipids, especially for phosphatidylserine [55–57].

Membrane geometry and mechanical properties may also play a role in its subcellular preference profile, as amphipathic helices are known to 'sense' membrane curvature and defects (and/or induce them). The inner leaflet is not only a densely anionic membrane, but also a largely defect-free and 'flat' one due to its high sterol content [55–57]. Such membrane physical characteristics are well recognized by amphipathic helices with cationic residues surrounding bilayer-partitioning residues with large side-chains (like that of RGS-LOV), and support long-range electrostatic interactions between them [55,56]. These long-range electrostatic interactions may be critical to the signaling response in mammalian cells. For example, high-salinity prevents BcLOV4 from associating with *in vitro* membrane interfaces, and RGS-LOV that distribute to the nucleus in the dark-adapted state do not bind the inner nuclear membrane, the latter presumably due to the presence of a thick and dense nuclear lamina (see Figures S5 and S6 of Ref. [7••]).

While it is possible that BcLOV4 binds a plasma membrane-associated partner that could influence subcellular localization selectivity, the interaction is likely transient if such a mammalian partner exists; multiple attempts at (AP/MS) affinity purification mass spectrometry-based interactome analyses of stably transducing BcLOV4-HEK293 cells showed no preferential partner in a light-dependent manner in our hands. One consequence of this preliminary insight is that BcLOV4 may be fairly 'inert' as an optogenetic tool, devoid of spurious protein–

protein interactions with basal levels of mammalian proteins. The membrane undocking kinetics of BcLOV4, though, which lags the photoadduct thermal reversion timescale by typically approximately one minute in cells and in lipid-stabilized emulsions, does suggest the existence of unidentified lipid-interaction(s) across the multi-domain protein once it is membrane-localized (or possibly a slower photoadduct reversion for the lipid-bound state than in the bulk solution). In other non-mammalian cellular and *in vitro* model systems, the distribution profile and determinants will certainly be influenced by the respective membrane compositions (lipids, proteins, matrices, etc.), membrane structural properties (curvature, defect-density, fluidity, etc.), intracellular milieu (salinity, oxidation-reduction environment, pH, molecular crowding, etc.), and post-translational modifications, and thus, it is important to note that the commonalities of RGS-LOV signaling characteristics observed across model systems may be coincidental.

A high-resolution structure of the lipid-bound state is likely needed to conclusively determine what governs its plasma membrane preference and to refine our proposed multi-domain rearrangement and signal transmission mode. A high-resolution structure would also inform the rational design of a monomeric form of the native dimeric BcLOV4 (although naturally monomeric RGS-LOV may exist), a truncated or 'minimal' BcLOV4 that preserves only the essential structural elements required for light-induced membrane binding, or variants with decreased membrane undocking timescales to improve overall temporal precision as a tool. However, given that the high-affinity protein-membrane interaction is near diffusion-limited, it is unlikely that meaningfully faster membrane recruitment times can be achieved by engineered RGS-LOV or any other optogenetic tool.

Conclusion and future directions: is it a feature or bug?

Despite the comparative advantages respectively reported for existing optogenetic systems, there is no single solution for an ideal tool for membrane recruitment and signaling. Shortcomings of any particular system are typically attributable to inherent biophysical arguments or signaling responses downstream of optical induction, not to poor protein engineering. For example, speed comes at the expense of optical induction efficiency; fast undocking benefits temporal resolution of a tool but necessitates more light to sustain signaling because the activated protein thermally reverts quickly post-induction, whereas a 'slow' tool is more efficient because it better sustains signaling post-induction [58], even if the chromophore extinction coefficient is the same between the two scenarios. In our own work in studying calcium signaling dynamics, the intrinsic 'slowness' of the CRY2:CIBN system confers *quasi* bi-stability that is helpful in limiting photobleaching when performing second messenger signaling assays with fluorescent reporters [23].

In another example of contextual dichotomy, because no transition between biological states is infinitely steep, high-affinity light-activated binding that promotes rapid membrane recruitment and robust association between partners places an upper bound on protein expression level, in order to avoid permanent association in the dark (i.e. when expression level exceeds the binding affinity in the dark-adapted state), as described with iLID [38•]. Similarly, the strong intrinsic preference of RGS-LOV for the inner leaflet is advantageous for rapid plasma membrane recruitment, but could hinder applications that target other subcellular structures.

The proverbial quip from computer engineering of 'It's not a bug, it's a feature' is perhaps apropos for any characteristic of any optogenetic tool. Accordingly, it is useful to consider an application-specific operating window in optogenetics, or a useful dynamic range in analogy to the therapeutic index in pharmacology. The successful implementation of optogenetic tools for membrane recruitment and hetero-dimerization often necessitates extensive side-by-side comparisons of multiple technologies in context of the specific end-application to identify such windows; some examples of thorough characterization on application-specific kinetics [59•,60] and expression level-based performance [38•] in subcellular optogenetics can be found in works by others.

The BcLOV4 protein discussed here possesses functional windows well-suited for optically inducible membrane recruitment-based signaling in mammalian cells with respect to temporal precision, signaling induction efficiency by sparse illumination, and signaling contrast ratio between the photoactivated and dark-adapted states. It is possible that these characteristics were evolutionarily optimized for membrane recruitment-based signaling as natural proteins, but as engineered optogenetic tools, the RGS-LOV proteins studied to date were not intentionally designed so, beyond mammalian codon optimization to increase expression level and the rational engineering of the aforementioned bi-stable mutant by lengthening the flavin photocycle duration. Thus, further engineering, structure-function analyses, and/or experimental characterization of other homologous RGS-LOV proteins [7••,8•] will likely prove to be fruitful endeavors with exciting and valuable outcomes.

Conflict of interest statement

Nothing declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.sbi.2019.01.017.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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