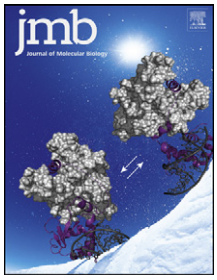
Review



Applications of Optobiology in Intact Cells and Multicellular Organisms

John S. Khamo [1], Vishnu V. Krishnamurthy [1], Savanna R. Sharum [1], Payel Mondal [1] and Kai Zhang [1,2,3]

*1 - **Department of Biochemistry,** University of Illinois at Urbana–Champaign, Urbana, IL, USA*
*2 - **Neuroscience Program,** University of Illinois at Urbana–Champaign, Urbana, IL, USA*
*3 - **Center for Biophysics and Quantitative Biology,** University of Illinois at Urbana–Champaign, Urbana, IL, USA*

***Correspondence to Kai Zhang:** 600 South Mathews Avenue, 314B Roger Adams Laboratory, Urbana, IL 61801, USA*
kaizkaiz@illinois.edu
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Abstract

Temporal kinetics and spatial coordination of signal transduction in cells are vital for cell fate determination. Tools that allow for precise modulation of spatiotemporal regulation of intracellular signaling in intact cells and multicellular organisms remain limited. The emerging optobiological approaches use light to control protein–protein interaction in live cells and multicellular organisms. Optobiology empowers light-mediated control of diverse cellular and organismal functions such as neuronal activity, intracellular signaling, gene expression, cell proliferation, differentiation, migration, and apoptosis. In this review, we highlight recent developments in optobiology, focusing on new features of second-generation optobiological tools. We cover applications of optobiological approaches in the study of cellular and organismal functions, discuss current challenges, and present our outlook. Taking advantage of the high spatial and temporal resolution of light control, optobiology promises to provide new insights into the coordination of signaling circuits in intact cells and multicellular organisms.

Introduction

Spatiotemporal coordination of gene expression and signal transduction during developmental processes

Despite the variety of cellular processes occurring during embryonic development, only a handful of signaling pathways, namely, the Notch, Wnt/β-catenin, Sonic Hedgehog, transforming growth factor β, bone morphogenetic protein, and fibroblast growth factor (FGF) signaling pathways, are repeatedly used to regulate early embryonic development and differentiation [1]. How can a limited number of signaling pathways regulate such diverse cell behavior? Mounting evidence suggests that spatial and temporal regulation of these signaling pathways is crucial to cell fate determination during development. The same pathways can be turned on or off at different times and locations to regulate distinct cell functions.

Circadian oscillators play a critical role in coordinating temporal kinetics of gene expression and signal transduction, responding to signals with a periodicity of 10–24 h. In contrast, oscillation with a shorter periodicity, referred to as ultradian oscillation, determines many biological events at shorter timescales [2]. Temporal coordination of ultradian oscillation often correlates with the formation of spatial patterns of tissue structures during development. For instance, cyclical activation of the Notch pathway is crucial for the formation of a “salt and pepper” pattern of ciliated cells during ciliogenesis [3]. Similarly, oscillation of Notch activation is required for the formation of somites, the precursors to a variety of segmental structures such as the peripheral spinal nerves, vertebrae, axial muscles, and early blood vessels [4,5]. The period of the ultradian oscillations in somitogenesis varies from 20 min in zebrafish to 4–6 h in humans [6]. Besides oscillation, variations in the duration of signals can also lead to distinct cell fates. For instance, transient and sustained activation of the

extracellular signal-regulated kinase (ERK) pathway leads to PC12 cell proliferation and differentiation, respectively [7,8]. In pairs of genetically identical MCF-10A sister cells treated with EGF, cells entering S phase experience sustained ERK activity, whereas the lagging sister cells exhibit pulsatile ERK activity [9]. In cultured rat hippocampal neurons, acute delivery of brain-derived neurotrophic factor (BDNF) elicits transient TrkB signaling and promotes neurite elongation, whereas gradual delivery of BDNF elicits sustained TrkB signaling and promotes neurite branching [10]. Interestingly, high-frequency neuronal stimulation can convert a transient BDNF–TrkB activity into a sustained one [11]. Thus, spatiotemporal coordination of gene expression and signal transduction provides a fundamental molecular mechanism to regulate cell fates.

Both biochemical feedback and molecular stability influence signal oscillation and duration. For instance, negative feedback between the Hes1 protein and its mRNA leads to oscillatory or sustained Hes1 protein expression, resulting in the proliferation and differentiation of neural progenitor cells, respectively [12,13]. Negative feedback loops in the mitogen-activated protein kinase pathway can lead to sustained oscillation in kinase activity [14–16]. In the case of molecular stability, an increase in the half-life of *Hes1* mRNA leads to an extension of the Hes1 protein oscillation period [17]. Unraveling such intricate signaling mechanisms demands tools to probe these dynamic regulatory processes with spatiotemporal precision.

Challenges in spatiotemporal control of signal transduction and gene expression

Our current understanding of gene expression and signaling mechanisms has primarily relied on conventional genetic and pharmacological approaches. Commonly used genetic approaches such as gain- and loss-of-function mutagenesis often lead to constitutive activation or inactivation of signaling activity. To address this issue, alternative chemical and genetic tools have been developed. For instance, several inducible systems are available to activate or repress protein expression in yeast [18]. Chemical regulation of gene expression has been achieved using promoters that respond to molecules such as galactose, methionine, and copper. Alternatively, engineered gene regulatory systems that employ estrogen or doxycycline can be utilized for orthogonal gene transcription control. While these inducible systems have greatly facilitated the control of gene expression, their off-target effects, ineffective delivery, and limited reversibility have restricted their use in live cells and multicellular organisms [19–21]. Moreover, it remains challenging for these conventional approaches to precisely perturb gene expression and signal transduction at a resolution that matches the spatial and temporal scales of endogenous developmental events, which can occur in minutes within space of several microns.

Emerging optobiological approaches

Light can be confined to a sub-micron space and precisely tuned in time, with minimal invasiveness to biological organisms. These advantages initially enabled the interrogation of neuronal firing by light-sensitive synthetic ion channels [22] and channelrhodopsin [23,24] in genetically dissected circuits, which led to the coining of the term “optogenetics” [25]. By modulating neuronal firing, optogenetics has provided a new way to investigate neuronal circuits and to establish causal relationships between brain activity and health and disease [26,27]. These powerful tools not only help researchers in basic science understand signaling circuits in brain functions such as learning and memory, but also show promising preclinical and clinical potential for rewiring neuronal circuits to amplify or override specific neuronal phenotypes. Indeed, optogenetics has been extended to basic and preclinical research on a wide spectrum of topics such as Parkinson's disease [28], sleep [29], cardiac function [30,31], neuropsychiatric diseases [32], epilepsy [33], and sight-restoring therapy [34,35]. This new modality provided by light has been transforming neuroscience research, as evidenced by its explosive growth in recent years.

The power of light to interrogate cell functions, however, is not limited to the modulation of the membrane potential of excitable cells [36]. Light has also been used to control a variety of signaling processes [37–43]. As pointed out earlier by Kim and Lin [44], we believe that it is more appropriate to refer to this broader emerging field as optobiology, in which light enables new modalities to study biological processes in intact cells and multicellular organisms, including in situations where genetic dissection is not essential. In optobiology, light serves not only as an observational tool to follow biological events but also as a manipulative device to modulate activation states of signaling components with high spatial and temporal resolution.

Over the past decade, a portfolio of optobiological tools has emerged to regulate protein–protein interactions in live cells. The core components of optobiology are photoactivatable proteins, which respond to visible or infrared (IR) light stimulation by changing their conformations [45]. These light-triggered conformational changes can induce inter- or intra-molecular interactions. When linked to specific signaling components, photoactivatable proteins allow for light-controlled activation or inactivation of target signal transduction. To date, light has been used to control cellular processes such as gene transcription, translation, protein degradation, differentiation, apoptosis, and migration, as discussed in the following sections. Photocaged small molecules or amino acids also enable light-mediated control of cell functions, a process often referred to as photopharmacological or optochemical control.

Recent optobiological work has expanded its host system from cultured cells to multicellular organisms. Compared to cultured cells, multicellular organisms require better quality control in genetic and protein engineering, as well as material and light delivery. In this review, we outline recent advances in optobiology, focusing primarily on accomplishments made in the past 3 years. For earlier work, interested readers are encouraged to refer to previous reviews [46–54]. In the following sections, we describe optobiological tools with new photophysical properties, update new optobiological applications in intact cells and multicellular organisms, discuss challenges in optobiology, and present our outlook. Because many reviews have covered optogenetic control of neuronal activity based on channelrhodopsin and their derivatives [55–61], we will not repeat that aspect of optobiology in this work.

New features of second-generation optobiological tools

Optobiology offers a repertoire of light responsive modules. Because the commonly used phytochrome, cryptochrome, and light-oxygen-voltage (LOV) domains have been extensively reviewed [45–49], we only list new members of photoactivatable proteins discovered within the past 3 years (Table 1). We summarize key characteristics of these proteins, including their sizes and photophysical properties, namely, the excitation wavelength, association and dissociation kinetics, and binding affinity. Most of these new members evolved from their first-generation parental proteins. Like their parental proteins, these new photoactivatable proteins alter their intra- or intermolecular interactions upon light illumination. Their mechanisms of action and new features are depicted in Fig. 1.

New members of LOV-based tools

Avena sativa light-oxygen-voltage 2 (AsLOV2), a photoactivatable protein commonly used in caging a protein of interest, has been engineered to create light-inducible dimerizers, including (1) the tunable light-controlled interacting protein tags (TULIPs) [43] and (2) light-inducible dimers (LIDs). Both utilize the blue light-induced uncaging of a peptide concealed in the C-terminal Jα helix of AsLOV2. TULIPs consist of a peptide epitope attached to serial truncations of the Jα helix (LOVpep), which binds to an engineered Erbin PDZ domain upon blue light illumination. Binding affinity between LOVpep and engineered Erbin PDZ domain can be tuned by AsLOV2 mutants that either increase or decrease helix docking. The original LID [73] utilized the similarity between a bacterial peptide, SsrA, and the Jα helix in AsLOV2 to cage SsrA from its binding partner protein, SspB. Blue light-induced conformational changes in AsLOV2 exposed SsrA, which was then free to interact with SspB. The original LID system is limited by a relatively small dynamic range (8-fold change in affinity between lit and dark states) and a high dark-state affinity (800 nM). Guntas et al. [66] sought to improve the dynamic range of LID

Table 1. Collection of photoactivatable proteins and their photophysical properties

Photoactivatable protein	Parental protein	Excitation λ (nm)	Size (aa)	Asso. time	Disso. time	K_d	Ref.
pdDronpa1 variants[a]	Dronpa K145N	500/400	224	3 s[b]	8 s	4 μM	[62]
CRY2–CIB1 variants[c]	CRY2wt	450–490	535/81 or 170	ms	2.5–24 min	Not reported	[63]
CRY2E490G	CRY2wt	450–490	498	ms	23.1 min	Not reported	[64]
CRY2clust	CRY2wt	450–490	507	<1 s	~220 s	Not reported	[65]
iLID nano and micro	*As*LOV2–SsrA SspB	450–490	149/112	<30 s	<30 s	0.1–47.0 μM[d]	[66]
LOVTRAP	*As*LOV2 Zdk	450–490	146/65	<30 s	1.7–496 s	17 nM – >4 μM[e]	[67]
Magnets	VVD	450–490	150	1.5 s	6.8 s–4.7 h	Not reported	[68]
CBD	CarH	545	200	Not reported	Not reported	Not reported	[69]
VfAU1–LOV	n/a	470	150	<1 min	625 s	Not reported	[70]
CPH1S	CPH1	660/720	513	Not reported	Not reported	Not reported	[71]
BphP1–PpsR2	BphP1 and RpPpsR2	740/636	731/464	3.5–28 s[f]	3–21 s[g] and 170 [h]	Not reported	[72]

[a] pdDronpa1: Dronpa M40I V60A E121R V123 T K145 N N158 V; pdDronpa1.2: pdDronpa1 V158I.
[b] Kinetics based on fluorescence readout in live cells.
[c] CRY2 535 (aa 1–535 of CRY2), tighter light control than CRY2PHR; CIB81 (aa 1–81 of CIB1) smaller fragment that retains interaction with CRY2; CRY2(L348F), prolonged dissociation half-life of 24 min; CRY2(W349R), shortened dissociation half-life of 2.5 min.
[d] iLID nano, blue light lit affinity 132 nM, dark affinity 4.7 μM; iLID micro, blue light lit affinity 800 nM, dark affinity 47 μM.
[e] Zdk1 blue light lit affinity >4 μM, dark affinity 26.2 nM; Zdk2 blue light lit affinity 761 nM, dark affinity 17 nM; Zdk3 blue light lit affinity 537 nM, dark affinity 11.4 nM.
[f] Exact association kinetics depends on the power of 740-nm light. Stronger excitation light reduces the half-time.
[g] Exact dissociation kinetics depends on the power of 636-nm light. Stronger excitation light reduces the half-time.
[h] Values are based on dark-conversion kinetics. Irradiation with 636-nm light restores 80% Pfr absorbance, and the rest, 20%, depends on dark conversion.

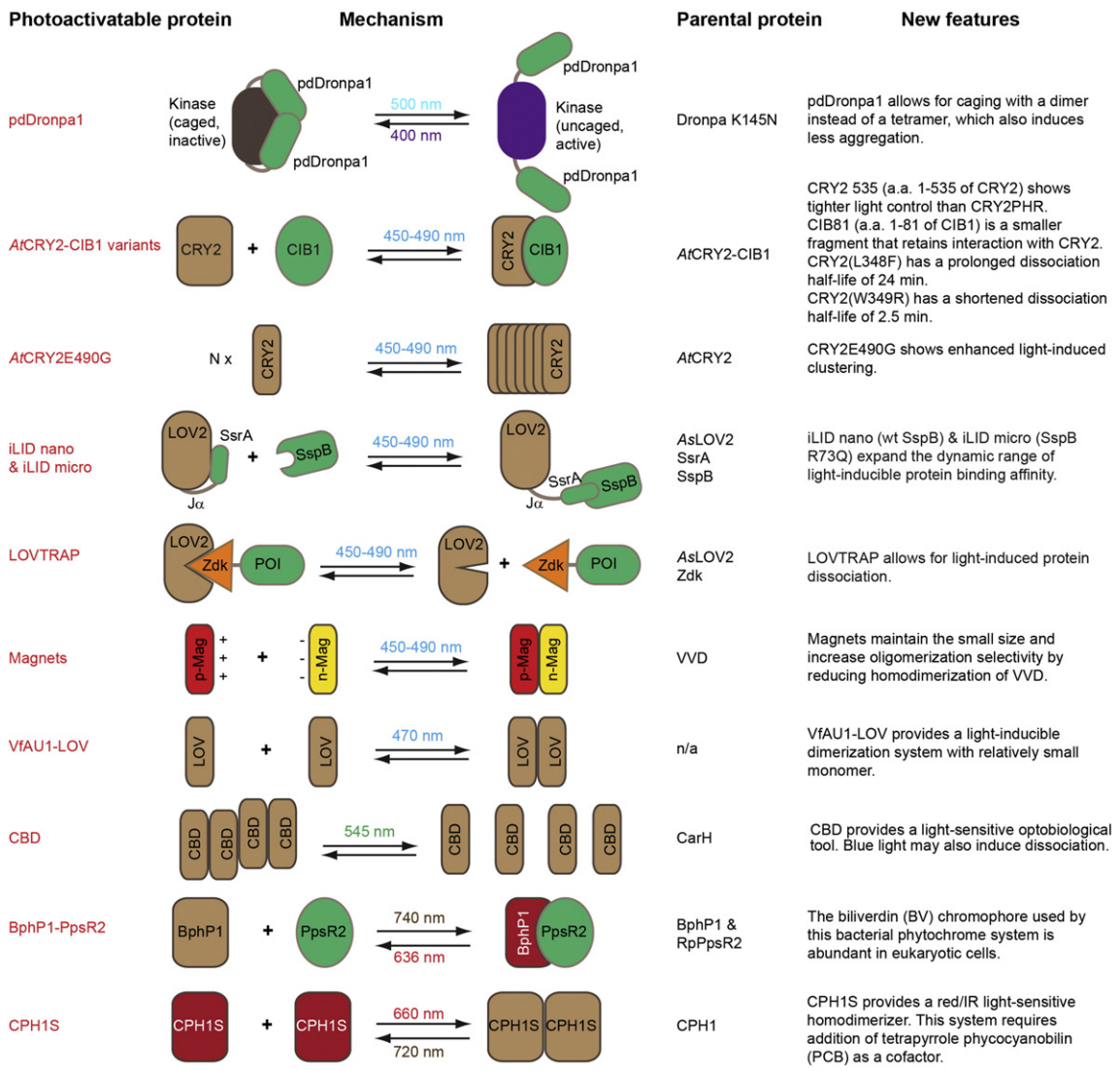

Fig. 1. Mechanism of action and new features of various newly developed photoactivatable proteins. Engineered through their parental proteins, these new optobiological tools retain their responses to visible and near IR light stimulation.

by phage display screening and found two improved LIDs, iLID nano and iLID micro. Together with TULIPs, iLID nano and micro provide a wide range of lit and dark state affinities to be utilized in specific cellular applications [74].

Another new application of the AsLOV2 is to induce protein *dissociation* by light as opposed to other optogenetic systems, which generally use light to induce protein *association*. Wang et al. [67] developed the LOV2 trap and release of protein system (LOV-TRAP) where proteins of interest were fused to a small protein Zdark (Zdk). Zdk was generated by screening a library of amino acid variants of the Z domain from staphylococcal protein A. In the dark state, Zdk domain bound to LOV2 with high affinity, which sequestered the fusion protein to LOV2. Upon blue light illumination, conformational changes in LOV2 promoted their dissociation to release the proteins of interest.

The Aureochrome-1 (AUREO1) transcription factor protein from the alga *Vaucheria frigida* consists of a blue-light-sensing LOV domain in its C-terminal region and a central basic region/leucine zipper (bZIP) domain. Blue light-induced dimerization of monomeric AUREO1 increases its affinity for target DNA and enables its functionality in transcriptional control. The functionality of AUREO1 as a transcriptional switch was proposed to arise from synergistic interactions of its LOV–LOV and bZIP–bZIP domains, which stabilized the monomeric form in the dark and the dimeric form in the lit state [75]. Blue light-induced dimerization of VfAU1–LOV alone, however, is also sufficient to activate fused receptor tyrosine kinases (RTKs) in mammalian cells [70].

The 150-amino-acid vivid (VVD) protein from the filamentous fungus *Neurospora crassa* undergoes homodimerization upon light stimulation. To improve

upon its low affinity of homodimerization, slow switch-off kinetics, as well as its selectivity, Kawano et al. [68] developed a series of oppositely charged VVD pairs referred to as "Magnets". Charged residues were introduced in the VVD homodimerization interface comprising residues 47–56. Similar to the attraction between opposite magnetic poles, attraction only occurs between negative-charge-bearing "n-Magnets" and the positive-charge-bearing "p-Magnets", but not between n–n or p–p Magnet pairs. Magnet dissociation times can be tuned by combining different Magnet mutants that have either high affinity or fast dissociation kinetics.

Single-chain photodissociable Dronpa

Zhou et al. [62] developed photodissociable dimeric Dronpa domain, photodissociable dimeric Dronpa (pdDronpa1), which can be photoswitched between monomeric and dimeric configurations by cyan and violet light, respectively. Compared with its parental tetrameric protein DronpaK145 N, pdDronpa1 utilizes a dimer-to-monomer mechanism to cage/uncage the protein of interest. In addition, pdDronpa1 is less likely to aggregate because the dimer has its valency requirement met by its own intramolecular interaction.

Second-generation tools based on cryptochrome

Cryptochrome 2 (CRY2) and cryptochrome-interacting basic-helix–loop–helix 1 (CIB1) is a heterodimerizing pair that has been widely used in optobiology. The first-generation optogenetic tools utilized either full-length CRY2 or its photolyase homology region (CRY2PHR; 1–498 aa) to interact with CIB1 or its N-terminus (CIBN). The system does not require addition of exogenous cofactors, but suffers from a relatively slow dissociation time ($t_{1/2} \approx 5.5$ min) [41]. Taslimi et al. sought to generate improved CRY2–CIB1 variants of smaller size, altered interaction lifetimes, and reduced dark-state interactions. A truncated version of CRY2 containing residues 1–535 was identified to possess higher dynamic range and less self-interaction in the dark compared to CRY2PHR. Truncations in CIB1 revealed that only the first 81 residues of CIB1, termed CIB81, were functionally at par with CIBN (170 aa) in its capacity to recruit a cytosolic CRY2PHR to the plasma membrane under blue light. By mutating the photocycle-impacting residues in CRY2(535), Taslimi et al. developed two mutants, which had shorter ($t_{1/2} \approx 2.5$ min, W349R) and longer ($t_{1/2} \approx 24$ min, L348F) dissociation times [63]. In addition, a CRY2 mutant (CRY2olig, E490G) with a stronger oligomerizing potential has been found by the same group [64]. Very recently, Park et al. [65] discovered a short peptide (9 aa), which significantly enhanced CRY2PHR clustering when appended to its C-terminus. When compared with other CRY2-based clustering tools, this enhanced reversible clustering tool, named "CRY2clust" exhibited lesser concentration dependence for clustering, higher light sensitivity, faster assembly and disassembly dynamics, and no undesired accumulation in subcellular compartments. Interestingly, the authors report that a 9-aa extension of the intrinsic PHR domain in CRY2, CRY2[1–507], also increased the efficiency of light-dependent CRY2 clustering.

Optobiological modules responsive to green light

Optobiological tools that respond to green light have been largely unavailable. Having another excitation wavelength would facilitate multiplexing optobiological applications. Kainrath et al. [69] engineered bacterial cobalamin (vitamin B_{12}) binding domains (CBDs) as a green light-mediated optobiological tool for use in cultured cells and zebrafish. CBD uses a 5′-deoxyadenosylcobalamin (AdoCbl) cofactor, which absorbs green light. In the dark, CBD forms dimers of dimers, which dissociate into monomers under green light irradiation. When fused to the intracellular domain of a membrane-anchored murine fibroblast growth factor receptor mFGFR–MxCBD and expressed in mammalian cells, the construct induced elevated ERK activity, which was then reduced by green light-induced CBD dissociation. Similarly, hyperactive FGFR signaling could also be reduced by green light in zebrafish.

Optobiological modules responsive to red and near IR light

As we have discussed so far, most optobiological tools are biased toward the blue end of the visible spectrum. Blue light, however, is suboptimal for deep tissue penetration. An attractive photoactivatable protein is phytochrome, which can be switched between the Pfr (absorbing IR) and Pr (absorbing red light) states using IR and red light. Such a bidirectional control makes phytochrome useful in studying processes with fast association and dissociation kinetics. While *Arabidopsis thaliana* phytochrome B (PhyB)-based systems can outshine blue light-inducible optogenetic systems *in vivo* due to the better tissue penetrance of red light, such systems require the incorporation of an exogenously supplied chromophore, phycocyanobilin, which severely restricts their use [39]. Nonetheless, Buckley et al. [76] successfully used this system to control subcellular protein localization in zebrafish embryos.

The recently discovered *Rhodopseudomonas palustris* bacterial bathy phytochrome, BphP1, has optogenetic properties that are similar to plant phytochromes. More attractively, BphP1 uses a biliverdin cofactor that can be produced by mammalian cells. Kaberniuk et al. [72] characterized the *in vitro* and *in vivo* interactions between BphP1 and its partner protein PpsR2 and developed an improved optogenetic system which

responds to 740- and 636-nm light. This bidirectional control was tapped in a variety of applications ranging from protein translocation to gene transcription *in cellulo* and *in vivo*. To further improve the functionality and dynamic range of the BphP1–PpsR2 pair, Redchuk and colleagues [77] identified a minimal BphP1-interacting Q-PAS1 fragment (17 kD). Combining this system with blue light-activated AsLOV2 uncaging, the authors constructed a dual-color optogenetic system called near-IR (NIR)–blue-light-inducible shuttle, which exhibited minimal spectral crosstalk. Another homodimerizer based on cyanobacterial phytochrome 1 (CPH1) has also been developed [71]. This system still requires addition of the tetrapyrrole phycocyanobilin as a cofactor.

New photo-pharmacological tools

Photo-pharmacological approaches combine the spatiotemporal precision offered by light with the diversity and versatility of chemicals to control cell functions [78–80]. The well-established rapamycin-mediated dimerization of FKBP- and FRB-fusion proteins has been widely used in controlling protein dimerization [81]. A light-controllable rapamycin-FKPB–FRB system was developed by constructing a photocaged analog of rapamycin [82]. Alternatively, Brown et al. [83] utilized a photocleavable dimerized rapamycin (dRap) to form inactive FKBP12–dRap–FKBP12 complexes, which could not bind FRB. UV light irradiation cleaved dRap into two functional Rap molecules and enabled the formation of the FKBP12–Rap–FRB complex. Other UV light-mediated dimerizers include caged abscisic acid [84], cTMP–Htag [85], a photoactivatable crosslinker for SNAPTag and HaloTag [86], and gibberellic acid [87].

Aside from photocaged small molecules, photocaged amino acids form another critical group of photopharmacological reagents [46]. Importantly, photocaged unnatural amino acids can be incorporated into any protein in response to an amber codon, UAG, through a protocol pioneered by Young and Schultz [88]. To do so, a synthesized pyrrolysyl-tRNA synthetase/tRNA pair was optimized to incorporate a photocaged lysine in response to an amber codon in mammalian cells. This system has been used to control protein localization [89], kinase activity [90], gene expression [91], and CRISPR/Cas9 activity [92]. A similar strategy was used to generate photocaged tyrosine, which was incorporated into the Cre recombinase to achieve light-controllable DNA recombination [93]. Recently, a caged methionine, (*S*)-*N*-(4,5-dimethoxy-2-nitrobenzyloxycarbonyl)-methionine, was synthesized through a low-cost, one-step reaction [94]. UVA (365-nm) light illumination removed NVOC to release methionine. Instead of incorporating the photocaged methionine into a protein, the authors used it directly to achieve light-mediated repression of gene expression through a pMET17 promoter.

Optobiological control of cellular functions

Parallel to the development of new photoactivatable proteins, optobiology continues finding new applications in the modulation of cellular and organismal functionality though light-induced uncaging, protein translocation, modulation of concentration and avidity, and allosteric control (Fig. 2). In this section, we summarize recent advances in optobiological control of cellular functions. We use subcellular locations (plasma membrane, cytoplasm, and nucleus) to group these cases, highlighting the capacity of light to modulate cellular events with high spatial resolution. We also itemize these findings in Table 2.

Controlling molecular machinery at the plasma membrane

Calcium signaling

Calcium signaling is essential for cellular function and is involved in a variety of cellular processes such as exocytosis, excitability, and motility [129]. Calcium signaling is also diverse in both space and time, requiring precise spatiotemporal control for accurate study [130]. Optobiological systems have been developed to address this need, but some of the first were not specific for calcium [131] and provided modest changes in the intracellular calcium concentration [132], which did not reach the physiological range. To produce more robust optobiological tools for calcium signaling, Ishii et al. [95] engineered a photocaged fragment of stromal interaction module 1 (STIM1), the gating factor for the ORAI1 Ca^{2+} release-activated Ca^{2+} channel. The authors named this system "blue light-activated Ca^{2+} channel switch." The human or *Drosophila* minimal STIM1 fragment was fused to the C-terminus of AsLOV2, which caused steric hindrance in the dark. Blue light-stimulated unfolding of the LOV Jα helix led to the exposure of the STIM1 fragment, which allowed it to interact with endogenous or exogenous ORAI1 channels to promote reversible extracellular Ca^{2+} influx within the physiological range. The authors demonstrated that the degree of Ca^{2+} influx was tunable based on the intensity of stimulating light, and that the induction could be spatially confined to axon tips and dendrites. Using a similar approach, He et al. [96] developed a NIR-inducible optogenetic system (Opto-Ca^{2+} release-activated Ca^{2+}) to manipulate Ca^{2+} influx and Ca^{2+}-dependent gene expression in immune cells to promote heightened immune responses and tumor suppression in a melanoma mouse model. In line with these studies, Kyung et al. [97] engineered OptoSTIM1, a light-inducible system to reversibly control Ca^{2+} influx through endogenous ORAI1 channels. By fusing CRY2PHR to a truncated form of cytosolic STIM1, the authors used blue light to form OptoSTIM1 clusters, which translocated to the plasma membrane to activate ORAI1 channels. More

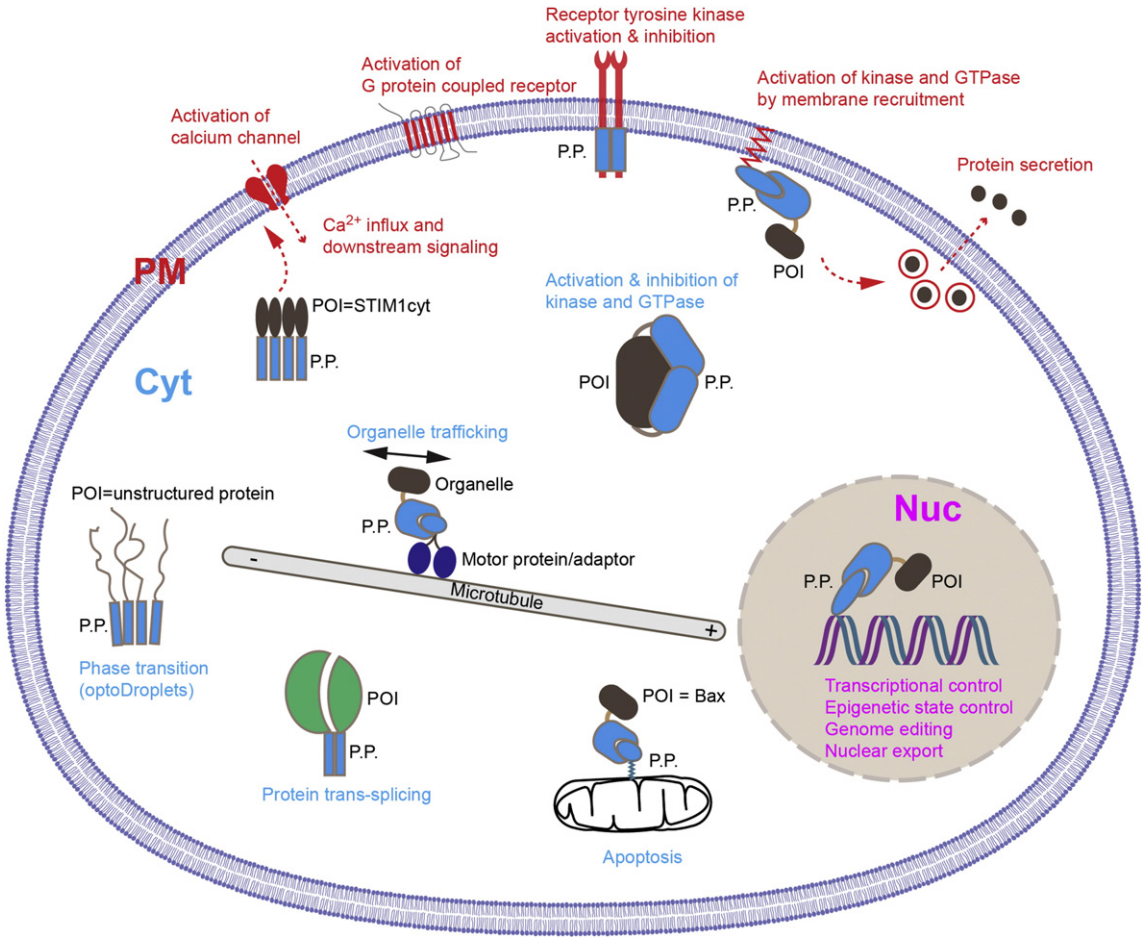

Fig. 2. Expanded applications of optobiology in intact cells and multicellular organisms. Optobiology has been utilized to control molecular machinery within distinct subcellular locations such as the nucleus, the cytoplasm, the plasma membrane. Functionality of protein of interest (POI) can be modulated by light-mediated conformational changes in photoactivatable protein (P.P.).

recently, Park et al. [65] deployed the earlier-described CRY2clust tool in OptoSTIM1 to obtain two-fold faster changes in intracellular Ca^{2+} levels.

Modification of phosphoinositides

Phosphoinositides in the plasma membrane are spatiotemporally regulated, and they play a key role in the regulation of integral membrane proteins and a variety of cell signaling events [133]. Xie et al. [98] used the CRY2–CIBN protein pair to investigate how membrane depletion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) affects the Ca^{2+}-triggered secretion of insulin from cultured pancreatic β cells. CIBN was anchored to the plasma membrane by a farnesylation motif, and CRY2 was fused to a 5′-phosphatase. Blue light illumination induced membrane recruitment of the 5′-phosphatase, where it could catalyze the conversion of PI(4,5)P2 to phosphatidylinositol 4-phosphate. PI(4,5)P2 depletion reduced Ca^{2+} influx and suppressed insulin secretion, thereby highlighting PI(4,5)P2 as a key regulator of voltage-dependent Ca^{2+} channels and Ca^{2+}-triggered insulin secretion from pancreatic β cells. PI(4,5)P2 is also known to regulate actin polymerization at the plasma membrane [134]. Because cell contractility depends on cortical actin dynamics, which in turn relies on the plasma membrane levels of PI(4,5)P2, membrane depletion of PI(4,5)P2 arrested ventral furrow formation in developing *Drosophila* embryos [99].

Phosphatidylinositol 3,4,5-triphosphate is regarded as a secondary messenger downstream of phosphatidylinositol 3-kinase (PI3K) that regulates actin polymerization and cell polarity [135]. Kawano et al. [68] induced spatially confined generation of phosphatidylinositol 3,4,5-trisphosphate on the plasma membrane of NIH3T3 cells. By pairing the Magnet variants nMagHigh1 and pMagFast2, where the former was membrane anchored and the latter cytosolic, they used blue light to recruit the pMagFast2-fused, P110 catalytic subunit of PI3K to the plasma membrane. Localized lamellipodia protrusion and plasma membrane ruffles

Table 2. Emerging cellular and organismal functionality controlled by photoactivatable modules

Site	Function	Module	Target	MOA	Organism	Ref.
PM	Ca^{2+} influx	AsLOV2	STIM1	Caging	Mammalian and hippocampal neurons, *Drosophila* S2 cells, transgenic mice	[95]
PM	Ca^{2+} influx	AsLOV2	STIM1	Caging	Mammalian cells, mice	[96]
PM	Ca^{2+} influx	CRY2	STIM1	Association	Mammalian cells zebrafish embryos, mice hippocampi	[97]
PM	PI(4,5)P2 regulation	CRY2–CIBN	Phosphatase	Association	Mammalian cells	[98]
PM	Cell contractility, PIP2 depletion	CRY2–CIBN	OCRL	Association	*Drosophila* embryo	[99]
PM	Phosphoinositide production	Magnets (VVD)	iSH2, Rac1	Association	Mammalian cells	[68]
PM	Cytoskeletal remodeling	AsLOV2	ITSN, Tiam1	Caging, association	Mammalian cells	[66]
PM	Membrane protrusion	AsLOV2	GTPase	Caging, association	Mammalian cells	[67]
PM	Cytokinetic furrowing	LOV–PDZ	LARG	Association	Mammalian cells	[100]
PM	ERK signaling	AsLOV2	SOS	Caging, association	*Drosophila* embryos	[101]
PM	Differentiation	CRY2–CIBN	Raf1	Association	Mammalian cells, *Xenopus* embryos	[102]
PM	GSV membrane translocation	CRY2–CIBN	PI3K, Akt	Association	3T3-L1 adipocytes	[103]
PM	RTK signaling	CRY2	SH2 of PLCγ	Association	Mammalian cells, rat hippocampal NSCs	[104]
PM	Polarity signaling	PIF6–PhyB	Pard3	Association	Zebrafish embryos	[76]
PM	Release of hormones	BphS	BldD	c-di-GMP synthesis	Mammalian cells, C57BL/6 J mice	[105]
PM	Insulin secretion	PhotoETP	GLP-1R	Allosteric switch	Mammalian cells, C57BL6 mouse islets	[106]
Cyt	Cargo transport	LOV–PDZ/ CRY2–CIBN	Motor proteins	Association	Mammalian cells, rat hippocampal neurons	[107,108]
Cyt	Peroxisome transport	AsLOV2	PTS	Caging	Mammalian cells	[109]
Cyt	Enzymatic activity	AsLOV2	Src, Rac1	Allostery	Mammalian cells	[110]
Cyt	Inhibition of JNK and p38MAPK	AsLOV2	Peptides	Caging	Mammalian cells, cerebellar granule neurons	[111]
Cyt	Inhibition of CaMKII	AsLOV2	Peptides	Caging	Mammalian cells, mice hippocampal slices, mice	[112]
Cyt	Apoptosis	CRY2–CIBN	Bax	Association	Mammalian cells	[113]
Cyt	Kinase activity, synaptic vesicle trafficking	pdDronpa	Kinases	Caging	Mammalian cells, *C. elegans*	[62]
Cyt	Intracellular phase transition	CRY2	IDRs	Clustering	Mammalian cells	[114]
Cyt	Protein trans-splicing	AsLOV	Intein	Caging	Mammalian cells	[115]
Nuc	Transcription	CRY2–CIBN	Cre	Association	Ai9 mice cultured neurons	[116]
Nuc	Transcription	NVOC–Met	pMET-17	Caging	Yeast	[94]
Nuc	Transcription	TAEL	Sox32, lefty1, Cas9	Caging, association	Zebrafish embryos	[117]
Nuc	Transcription	CRY2–CIBN	dCas9	Association	Mammalian cells	[118]
Nuc	Transcription	CRY2–CIBN	Il1RN, HBG1, HBG2, ASCL1	Association	Mammalian cells	[119]
Nuc	Transcription	CRY2–CIB1 (variants)	Cre	Association	Yeast, mammalian cells	[63]
Nuc	Transcription, translocation	DMNB	ABA	Caging	Mammalian cells	[84]
Nuc	Transcription, behavior	CRY2–CIBN	GCaMP3.0, TRPA1	Association	*Drosophila* S2 cells, *Drosophila* embryos	[120]
Nuc	Epigenetic perturbations	CRY2–CIB1	Grm2, histone	Association	Mammalian cells, cortical and prefrontal cortex neurons	[121]
Nuc	Protein shuttling, transcription	PIF3–PHYB	VP-16/TetR	Association	Mammalian cells, zebrafish embryos	[122]
Nuc	Nuclear export	AsLOV2	CRM1	Caging	Mammalian cells	[123]
Nuc	Genome editing	Magnets	Cre	Association	Mammalian cells	[124]
Nuc	Genome editing	Magnets	Cas9	Association	Mammalian cells	[125]
Nuc	Genome editing	Caged lysine	Cas9	Caging	Mammalian cells	[92]
Nuc	DNA recombination	dRap	Cre	Cleavage	Mammalian cells	[83]
Nuc	Histone acetylation	COMET probes	HDAC	Isomerization	Mammalian cells	[126,127]
Nuc	Histone modification	AsLOV2	Bre1	Caging, association	Yeast	[128]
Nuc	DNA recombination	Caged aa	Cre	Caging	Mammalian cells	[93]

ABA, abscisic acid; COMET, chemo-optical modulation of epigenetically regulated transcription; SOS, Son of Sevenless.

occurred on the illuminated portion of the cell and membrane retraction occurred on the non-illuminated rear side.

Modulation of GTPase activity

Small Rho GTPases including RhoA, Rac1, and Cdc42 are well known as regulators of cytoskeletal dynamics in processes such as cell motility, polarity, cell cycle, and many others [136,137]. Therefore, controlling and perturbing their activity with spatiotemporal precision is of great interest and use in biological research. Guntas and colleagues [66] used the iLID system to recruit the guanine nucleotide exchange factors (GEFs) ITSN and Tiam1 to user-defined spots on the plasma membrane of fibroblasts, allowing control of local GTPase activity in a blue-light dependent manner. Membrane recruitment of GEF evoked lamellipodia formation and membrane ruffling in a localized fashion. Wang et al. [67] used the LOVTRAP system to drive cell edge oscillations by mimicking the natural oscillatory pattern of the underlying signaling pathways. Constitutively active forms of Rac1, RhoA, and their upstream GTP exchange factor Vav2 were fused to the Zdk protein and sequestered on mitochondria containing LOV2. Interestingly, Vav2 activation generated a sustained increase in protrusion and extension velocities.

Successful cell division relies on spatiotemporal precision in the assembly and function of the actomyosin-based contractile ring [138]. To investigate the spatial and temporal regulation of cytokinesis, Wagner and Glotzer developed PR_GEF, a cytosolic tandem PDZ-tagged catalytic domain of a RhoA GEF, for the specific activation of RhoA, a membrane-bound GTPase functional in contractile ring assembly during cytokinesis [100,139]. By anchoring a LOV-caged PDZ binding epitope at the plasma membrane, light-induced uncaging of the epitope allowed membrane recruitment of PR_GEF and subsequent RhoA activation. When tested in non-contractile early anaphase cells with a blocked endogenous activating pathway for RhoA, PR_GEF successfully induced cytokinetic furrows within the spatially defined illumination zones spanning both the cell midzone and the poles, but ingression was incomplete.

Modulation of kinase signaling

Kinase signaling pathways underlie all major cellular processes such as growth, proliferation, differentiation, migration, polarization, and apoptosis. Achieving precise control over the activation of these pathways has been technically challenging. In addition, ligand dose–response relationship is often non-linear, which is further confounded by adaptive responses and oscillatory responses [140]. Since embryonic development requires inviolable spatiotemporal control, perturbing developmental signaling remains a prime challenge. Optobiology tackles this challenge by appending photoactivatable proteins to the target and rendering it responsive to light, the application of which can be controlled with spatiotemporal precision.

RTKs can activate spatially and temporally distinct signaling networks to mediate cellular processes such as cell survival, migration, proliferation, and differentiation [141]. To bypass ectopic overexpression of receptors, Bugaj et al. [104] developed a light-activated system called Clustering Indirectly using Cryptochrome 2 to precisely control endogenous RTK signaling in space and time. CRY2 was fused to the src homology 2 (SH2) domain of PLCγ, an RTK binding domain (BD) that has limited affinity as a monomer. Blue light-induced cytosolic clustering of the BD-CRY2s increased their avidity to associate with and consequently activate endogenous RTKs. Blue light illumination resulted in successful RTK targeting in several cell types expressing the Clustering Indirectly using Cryptochrome 2 system. Specifically, the system was found to modulate endogenous PDGFRβ, a mediator of fibroblast chemotaxis.

Our recent work investigated the effect of timed kinase activity on embryonic development. Using the CRY2PHR–CIBN system, we activated ERK *via* membrane recruitment of Raf1 in PC12 neuroblasts and in *Xenopus* embryos. It was known that hyperactive ERK results in the formation of ectopic tail-like structures in developing *Xenopus* embryos [142]. It was unclear whether ectopic tail induction was restricted to any specific developmental time window. By optically hyperactivating ERK during and after gastrulation, we demonstrated that these structures can be induced by hyperactive ERK even after gastrulation [102,143]. Johnson et al. [101] used the iLID system to probe the importance of the signaling precision of ERK in *Drosophila* embryonic development. Blue light-induced uncaging of a plasma membrane anchored iLID was used to recruit Son of Sevenless, a Ras activator, to the membrane, which then activated ERK signaling. Two different regions in the embryo exhibited differential sensitivity to the same ERK signal – the middle of the embryo was most sensitive and the poles were unaffected. Interestingly, hyperactive ERK-induced segmentation defects and subsequent lethality occurred only during a brief window of ERK hyperactivation.

The PI3K signaling pathway is another primary pathway downstream of RTK activation. In response to insulin, PI3K signaling regulates the membrane translocation of glucose transporter 4 storage vesicles (GSVs) [144,145]. Akt, or protein kinase B, is downstream of PI3K activation and is a spatiotemporally-regulated kinase with a variety of intracellular substrates that mediate diverse cellular functions [146]. To delineate PI3K and Akt's contributions to insulin-regulated glucose transporter 4 trafficking, Xu et al. used the CRY2–CIBN [103] system to recruit either the PI3K catalytic domain (Opto-PIP3) or Akt (Opto-Akt) to

the plasma membrane. In adipocytes, compared with insulin stimulation, light-mediated activation of Opto-PIP3 induced a comparable magnitude of PIP3 generation, Akt phosphorylation, and GSV membrane translocation. Compared to Opto-PIP_3, Opto-Akt showed reduced GSV translocation.

Allosteric activation of G-protein-coupled receptor

Allosteric regulation has potential to increase drug specificity as drug discovery is presently hampered by the inability to control allosteric sites. The ligand-dependent allosteric activator 4-(3-(benzyloxy) phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine is known to modulate activity of glucagon-like peptide-1 receptor (GLP-1R), a class B G-protein-coupled receptor that is involved in maintenance of glucose levels. Broichhagen et al. [106] constructed PhotoETP, a photoswitchable moiety that achieved light-mediated allosteric control of GLP-1R. Isomerization of photoETP between its *trans* and *cis* states can be induced by blue (440 nm) and UV (330 nm) light. Similar to 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine, *trans*-PhotoETP potentiates GLP-1R through ligand-induced increase of cAMP and Ca^{2+} levels. This functionality can be switched off through UV illumination, which converts *trans*-PhotoETP to its *cis* form.

Controlling molecular machinery in the cytoplasm

Control of organelle and protein trafficking

Precise positioning of organelles at specific subcellular locations plays a crucial role in coordinating signal transduction and cell fate determination, particularly in polarized neuronal cells. For instance, mitochondrial positioning near the plasma membrane is crucial to maintain Ca^{2+} influx and T-cell activation [147,148], and specific endosome localization leads to neuron polarization and outgrowth [149]. Not surprisingly, defective organelle trafficking is involved in multiple neurological diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [150]. A key question is whether defective organelle trafficking is a cause or consequence of neurological disorders. Answering this question requires tools that can control organelle trafficking in live cells, and recent developments in optobiology provide such tools. Recent work on optogenetic control of cargo trafficking has been reviewed [151]. Attaching photoactivatable proteins such as LOVpep–PDZ [107] or CRY2PHR–CIBN [108] to motor proteins and cargoes allows for the control of organelle trafficking by light. In addition, light can also be used to control protein localization to a specific organelle. For instance, Spiltoir et al [109] fused GFP to AsLOV2, which caged a peroxisomal-targeting sequence (PTS) in the dark. Unwinding of the ASLOV2 Jα helix under blue light exposed the PTS, which then bound to the Pex5 peroxisomal import receptor, thereby allowing for translocation of the protein to the peroxisome. In addition, light-induced trafficking of the pro-apoptotic protein Bax to the outer mitochondrial membrane was used to initiate apoptosis [113].

Control of enzymatic activity

Precise spatiotemporal control of enzymatic activity is necessary to better investigate the biochemical complexity of living cells. As a recurring theme in optobiology, one method to achieve this relies on engineered allosteric control of the target enzyme of interest. Dagliyan et al. [110] described a generalizable approach for genetically inserting light-sensitive allosteric switches into enzymes to control their activity using light. The authors inserted an AsLOV2 as the photoswitch into nonconserved surface "tight loops" that were mechanically coupled to the active site of a constitutively active Src kinase and the Rac1 GTPase. In the dark, enzyme structure and function was unhindered. Upon blue light illumination, a conformational change was induced in the LOV2 domain, which was allosterically propagated to the host protein to disrupt its structure and function. Interestingly, the photoswitching in each engineered enzyme highly resembled the conversion between the natural active and inactive states of the wild-type enzymes as revealed by comparison to known crystal structures.

Enzymatic activity can be controlled by caging the catalytic domain of the enzyme of interest. By inserting a monomeric pdDronpa1 at the N-terminus of the kinase domain and within the FG loop, Zhou et al. [62] reported light-controllable kinase activity in MEK1, MEK2, and Raf1. The kinase domain is caged by the dimeric pdDronpa, whereas in the monomeric state, it is exposed. Thus, kinase activity can be controlled by switching between cyan and violet light. The authors discovered a new negative feedback loop in the Raf/MEK/ERK pathway, where ERK activation induces phosphatase PP1 or PP2A to dephosphorylate MEK1/2. Using a similar strategy, the authors developed a photo-switchable cyclin-dependent kinase 5, the activation of which relocated synaptic vesicles from dendrites to axons in *Caenorhabditis elegans.*

Enzymatic activity can also be controlled by engineering photocaged peptide inhibitors for the enzyme of interest. Melero-Fernandez de Mera et al. [111] caged the C-terminal residues of the Jun amino-terminal kinase (JNK) inhibitor JIP1 using AsLOV2 (OptoJNKi). Uncaging of the inhibitor peptide under white or blue light enabled it to interact with and inhibit JNK, allowing for precise regulation of JNK activity in mammalian cells. Based on molecular dynamic simulations of OptoJNKi, the authors proposed adding a terminal phenylalanine residue as a simple method for effectively caging certain C-terminal peptides using AsLOV2. Using this approach, the authors also

generated an inhibitor for p38MAPK, showcasing the method's general applicability to other peptide-based inhibitors. Similarly, Murakoshi et al. [112] developed photoactivatable autocamtide inhibitory peptide 2 (paAIP2), an AsLOV2-caged inhibitor for endogenous Ca^{2+}/calmodulin kinase II (CaMKII). Activation of paAIP2 during the first 1–2 min of the induction of dendritic long-term potentiation inhibited the process in hippocampal slices and amygdalar neurons of mice.

Enzyme activity can be regulated by the complementation of its catalytically inactive fragments. Wong et al. [115] engineered a photoactivatable protein trans-splicing system that allowed light-induced restoration of enzymatic activity. A photoactivatable trans-splicing intein (LOVInC) was generated by caging one half of the naturally split DnaE intein from *Nostoc punctiforme* using AsLOV2. Light-induced uncaging allowed the split inteins to recombine and enable splicing between split enzyme fragments fused to the inteins to reconstitute enzymatic activity. This system was applied to activate enzymes including RhoA and the apoptotic protein Caspase-7.

Control of phase separation of liquid droplets

Although many biochemical interactions in cells occur within membrane-bound organelles such as mitochondria and the endoplasmic reticulum, they have also been observed in a variety of membraneless organelles. For instance, ribonucleoprotein granules are one type of membrane-less organelles that harbor RNA–protein interactions. A crucial driving force for these interactions is the concentration-dependent association between intrinsically disordered protein/regions. RNA-binding proteins often have intrinsically disordered protein/regions that facilitate their condensation into liquid-like droplets within cells. However, understanding of the physiological assembly of these granules largely depends on *in vitro* reconstitution, due to a lack of tools to probe protein phase transitions in live cells. Using an optogenetic assay, Shin et al. [114] fused CRY2PHR to various sticky IDRs and used blue light to modulate their phase separation between liquid and gel-like structures in the cytoplasm of live cells. Depending on the proximity to the phase boundary, these optoDroplets display distinct assembly kinetics—from fully reversible structures to irreversible aggregates. Thus, optoDroplets facilitate studies of physiological assembly and pathological aggregates of liquid droplets in cells.

Controlling molecular machinery in the nucleus

Control of nucleocytoplasmic protein shuttling

Protein transfer into and out of the nucleus is critical for the tight regulation of gene expression and cell fate [152]. Beyer et al. [122] developed a light-inducible system to control the nuclear localization of transcription factors in zebrafish. By taking advantage of the intrinsic nuclear localization signal (NLS) of phytochrome interacting factor 3 (PIF3), synthetic transcription factors fused to PHYB with a nuclear export signal (NES) were translocated to the nucleus upon illumination with 660-nm light. The transcription factor activity was reversed using 740-nm light, leading to PHYB dissociation from PIF3 and nuclear export of the transcription factor.

To control nuclear export of proteins initially residing in the nucleus, Niopek et al. [123] developed a reversible light-inducible nuclear export system using an AsLOV2-caged NES. An N-terminal NLS was also introduced into the construct to allow for nuclear accumulation prior to illumination. In mammalian cell culture, blue light exposure uncaged the NES, allowing it to interact with the endogenous nuclear export receptor, CRM1, to mediate nuclear export of the construct through nuclear pores. Harnessing this mechanism, the authors created a chromatin-anchored light-inducible nuclear export system variant through fusion with histone H2B to sequester CRM1 and reversibly inhibit endogenous nuclear export. Similarly, Yumerefendi et al. [128] introduced a light-inducible nuclear exporter (LINX) by caging a super-PKI-2 NES within the Jα helix of AsLOV2. NLS sequences with varying strengths were used to direct the proteins to nuclei in the dark. The tools developed by both groups showed rapid export–import kinetics with half-lives of seconds to minutes.

Control of gene expression

The Cre–LoxP system is commonly used to alter gene expression. A split Cre recombinase has been used with CRY2–CIBN to control gene expression with high spatiotemporal resolution in cell cultures [41], *Drosophila* [153], and mice [116]. This photoactivatable split Cre recombinase (PA-Cre 1.0) was constructed by fusing CRY2 and CIBN to the N- and C-half, respectively. A second-generation PA-Cre (PA-Cre2.0) was generated based on a CRY2 mutant, L348F, a slow cycler with a dissociation half-life of 24 min [63]. Compared with PA-Cre1.0, PA-Cre2.0 provided a five-fold increase in the dynamic range. Using Magnets, Kawano et al. [124] also engineered a light-inducible split Cre recombinase system, where an nMag was fused to an N-Cre and a pMag was fused to a C-Cre. Because Magnets have slow photocycle kinetics (half-life 1.8 h), this split Cre system enabled DNA recombination in cells and in mice with a 30-s blue light pulse illumination.

Achieving simultaneous targeted transcriptional activation of multiple endogenous genes to perturb cells has been technically challenging. Two independent groups concurrently addressed this challenge by combining the versatility of the CRISPR–Cas9 system with optobiology to create similar light-activated CRISPR–Cas9 systems [118,119]. In both designs, a

catalytically inactive dCas9 fused to CIB1/CIBN served as a customizable genomic anchor; a fusion containing CRY2PHR and a transactivation (TA) domain (P65/Vp64) was the activator. Blue light recruited the freely diffusive activator to the genome anchor, which constitutively occupied the promoter region of target genes. Both research groups demonstrated spatially defined gene expression that correlated with the spatial pattern of illumination. Importantly, both groups achieved multiplexed photoactivation of genes in cultured cells using multiple sgRNAs to target these different genes simultaneously. Also by using the CRY2–CIBN system, Chan et al. [120] achieved spatiotemporal control of gene expression in both embryonic and adult *Drosophila* tissues. By using two-photon excitation, the authors restricted gene expression to a 20-μm region of interest, demonstrating excellent spatial control.

Transactivation domains commonly used in cultured cells could lead to toxicity when applied in certain multicellular organisms such as zebrafish. Reade et al. [117] utilized a more tolerable KalTA4 TA domain to develop a light-inducible gene expression system for use in zebrafish embryos. The authors fused the KalTA4 TA to EL222, a naturally occurring LOV-based transcription factor, to create a light-gated transcription factor (TAEL). Using TAEL, a single 2-min blue light pulse was sufficient to drive light-specific expression, and the system inactivated within 30 min after light was turned off. The authors demonstrated spatial control of TAEL by generating ectopic endoderm from ectoderm by driving Sox32 expression locally in the presumptive ectoderm. They then used TAEL to dissect the distinct roles of nodal signaling in early development (mesendoderm specification) and later development (left–right axis patterning).

Control of epigenetic states

Konermann et al. developed a modular two-component optogenetic system to facilitate control of gene transcription and epigenetic states in mammalian cells [121]. The first component is a genomic anchor generated by fusion of a customizable DNA-BD to CRY2. The second component is an effector domain fused to CIB1. Blue-light rendered the association of CRY2 and CIB1 and recruited the effector domain to the target region enabling transcriptional repression or activation, depending on the nature of the effector. The modular nature of these light-inducible transcriptional effectors (LITEs) allows for flexibility in the DNA-binding region as well as the effectors thereby enabling a multitude of genomic or epigenetic modifications. Epigenetic mark-modifying LITEs were developed by including histone effectors in LITE to be used for locus-specific histone modification.

Controlling the subcellular localization of epigenetic modifiers is another powerful way to interrogate epigenetic states. The LINXa4 construct discussed earlier was used by Yumerefendi et al. [128] to control a histone modifying E3 ubiquitin ligase Bre1, which monoubiquitylates (ub1) the histone H2B in yeast and promotes transcription. Fusing an NLS-silenced Bre1 to LINXa4 in a BRE1 deletion strain, they demonstrated light-induced reversible loss of H2Bub1, which occurred within minutes. The kinetics of the accompanying H2Bub1-dependent histone modifications such as tri-methylation of lysine 4 on histone H3 and tri-methylation of lysine 79 on histone H3 were monitored, where the latter was observed to be much slower than the former, providing mechanistic insights into these epigenetic processes.

Reis et al. developed a method called chemo-optical modulation of epigenetically regulated transcription to generate a blue light-dependent histone deacetylase (HDAC) inhibitor [126,127]. The authors amalgamated a fast-relaxing photochromic ligand para-methyl red (DABCYL) with an ortho-amino anilide HDAC inhibitor that targets the catalytic zinc in the HDAC active site *via* electrostatic interactions. Blue light promoted *trans–cis* photoisomerization of DABCYL, which transmitted the electronic alteration to the attached zinc-chelating anilide for HDAC inhibition.

Genome editing

Attaining spatiotemporal and reversible control over genome editing with minimal cell invasiveness is a challenging goal. Nihongaki et al. [125] used magnets to develop a photoactivatable RNA-guided endonuclease Cas9 (paCas9) for applications in CRISPR-mediated genome editing. A split pair of Cas9 (N713, C714) was fused to pMag and nMag, respectively, creating paCas9–1. This construct showed no reduction in specificity compared to full-length Cas9 in homology-dependent repair assays and could cleave endogenous mammalian genomic loci with 60% efficiency of that of Cas9. PACas9 could also be used in targeted genome editing *via* homology-dependent repair when a donor repair template was supplied, with an incorporation frequency of 7.2%. Moreover, two additional functionalities were developed using paCas9–1: first, a single point mutation D10A converted paCas9–1 from an endonuclease to a nickase, which could reduce off-target genome modifications by making Cas9 activity dependent on a pair of guide RNAs instead of one; second, two point mutations D10A and H840A abolished Cas9 cutting activity, while retaining its targeting activity, which could be used to sterically block the transcription of a target-gene in an application termed CRISPR interference, which was also demonstrated with luciferase reporters. Similarly, Reade et al. [117] used TAEL to drive Cas9 expression in zebrafish, which led to tyrosinase gene disruption, demonstrating the CRISPR integration of TAEL for gene editing in multicellular organisms.

Using a photopharmacological strategy, Hemphill et al. [92] engineered two Cas9 variants with photo-caged

lysines at positions K163 and K866, which could be spatiotemporally activated with UV light. The photocaged Cas9 variants were completely inactive in the dark and achieved wild-type activity with 120 s of exposure to 365-nm light. Optical control of CRISPR/Cas9 was used to silence the endogenous transmembrane transferrin receptor, CD71, in HeLa cells.

Challenges and emerging new technologies

A major challenge in optobiology, both in basic sciences and in clinical research, is light delivery. Most photoactivatable proteins respond to visible light. The penetration depth for red light in biological tissues is around 0.2–2 mm [154]. Furthermore, as the light wavelength decreases, its penetration depth is reduced, severely limiting the use of visible light-responsive photoactivatable modules in deep tissues. Most *in vivo* optobiological applications have required the insertion of optical fibers to deliver visible light. This approach introduces a two-fold challenge: fibers impede free movement of the tethered animal and the insertion procedure may cause an inflammatory response in tissues.

Recent developments in nanotechnology have provided soft, stretchable, and implantable microLEDs that allow for wireless optobiology. These devices use miniaturized light-emitting diodes (LEDs) that are powered by radio-frequency scavengers [155–157]. To improve the chronic biocompatibility and integration with soft neuronal tissues and to use these devices in challenging areas such as the spinal cord and peripheral nervous system, fully implantable internal devices were made [158]. An improved multi-functional device, which included both optogenetic and microfluidic channels, allowed for the co-delivery of light and pharmacological reagents [159]. Organic LEDs have also been designed for high-resolution optogenetics [160] (Fig. 3A).

Parallel to the development of microLEDs, researchers used upconversion nanoparticles (UCNPs) to convert NIR light (800–980 nm) to visible light, which shifted the spectrum of illumination toward the NIR wavelength [161,162]. IR light has a higher penetration depth (~3–4 mm) than visible light [154]. For small animals, such as mice, this penetration depth can greatly help light reach deeper brain regions (Fig. 3B). However, it remains challenging for IR light to penetrate deep brain regions of larger animals including humans. Biocompatible UCNPs have been used in photodynamic therapy in deep tissues [163]. Recent

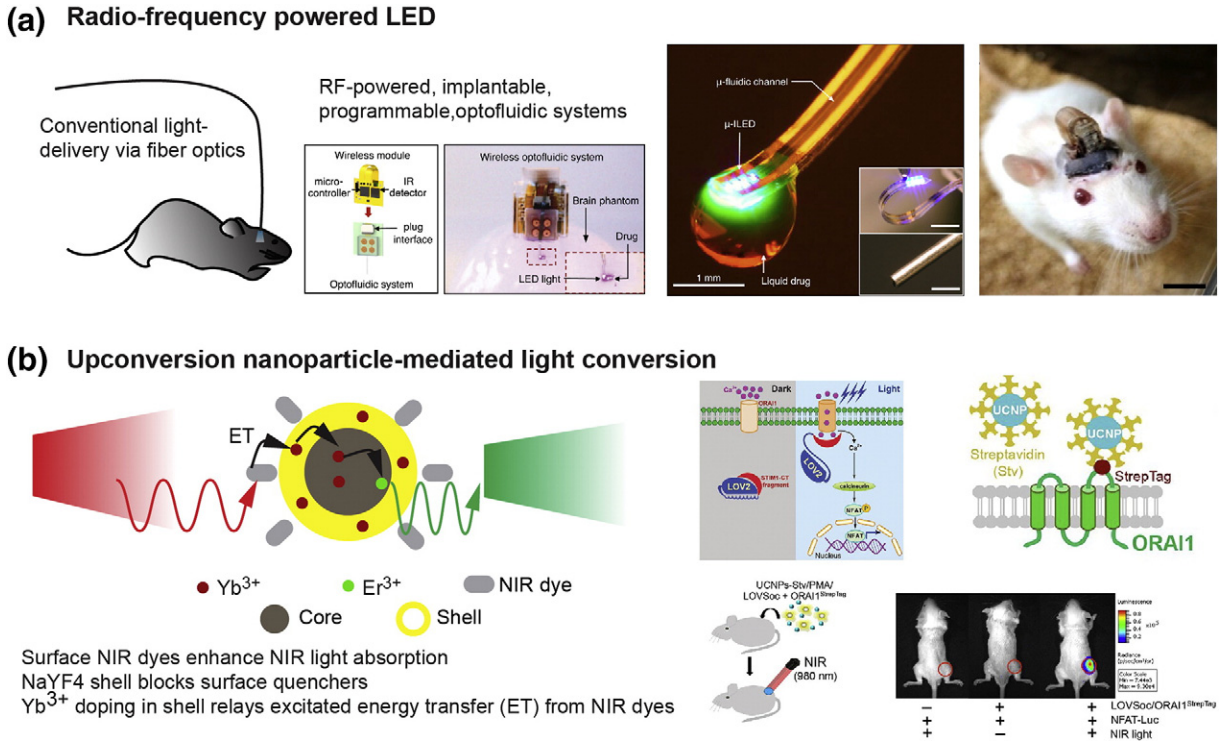

Fig. 3. Emerging technologies for achieving wireless optobiological control in multicellular organisms. (A) Wireless optobiology based on radio-frequency powered microLEDs. By integrating light source and microfluidic channels, these multiple-functionality devices can light up deep tissues in live animals and deliver pharmacological reagents. Reprint with permission from Jeong et al. [159] (B) UCNPs convert NIR light, which has deeper penetration depth, to visible light through energy transfer from excited, surface-bound NIR dyes to the cores of UCNPs. The output visible light can be used to excite photoactivatable proteins. Reprint with permission from He et al. [96].

proof-of-principle work showed that Er/Yb UCNPs coated with IR-sensitive dyes can be used to achieve optogenetic stimulation with 980-nm light in cell culture [164,165], *C. elegans* [166], and mice [96].

Outlook

Using photoactivatable moieties derived from nature, optobiology utilizes photoactivatable molecules in conjunction with proteins of interest to genetically engineer live cell systems. Parallel to the emerging field of synthetic biology, optobiology offers attractive approaches to customize cell and tissue responses. Orthogonal and multiplexed control of signaling circuits would help delineate endogenous signaling mechanisms. In addition, novel functionality can potentially be created by exerting light control over proteins that normally do not interact with each other. From a molecular perspective, successful design of an optobiological system relies on insights from both molecular modeling and experimentation. Although several optobiological modules are available to choose from, benchmarking experiments have revealed that tool efficacy varies depending on the cellular context and application [74,167]. In general, trial and error investigation to identify the appropriate tool is often required. Reproducible strategies to insert, remove, modulate, or split modules in target proteins call for the development of universal design principles and utilities.

Beyond the bench, optobiology has also been applied in pre-clinical research using live animals. Recent work demonstrated a cell-based therapy that made use of smartphones to induce secretion of insulin *via* an optogenetic approach [105]. In this work, optogenetically engineered cells that allowed for light-triggered release of glucose-lowering hormones were implanted in diabetic mice. The implanted cells responded to far-red light, which could be wirelessly controlled by a smartphone. Mouse insulin produced *in vivo* was able to maintain glucose homeostasis over several weeks in the diabetic mice. With superior spatiotemporal resolution, minimal invasiveness, and reversible operation, optobiological techniques promise to usher in a new era of molecular biology and biomedical research.

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Abbreviations used:
FGF, fibroblast growth factor; ERK, extracellular signal-regulated kinase; BDNF, brain-derived neurotrophic factor; AsLOV, *Avena sativa* light-oxygen-voltage; TULIPs, tunable light-controlled interacting protein tags; LIDs, light-inducible dimers; LOVpep, peptide epitope attached to AsLOV2 Jα helix; iLID, improved light-inducible dimers; LOVTRAP, LOV2 trap and release of protein system; Zdk, Zdark; AUREO1, Aureochrome-1; bZIP, basic region/leucine zipper domain; RTK, receptor tyrosine kinase; VVD, vivid; pdDronpa1, photodissociable dimeric Dronpa; CRY2, Cryptochrome 2; CIB1, cryptochrome-interacting basic-helix–loop–helix 1; CRY2PHR, CRY2 photolyase homology region; CIBN, CRY2 N-terminus; PhyB, *Arabidopsis thaliana* phytochrome B; BphP1, *Rhodopseudomonas palustris* bacterial bathy phytochrome; dRap, dimerized rapamycin; GLP-1R, glucagon-like peptide-1 receptor; PhotoETP, photoswitchable moiety for light-mediated allosteric control of GLP-1R; STIM1, stromal interaction module 1; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphatidylinositol 3-kinase; GEFs, guanine nucleotide exchange factor; SH2, Src homology domain; BD, binding domain; GSV, GLUT4 storage vesicles; PTS, peroxisomal-targeting sequence; JNK, Jun N-terminal kinase; PIF3, phytochrome interacting factor 3; NLS, nuclear localization signal; NES, nuclear export signal; LINX, light-inducible nuclear exporter; PA-Cre 1.0, photoactivatable split Cre recombinase; TAEL, TA EL222; LITE, light-inducible transcriptional effectors; ub1, monoubiquitin; HDAC, histone deacetylase; paCas9, photoactivatable Cas9 nuclease; LED, light emitting diodes; UCNP, upconversion nanoparticles; NIR, near-infrared; IR, infrared.

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