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Illuminating developmental biology through photochemistry

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Abstract

Developmental biology has been continually shaped by technological advances, evolving from a descriptive science into one immersed in molecular and cellular mechanism. Most recently, genome sequencing and “omics” profiling have provided developmental biologists with a wealth of genetic and biochemical information; however, fully translating this knowledge into functional understanding will require new experimental capabilities. Photoactivatable probes have emerged as particularly valuable tools for investigating developmental mechanisms, as they can enable rapid, specific manipulations of DNA, RNA, proteins, and cells with spatiotemporal precision. In this perspective, we describe optochemical and optogenetic systems that have been applied in multicellular organisms, insights gained through these probes, and their current limitations. We also suggest how chemical biologists can expand the reach of photoactivatable technologies and bring new depth to our understanding of organismal development.

How cells organize themselves into tissues and organs has intrigued scientists since Hippocrates and Aristotle began studying chicken embryogenesis over two millennia ago. Throughout its rich history, developmental biology has taken advantage of continual technological advances to gain new conceptual ground. When theory has significantly outpaced experimental capability, the discipline has lain in wait. For example, embryologists debated whether gametes contained preformed organisms until microscopes with sufficient resolving power were invented. Embryonic induction was first demonstrated after the introduction of micro-surgical methods, and developmental biology became a molecular science through organismal mutagenesis screens and positional cloning.

Modern developmental biology now faces a similar moment of reflection and inflection. Genome sequencing and “omic” approaches have helped to unveil the molecular complexity of tissue formation, homeostasis, and regeneration. Yet paradoxically, these recent advances evoke the descriptive past, with genome-wide parts lists and profiles replacing the hand-drawn illustrations by early embryologists. While correlations within these “omic” landscapes can inspire new hypotheses, translating this wealth of information into

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mechanistic understanding remains a major challenge. In addition to knowing which genes are required to form a specific tissue, we must determine how the proteins they encode function in space, in time, and with each other.

Achieving this next level of insight will require new scientific approaches. The glass needles and baby hairs wielded by early embryologists have been now largely replaced by molecular tools. Genomes have been altered through chemical mutagens, homologous recombination, and various editing tools. Transcripts have been suppressed through RNA interference and anti-sense oligonucleotides, and proteins have been modulated with small molecules and antibodies. Yet these modern technologies still cannot match the spatial and/or temporal scales of developmental processes, and as a result, their utility for testing morphogenetic models is limited. Chemical biologists can help bridge this theory-capability gap, empowered by their ability to create synthetic systems with unique biophysical properties. In particular, photoresponsive compounds and proteins are versatile tools for probing tissue patterning mechanisms *in vivo*, as they can be actuated within seconds and with single-cell resolution. These technologies promise to transform our models of tissue morphogenesis, synergizing with recent advances in imaging and computational modeling. We describe here how photoactivatable systems have been applied in multicellular organisms and lessons learned from these studies. We also discuss how chemical biologists can help advance optical technologies and catalyze the next renaissance of developmental biology.

Translating light into molecular action

Optical control is an attractive strategy for studying developmental biology, as light pulses can be delivered within the micrometer and sub-millisecond scales that bound most patterning processes. Natural evolution has produced a myriad of photoresponsive proteins, many of which can be re-engineered toward new functions, and chemists have added to this repertoire of light-sensitive molecules over the past 200 years. These natural and synthetic molecules have provided scientists with both inspiration and building blocks for new tools. We summarize here several key photoresponsive systems that have given rise to the optochemical and optogenetic technologies discussed in this Perspective.

Natural photoreceptors include G protein-coupled receptors (GPCRs) such as rhodopsin and other opsins, which translate the photoisomerization of 11-*cis* retinal to the all-*trans* state into visual signaling cascades (Fig. 1a)¹. Archaeal microbes and algae use rhodopsin channels and pumps to transport ions across membranes, driven by the light-induced conversion of all-*trans* retinal into the 13-*cis* conformer². Repurposing of the latter proteins to control neuronal function has revolutionized neuroscience research. These advances have been reviewed extensively and will not be discussed here^{3,4}.

Phytochromes are used by bacteria, cyanobacteria, fungi and plants to respond to changes in light (Fig. 1b)⁵. These dimeric proteins are composed of an N-terminal conserved photosensory module with a covalently linked tetrapyrrole chromophore (predominantly biliverdin and phytychromobilin in microbes and plants, respectively) and a variable C-terminal signaling domain. The heme-derived cofactor interconverts between red light- and far-red light-absorbing states via photoisomerization of the C15-C16 double bond, which re-

orients the vinylpyrrolone ring and alters signaling activities. For example, bacterial phytochromes typically regulate C-terminal histidine kinases, and the photosensory module in *Arabidopsis thaliana* phytochrome B (PHYB) binds to several nuclear factors (e.g., phytochrome-interacting factors PIF3 and PIF6) when illuminated.

Three additional classes of photoreceptors utilize flavins, which are protein-bound in either mononucleotide (FMN) or adenine dinucleotide (FAD) states. First, bacteria, algae, and fungi use BLUF (blue light receptor using FAD) sensors fused to an adenylate/guanylate cyclase or phosphodiesterase (Fig. 1c)⁶. When irradiated, the FAD cofactor undergoes electron and proton transfer to form a neutral radical, altering its hydrogen-bonding interactions with neighboring residues and triggering enzyme activity. Second, plants rely on cryptochromes to entrain their circadian rhythms to daily light-dark cycles (Fig. 1d)⁷. These proteins are closely related to DNA photolyases but lack DNA binding or repair activities. Blue-light stimulation of cryptochromes leads to the 5,10-methenyltetrahydrofolate-assisted semireduction of FAD, resulting in conformational shifts that allow the recruitment of downstream effectors. For instance, the *Arabidopsis* cryptochrome CRY2 interacts with the transcription factor CIB1 and the E3 ubiquitin ligase COP1 in a light-dependent manner. Finally, both plants and microbes use photoreceptors composed of light-oxygen-voltage sensitive (LOV) domains (Fig. 1e)^{8,9}. These modules bind to flavin in its FMN and/or FAD state, and upon blue-light excitation, they form a cysteine-flavin adduct and undergo structural changes. For example, light activates the *Avena sativa* phototropin PHOT1 by unfolding a regulatory helix (Ja.) linking its LOV2 and kinase domains¹⁰.

Man-made photoresponsive molecules were first developed in the 1800s, as chemists pursued the synthesis of benzene derivatives. The ability of azobenzene to adopt *Z* and *E* conformations upon ultraviolet (UV)- and blue-light irradiation, respectively (Fig. 2a), has been exploited to create photo-switchable polymers and other synthetic materials^{11,12}. More recently, azobenzene-containing molecules have been developed that enable optical control of nucleic acid and protein function¹³. Azobenzenes that have red-shifted absorbances and varying rates of relaxation to the *E* state have also been synthesized, expanding the versatility of these tools¹⁴.

Other synthetic molecules undergo high-yielding irreversible reactions upon exposure to light. Biologically functional molecules can be caged by the covalent attachment of these compounds and then activated through bond photolysis. The most commonly used cages are 2-nitrobenzyl derivatives, originally conceived as protecting groups for chemical synthesis (Fig. 2b)¹⁵. These moieties are typically introduced through ester, carbonate, or carbamate linkages and contain a benzylic C–O bond that cleaves upon UV irradiation. As chemists began to delve into biological mechanisms, 2-nitrobenzyl moieties were used to cage biomolecules such as ATP and glutamate^{16,17}. Photocleavable groups based on 7-nitroindoline, coumarin, amino-1,4-benzoquinone, and cyanine chromophores have since been developed (Fig. 2c–d), enabling one- or two-photon uncaging at longer wavelengths^{18–20}.

Photoactivatable probes of cellular function

Caged fluorophores were among the first photoactivatable molecules to be applied *in vivo*. Developmental biologists had traditionally used lipophilic, non-diffusible dyes (e.g., the long-chain dialkylcarbocyanines DiO and DiI) to label specific cell populations for fate mapping studies. This procedure typically involves manual pressure- or electrical current-mediated delivery, making it challenging to implement as development proceeds and individual cells become smaller and less accessible. This practical limitation was bypassed by the synthesis of 5-carboxymethoxy-2-nitrobenzyl-functionalized forms of fluorescein and Q rhodamine, which are non-fluorescent until the caging groups are photolytically removed^{21,22}. When conjugated to high-molecular-weight dextrans to prevent their diffusion and then microinjected into early-stage embryos, these probes can be used to label cells of interest at later stages by focal UV illumination (Fig. 3a). Genetically encoded photoactivatable and photoconvertible fluorescent proteins have further expanded the palette and utility of lineage-tracing tools^{23,24}, facilitating probe delivery at later developmental stages and enabling the targeting of specific cell types through *cis*-regulatory elements. For example, the green-to-red fluorescent protein Kaede has been applied in zebrafish to follow retinal cell movements during eye morphogenesis (Fig. 3b–c)²⁵, and avian neural crest development has been tracked with the blue-to-green marker psCFP2 (photoswitchable cyan fluorescent protein)²⁶. Cell signaling histories can also be mapped with photoconvertible proteins, as demonstrated by studies of zebrafish spinal cord development using a Hedgehog pathway-dependent Kaede reporter²⁷.

In addition to visualizing cells, fluorophore photoexcitation has been used to ablate specific populations, taking advantage of the triplet state-dependent generation of singlet oxygen and other reactive species. One of the first examples of this approach utilized fluorescein di- β -D-galactopyranoside, a non-fluorescent compound that is selectively hydrolyzed by β -galactosidase to liberate fluorescein²⁸. Tissues engineered to express β -galactosidase in specific cell types can be treated with this reagent and then irradiated with blue light in the presence of a singlet oxygen amplifier such as 3-amino-9-ethylcarbazole. The resulting cytotoxic products ablate the β -galactosidase-positive cells without damaging neighboring populations. Genetically encoded fluorophores that efficiently generate reactive oxygen species have also been developed. Membrane-bound KillerRed, a mutated form of the hydrazoan green fluorescent protein (GFP)-like protein anm2CP (Anthomedusae non-fluorescent chromoprotein-like), has been coupled with green-light illumination to induce apoptosis within the developing eye and pronephric kidney in *Xenopus* embryos²⁹. miniSOG (mini Singlet Oxygen Generator), an engineered LOV domain from *Arabidopsis* phototropin 2 (PHOT2), and subsequent variants such as miniSOG2 have similarly been used to photoablate cells in *Caenorhabditis elegans* and *Drosophila* (Fig. 3d–e)^{30–32}.

Photoactivatable probes of gene function

More intricate aspects of cell behavior can be optically controlled with photoactivatable tools that target specific genetic programs and signaling pathways. Light-induced genomic modifications have been achieved by re-engineering the Cre/*loxP* recombination system in two different ways. In one approach, the Cre recombinase is fused to the estrogen receptor

T2 variant (ER^{T2}), causing it to be sequestered in the cytoplasm by the Hsp90 chaperone machinery³³. A 4,5-dimethoxy-2-nitrobenzyl-caged form of the ER^{T2} agonist 4-hydroxycyclofen is then used to induce the nuclear translocation of Cre-ER^{T2} upon UV irradiation, promoting recombination of *loxP*-flanked genomic sites in the illuminated cells (Fig. 4a–b). This method has been used to activate Cre/*loxP* recombination in retinal and somitic cells of zebrafish embryos³³, as well as in skin and mammary gland cells of mice³⁴. An alternative strategy utilizes the CRY2-CIB1 system to regulate Cre recombinase activity (Fig. 4c)^{35,36}. The Cre protein is split into inactive N- and C-terminal fragments, which are respectively fused to CRY2 and CIB1 (either the full-length proteins or truncated minimal domains). Blue-light illumination then induces CRY2-CIB1 heterodimerization and reconstitutes split Cre activity. This optogenetic version of Cre recombinase has been applied in *Drosophila melanogaster* larvae, and two-photon excitation with infrared light has enabled its implementation in the mouse brain³⁷. Light-induced split protein reconstitution has also been employed to control CRISPR (clustered, regularly interspaced, short palindromic repeat)/Cas9-mediated genome editing in cultured cells³⁸, although *in vivo* applications of this technology have not yet been reported. In this approach, N- and C-terminal fragments of the Cas9 protein are individualized fused to VIVID LOV domain mutants designed to form obligate heterodimers (named “magnets” pMag and nMag³⁹). Interestingly, the same study found the CRY2/CIB1 system to be ineffective at reconstituting split Cas9 activity.

Photoactivatable transcription has been accomplished using several of the major optochemical and optogenetic platforms reported to date. For example, 4,5-dimethoxy-2-nitrobenzyl-caged doxycycline derivatives have been combined with the tetracycline transactivator system to attain light-gated transcription in *Xenopus* and mouse embryos⁴⁰. The PHYB/PIF, CRY2/CIB1, and VIVID LOV dimerization systems can be employed to create transcriptionally active complexes by assembling DNA-binding and activation domains from various proteins (Fig. 4d)^{35,41–46}. In addition, the LOV domain-containing transcription factor EL222 has been fused with heterologous activation domains to achieve light-dependent gene expression in zebrafish embryos^{47,48}. This bacterial photoreceptor is composed of LOV and DNA-binding domains that are connected through a regulatory J α helix, and the monomeric dark state is sterically occluded from complexing DNA. Blue-light illumination leads to J α helix unfolding, allowing EL222 to form a transcriptionally active homodimer, and thermal reversion follows within seconds. Such optogenetic tools have been used to compare the effects of sustained and oscillatory Notch pathway activation in cultured murine neural progenitors and to induce endodermal cell fates in zebrafish embryos^{48,49}.

Optical strategies for controlling RNA function have relied upon synthetic oligonucleotides, especially those containing methylenemorpholine- and phosphorodiamidate-based backbones⁵⁰. These “morpholino” oligonucleotides (MOs) are typically designed to target 25-base regions that overlap with or are adjacent to the start codon or intron-exon junctions in the targeted transcript, thereby suppressing RNA translation or splicing, respectively (Fig. 4e). Several approaches for caging morpholino activity have been developed, including the use of hairpin, duplex, base-modified, and cyclic structures to suppress MO/RNA hybridization^{51–55}. Each of these MO configurations is maintained by a photocleavable linkage, containing either a nitrobenzyl derivative for UV light-mediated activation or a functionalized coumarin for longer wavelengths⁵⁶. Caged MOs (cMOs) have been applied in

zebrafish embryos to uncover the dynamic, tissue-specific activities of several transcription factors, such as the T-box protein No tail-a (Ta), Tbx16/Spadetail, and E26 transformation-specific variant 2 (Etv2) (Fig. 4f)⁵⁷⁻⁶⁰.

Photoactivatable probes of protein function

Methods for regulating protein activity with light have been the most challenging to develop, due to the structural and functional diversity of polypeptides. An early attempt to achieve photo-control of protein function involved chromophore-assisted laser inactivation (CALI)⁶¹. In this technique, malachite green-conjugated antibodies that recognize the protein of interest are delivered to the target and then illuminated with a 620-nm laser to locally generate protein-inactivating free radicals. CALI was originally used to study signaling proteins in insect embryos, but it has not been widely applied to other model organisms. More recent photoresponsive technologies act through more selective mechanisms and can be grouped into three major categories: (1) optochemical modulators; (2) extrinsic optogenetic modulators of general protein function; and (3) intrinsic optogenetic modulators of specific protein activities.

The first category exemplifies the diverse ways that small molecules can respond to light. It includes caged inhibitors such as a Rockout, a nitropiperonyloxymethyl-functionalized Rho kinase inhibitor that was used to uncover left-right differences in *Xenopus laevis* gut patterning⁶², and caged proteins such as a 4,5-dimethoxy-2-nitrobenzyl-modified Gal4-VP16 transactivator that was applied in *Drosophila*, *Xenopus*, and zebrafish embryos⁶³. Endogenous photoresponsive molecules such as retinoic acid can be utilized as well. For example, retinoic acid signaling in zebrafish embryos has been controlled with light by chemically suppressing retinoic acid biosynthesis and introducing exogenous 13-*cis* metabolite (Fig. 5a)⁶⁴. Local UV illumination was then used to produce the all-*trans* isomer in anterior tissues, restoring retinoic acid receptor-dependent patterning of the hindbrain. Photoisomerization of synthetic compounds has similarly been exploited to control protein function, exemplified by the modulation of ion channels and GPCRs with azobenzene-functionalized ligands (Fig. 5b-c)⁶⁵⁻⁶⁷. These photochromic probes have imparted switchable neuronal activity in wild type zebrafish, mouse, and rat models, complementing the application of channelrhodopsins and halorhodopsins in transgenic organisms. Another intriguing example of ligand-based optical control is optovin, a photoactivatable agonist of the transient receptor potential A1 (TRPA1) channel (Fig. 5d)⁶⁸. Zebrafish treated with this rhodanine derivative exhibit light-dependent, reversible activation of sensory neurons and motor behaviors. Although the mechanistic details of optovin action remain unclear, its excited state may form a reversible, covalent bond with TRPA1, reminiscent of the cysteine-flavin adduct in light-activated LOV domains.

Optogenetic regulators of protein function can act through either extrinsic (general) or intrinsic (target-specific) mechanisms. The predominant strategy for the first objective has been to employ dimeric photoreceptor systems to alter protein localization. For example, the N-terminal PHYB photosensory domain fused to a CAAX prenylation site and PIF6 fused to Partitioning defective 3 (Pard3) have been used to regulate the membrane localization of this

apical polarity protein in zebrafish embryos⁶⁹. For these studies, phycocyanobilin (a close analog of phytochromobilin) was co-microinjected with *PHYB-CAAX* and *Pard3-PIF6* mRNAs into the zygote, since this chromophore is not synthesized by animal cells or readily taken up from embryo culture medium. The CRY2/CIB1 platform has been repurposed to recruit the catalytic domain of OCRL, an inositol polyphosphate 5-phosphatase, to the plasma membrane in *Drosophila* embryos (Fig. 6a–b)⁷⁰. In these experiments, blue light-induced recruitment of OCRL reduced PI(4,5)P₂ levels, disrupting actin-dependent apical constrictions that are required for ventral furrow formation and mesoderm invagination. As a third approach, the PHOT1 LOV2 domain has been engineered to embed peptide ligands for specific protein receptors within the J α helix (Fig. 6c)^{71–73}. These protein-binding motifs are sterically occluded in the LOV2 dark state and then liberated as the C-terminal helix unfolds upon blue-light illumination. This method has been applied in *C. elegans* to control nuclear localization of the transcription factor LIN-1/ETS⁷⁴, a key regulator of vulva development, and in *Drosophila* embryos to recruit the Son of Sevenless (SOS) catalytic domain to the plasma membrane, thereby activating Ras/Erk signaling⁷⁵.

Another general mechanism for regulating protein activity is the control of its degradation. Similar to the peptide-masking strategy described above, the PHOT1 LOV2 domain has been used to convey light-dependent proteolysis by incorporating a “degron” sequence into the J α helix⁷⁶. Proteins fused with the modified LOV2 domain are expressed at normal levels in the dark, since the degron is maintained in a cryptic state. Blue light-induced unfolding of the J α helix then exposes the degron and allows its recognition by ubiquitin-proteasome machinery. When expressed in zebrafish embryos, LOV2 fusion proteins could be significantly depleted after two hours of blue-light illumination. Other LOV domain-containing proteins, such as the *Arabidopsis* photoreceptor ZEITLUPE (ZTL) and its homologs, mediate the blue light-dependent degradation of circadian factors⁷⁷, suggesting that synthetic photoactivatable systems that regulate protein stability in *trans* may also be possible.

Achieving optogenetic control of intrinsic protein activity is significantly more daunting task. In some cases, a photoactivatable form of the desired activity has already evolved in bacteria, algae, or other microbes, allowing it to be utilized heterologously in multicellular organisms. Light-inducible adenylate cyclases from the unicellular flagellate *Euglena gracilis* and the *Beggiatoa* species of bacteria fall in this category, and these BLUF domain-containing proteins have been shown to be active in *Drosophila* embryos and *Xenopus* oocytes^{78,79}. More often than not, however, coupling photoresponsive modules to specific protein functions requires knowledge of the target's structure and biochemical mechanism. Adenylate cyclase and phosphodiesterase enzymes that are activated by red light have been developed in this manner^{80,81}. In both cases, the photosensory module from a bacterial phytochrome was fused to the corresponding enzyme, using helical extensions analogous to those connecting the photosensory and histidine kinase domains in the wild type photoreceptor. By expressing the photoactivatable adenylate cyclase in the cholinergic neurons of *C. elegans*, red light-inducible movement was observed. Red light-activated cAMP hydrolysis was similarly achieved by expressing the photoactivatable phosphodiesterase in zebrafish embryos.

LOV domains have been especially versatile platforms for protein engineering, due to their relatively small size and divergent structures outside of the flavin-binding site. A photoactivatable form of the small GTPase Rac1 was created by fusing the PHOT1 LOV2 domain and its J α helix to a constitutively active Rac1 mutant (Fig. 6d)⁸². In its dark state, the LOV2 domain sterically blocks the binding of Rac1 to downstream effectors, and light-induced unfolding of the J α helix liberates Rac1 function. Since Rac1 is a key regulator of directed cell movement, cells expressing this chimeric protein exhibit phototaxis toward blue light. Accordingly, photoactivatable Rac1 has been used to study neutrophil motility in zebrafish embryos and the collective migration of border cells in the *Drosophila* ovary^{83,84}. Photoactivatable growth factor receptors have also been constructed by removing their extracellular regions and coupling myristoylated versions of their intracellular signaling domains to the LOV module from *Vaucheria frigida* aureochrome1 (AUREO1), which homodimerizes upon blue-light illumination^{85,86}. This approach has been applied to members of the receptor tyrosine kinase family, including fibroblast growth factor receptor 1 (FGFR1), epidermal growth factor receptor (EGFR), and rearranged during transfection (RET). Surprisingly, six other dimerizing LOV domains proved to be ineffective for this application. The *V. frigida* AUREO1 LOV domain has also been combined with kinase domains from the type I and II activin B receptors, Acvr1b and Acvr2b, which mediate Nodal signaling (Fig. 6e–f)⁸⁷. By expressing these constructs in zebrafish embryos and irradiating cells within the embryonic shield, it was determined that Nodal pathway activation within this dorsal organizing center promotes prechordal plate and suppresses endoderm cell fates.

Emergent themes in optobiology

The examples described above illustrate our rapid progress toward new photoactivatable technologies and how these tools could transform the way we study development. Certain themes emerge from these studies, providing valuable lessons and goals for future investigations. For example, one immediate distinguishing feature among the myriad of approaches is whether they employ synthetic reagents, genetically encoded probes, or both. Optochemical strategies for a specific biological application have often preceded their optogenetic counterparts. Caged fluorescein was invented before photoactivatable or photoconvertible fluorescein proteins, and azobenzene-functionalized channel modulators were used to control neuronal activity before channelrhodopsins. This sequence of events reflects the facility with which chemists can synthesize new compounds that are not limited to nature's building blocks. It also reflects the efficiency with which chemical probes can be used to interrogate biological systems. Embryos can be cultured or directly injected with synthetic agents to obtain highly penetrant perturbations, and wild type animals are suitable for many optochemical experiments. In comparison, creating transgenic embryos for optogenetic studies can take days (e.g., worms) or months (e.g., mice and zebrafish), and these models are subject to Mendelian phenotypic distributions.

It is worth noting, however, that photoresponsive fluorescent proteins and channelrhodopsins have been more extensively utilized in biological studies than caged fluorescein and azobenzene-gated ligands. The preferential embrace of optogenetic technologies by the biological community does not detract from the value of optochemical tools. For example,

cell fate mapping with caged fluorescein dextran can be conducted by visualizing fluorescein emissions or immunostaining the deprotected fluorophore, whereas analogous studies with photoactivatable GFP are limited to fluorescence imaging. The application of caged morpholinos to directly control RNA function does not yet have an optogenetic alternative, and photoswitchable chemical modulators enable control of endogenous protein activities. Nevertheless, optochemical approaches face obstacles that hinder their widespread use. Most laboratories engaged in biological research lack the expertise and resources to prepare optochemical probes, while optogenetic tools can be obtained, re-engineered, and replenished with standard molecular biology techniques. As a result, the accessibility of these synthetic reagents is largely contingent on their commercialization. The delivery of optochemical reagents into developing organisms can also be challenging, especially at later developmental stages. Certain molecules must be microinjected into early-stage embryos, and others may exhibit non-uniform tissue distributions, unwanted diffusion, and biological instability. In contrast, optogenetic systems can be stably integrated into the genomes of model organisms and conditionally expressed in specific cells using *cis*-regulatory elements and recombinases. Finally, the transferability of chemical technologies between organisms is more encumbered than that of genetically encoded tools. An optochemical probe employed successfully in one species may be ineffective in another, due to differences in target (and off-target) structures or animal physiology, and re-designing such molecules for broader use is not always straightforward. In comparison, transferring DNA-encoded technologies between species is expedited by widely available bioinformatic and genetic engineering tools. Based on these factors, optical strategies that primarily rely upon genetically encoded components are advantageously positioned for universal use.

Yet previous studies also demonstrate that no single optogenetic platform is a universal solution. Excitation wavelengths for specific photoreceptors can preclude them from certain applications, particularly those that require fluorescence imaging. For instance, GFP reporters and flavin-based proteins are challenging to use in combination, as the blue light required to observe the biological system will also perturb it. Although all of the major optogenetic systems respond to light within seconds, their average excited-state lifetimes and mechanisms of ground-state reversion vary. After blue-light illumination, the cryptochrome excited state persists for only a few minutes, while red light-activated phytochrome family members require near-infrared illumination to return to their ground-state conformations. LOV domains and their structural variants have thermal reversion timescales ranging from seconds to days⁸⁸. Depending on the experimental application, specific light-state lifetimes or photochromic behavior may be preferred. Photoactivatable proteins with long excited-state lifetimes are useful tools for studying cell populations that cannot be continually targeted with light. Those with shorter lifetimes afford greater control of activity level and duration, and photoswitchable systems can convey even greater spatiotemporal precision. Certain excited-state lifetimes may also be required to efficiently engage the targeted signaling processes, as has been observed with the photoactivatable FGFR1 system⁸⁵.

Photoresponsive proteins are further differentiated by their divergent structures. Phytochromes and cryptochromes are significantly larger than LOV and BLUF domains, even when their minimum photosensory regions are employed, and all four photoreceptor

classes have distinct polypeptide folds. A remarkable degree of structural diversity exists within the LOV domain family alone, as sequences outside of the flavin-binding β -sheet scaffold are highly variable. Multiple optogenetic platforms can be used to regulate molecular proximity, such as the subcellular localization of signaling proteins or the assembly of transcriptionally active complexes. However, it is notable that of seven dimeric LOV domains, only the *V. frigida* AUREO1 LOV domain could successfully generate photoactivatable tyrosine receptor kinases⁸⁵, and although the CRY2/CIB1 system could be used to reconstitute split Cre recombinase^{35,36}, it was ineffective with split Cas9³⁸. The repurposing of photoreceptors to allosterically regulate protein function promises to be an even greater challenge, as exemplified by the photoactivatable Rac1 system, which was found to be profoundly sensitive to changes in PHOT1 LOV2-Rac1 connectivity⁸². Moreover, when the LOV2 domain was deployed to regulate the homologous GTPase Cdc42, the analogous chimeric construct was constitutively active, and structure-guided mutagenesis was required to achieve light-dependent function⁸².

The technological focus of these early lessons learned reflects the still-nascent state of optobiology. Most applications to date have been proof-of-concept studies. However, in a growing number of cases, optical tools have been utilized to gain new biological insights. These optical forays into experimental science highlight one final important theme: the power of photoactivatable probes to change our models of developmental biology. For example, optochemical technologies have revised our understanding of T-box transcription factor function during early vertebrate development. Zebrafish mutants lacking Tbx16/Spadetail function develop tail but not trunk somites, and their tailbuds become enlarged as mesodermal progenitor cells (MPCs) accumulate posteriorly⁸⁹⁻⁹¹. It was generally believed that Tbx16 regulates anterior-posterior fates by promoting morphogenetic cell movements during gastrulation, since *tbx16* mutant cells transplanted into wild type hosts fail to undergo dorsal convergence and accrue in the tailbud^{90,92}. In this model, the mislocalization of Tbx16-deficient cells leads to their acquisition of posterior fates. Our laboratory took advantage of the spatiotemporal control afforded by photoactivatable morpholinos to reexamine how Tbx16 regulates paraxial mesoderm patterning⁶⁰. In particular, we targeted MPCs derived from the ventral margin since these cells uniquely contribute to trunk somites without undergoing dorsal convergence⁹³, making them a convenient population for discerning Tbx16-dependent cell movement and fate choice. By specifically knocking down Tbx16 expression in ventral MPCs and assessing the transcriptional changes in these cells (Fig. 7a), we discovered that loss of Tbx16 function causes precocious expression of posterior Hox transcription factors, altering the Hox codes that establish rostrocaudal identity⁶⁰. We further demonstrated that these changes in *hox* gene expression can precede MPC mislocalization and that ectopic expression of a single posterior *hox* gene can alter MPC movement. Our findings therefore support an alternative model in which Tbx16-dependent fate choice determines cell position, rather than vice versa.

The spatiotemporal control afforded by optogenetic probes has similarly advanced our understanding of tissue patterning mechanisms. For instance, zebrafish mutants lacking the Nodal co-receptor tetracarboxy-derivative growth factor 1 fail to form endoderm and prechordal plate mesoderm, demonstrating a requirement for Nodal signaling for both cell fates⁹⁴. To determine whether the duration of Nodal pathway activity can influence cell fate

choice, the AUREO1 LOV domain-actuated Nodal receptors described above were expressed in zebrafish embryos and modulated by illumination regimens of varying lengths⁸⁷. Nodal pathway activation from 3-5 hours post fertilization (hpf) preferentially induced the expression of endodermal markers, whereas pathway induction from 3-6 hpf promoted prechordal plate specification and suppressed endoderm fates. The temporal pattern of Nodal signaling therefore is a key aspect of its embryonic function.

Optogenetic technologies have also been used to examine how the location and timing of developmental signaling impacts tissue formation. In *Drosophila* embryos, localized activation of the receptor tyrosine kinase Torso normally restricts Ras/Erk signaling to the anterior and posterior poles^{95,96}. Gain-of-function Torso mutations increase Ras/Erk pathway activity throughout the blastula, leading to body segment loss or fusion and embryonic lethality⁹⁵. These phenotypes could be due to either changes in the spatial distribution and/or level of Ras/Erk signaling, and the photoactivatable SOS system has allowed these mechanisms to be distinguished (Fig. 7b)⁷⁵. When the Ras/Erk pathway was photoactivated at the poles alone, the embryos developed normally. However, inducing Ras/Erk signaling in centrally located cells altered their fate specification and caused embryonic lethality, even when limited to a 40- μ m-wide domain (Fig. 7c). In addition, global increases in Ras/Erk signaling caused segmentation defects only when the optical perturbation occurred within the first 4 hours of *Drosophila* development; thereafter, tissue patterning was remarkably resistant to changes in Ras/Erk signaling. Taken together, these findings reveal tissue- and stage-specific consequences of Ras/Erk hyperactivation therefore has tissue- and stage-specific consequences that were not evident in previous studies.

Lighting the way forward with chemistry

As photoactivatable probes become an integral part of the developmental biologist's toolbox, it is essential that these technologies keep in step with new mechanistic models. Our current repertoire of photoresponsive molecules has limited spectral, structural, and functional diversity, and general methods for efficiently developing new photoactivatable compounds and proteins are lacking. Future progress will require not only an understanding of photochemistry, but also methods for probe discovery, such as synthetic chemistry, protein engineering, high-throughput screening, and directed evolution. With expertise spanning these diverse areas, the chemical biology community is well-suited to lead the way.

Expanding the biophysical landscape of photoactivatable probes would make them more versatile tools. For example, a broader spectrum of excitation wavelengths would facilitate their use with fluorescent probes and enable the simultaneous, independent control of multiple factors. Most commonly used photocleavable groups are sensitive to UV or blue light, and recent reports of red light-sensitive amino-1,4-benzoquinone and heptamethine cyanine derivatives are exciting steps toward a rainbow of optochemical tools. Non-rhodopsin optogenetic tools have limited spectral bandwidth as well; current bilin-based systems respond to red/far-red light and flavin-based systems only to blue. Mutagenesis has significantly expanded the palette of fluorescent proteins and channelrhodopsins^{4,24}, and it may be possible to similarly diversify phytochromes, cryptochromes, BLUF sensors, and/or LOV domains through directed evolution. In that iterative process, chromophore-interacting

residues in these photoreceptors could randomly mutagenized and spectral variants could be selected through biochemical or cell-based screens. Alternatively, synthetic and biosynthetic chemistry could help address this challenge through the development of bilin and flavin analogs with new spectral and photophysical qualities.

The structure-activity landscape of photoresponsive molecules is also underexplored. Phytochromes, cryptochromes, BLUF sensor-containing proteins, and LOV domains have primarily been repurposed as dimerizing agents in cells. Although such platforms enable us to control general, proximity-dependent aspects of protein function and reconstitute split enzymes in some cases, they do not allow the kind of photo-allosteric control that nature has evolved. Crystallographic structures are now available for each of these photoreceptor families, providing molecular insights that can inform optogenetic probe design. The re-engineering of bacterial phytochromes and the PHOT1 LOV2 domain to regulate heterologous effectors in *cis* exemplifies the remarkable tools that can be obtained through structure-guided trial and error. Yet realizing the full potential of “opto-developmental biology” cannot be achieved through that approach alone.

Chemical biologists could help catalyze the discovery process, again using standard tools of their trade. For example, directed evolution has been used to optimize LOV2 domain-caged peptide ligands and to obtain miniSOG mutants with greater *in vivo* efficacy^{32,73}. Large libraries of photoreceptor-effector chimeras with differing structures and connectivities could be similarly screened for light-dependent activities. Photoresponsive scaffolds could also be iteratively mutagenized and surveyed for variants that interact specifically in *trans* with proteins of interest, enabling the optical control of endogenous targets. It may even be possible to test such libraries in phenotypic cell-based assays to identify optogenetic probes that modulate developmental signaling pathways through unanticipated mechanisms. The potential value of high-throughput screens for new photoactivatable tools is not restricted to genetically encoded molecules. Synthetic chemical libraries could be based on optovin-like rhodanines or other photochromic scaffolds (e.g., azobenzenes, spiropyranes, and naphthopyrans¹²) and then screened for light-dependent interactions with key regulatory proteins and pathways.

A realistic near-term goal for the chemical and developmental biology communities would be to develop specific, photoactivatable tools for the major signaling pathways that regulate tissue patterning. The experimental capabilities empowered by this resource would help reveal how these pathways act in multiple dimensions to create manifold, complex forms, especially when combined with programmable illumination, *in toto* imaging, and computational modeling. They could also enable scientists to recreate these structures in the laboratory and perhaps even in the clinic. While this can be achieved through close collaborations between tool builders and developmental biologists, more chemical biologists who bridge both disciplines will be needed. As developmental biology continues to evolve into a science of molecular interactions and reactions, chemistry will be an increasingly important catalyst for discovery.

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Figure 1. Photoresponsive proteins evolved by microbes and plants

Representative protein structures and chromophore photochemistries for selected photoreceptor families. (a) Mammalian rhodopsin (PDB ID: 2J4Y) and algal channelrhodopsin (3UG9). (b) Bacterial (5AKP) and plant (4OUR) phytochromes and their respective chromophores: biliverdin (left), phytochromobilin (right). (c) BLUF sensor (3GG0). (d) Cryptochrome (1U3C). (e) LOV domains (3RH8, 3UE6, and 2V0W). Protein structures are shown as ribbon diagrams (blue) with surfaces (grey), and chromophores depicted as red spheres. Light-sensitive covalent and hydrogen bonds are colored red in the skeletal structures. Flavin structures: FMN, R = phosphate; FAD, R = ADP.

Figure 2. Photoresponsive synthetic compounds

(a) Azobenzenes. (b) Nitrobenzyl caging group. R_1 , R_2 , and R_3 groups can be varied to tune absorbance maxima and quantum efficiencies. (c) Representative coumarin caging groups with differing excitation wavelengths. (d) Heptamethine cyanine caging group. Caged/photoswitched chemical or biomolecules are depicted by the blue and red circles.

Figure 3. Photoactivatable probes of cell function

(a) Caged fluorescein. (b) Green-to-red photoconversion of the Kaede chromophore. (c) Kaede-based cell lineage tracing of zebrafish eye development, demonstrating the contribution of anterior ectoderm cells to both lens and non-lens tissues. Anterior ectoderm cells were irradiated at 12 hpf and then tracked for another 12 hours. The optic vesicle and optic cup are indicated by the dashed lines. Scale bar: 50 μ m. Adapted with permission from ref. 25 (Copyright 2012, Company of Biologists, Ltd.). (d) Light-dependent generation of reactive oxygen species by miniSOG2, a PHOT2 LOV2 domain variant. A SWISS-MODEL homology model of the miniSOG2 structure is shown as ribbon (blue) and surface (grey) representations. The FMN chromophore is depicted as red spheres, and point mutations introduced to promote singlet oxygen production are highlighted in yellow. (e) Selective photoablation of *decapentaplegic*-expressing cells in the *Drosophila* wing disc using the miniSOG2 system. Adapted with permission from ref. 32 (copyright 2017, Elsevier).

Figure 4. Optochemical and optogenetic probes of gene function

(a) Light-dependent control of Cre-ER^{T2} using caged 4-hydroxy-cyclofen (4-OHC). (b) Light- and caged 4-OHC-mediated Cre/loxP recombination in mammary gland cells of *CreERT²;tdTomato/enhanced GFP* mice. Scale bar: 100 μm. Adapted with permission from ref. 34 (copyright 2012, American Chemical Society). (c) Light-induced reconstitution of split Cre recombination using the CRY2/CIB1 system. Both full-length and truncated forms of CRY2 and CIB1 can be used to regulate Cre function. (d) Photoswitchable regulation of transcription using the PHYB/PIF system. (e) Chemical structure of MOs and photoactivation of a cyclic cMO to inhibit RNA function. (f) cMO-mediated knockdown of *Tbx16* in zebrafish mesodermal progenitors derived from the ventral margin (arrowheads), leading to the posterior mislocalization of these cells. The optically targeted cells were also fluorescently labeled using a Kaede reporter. Scale bars: 200 μm. Adapted with permission from ref. 60, (copyright 2015, Nature Publishing Group).

Figure 5. Optochemical probes of protein function

(a) Light-dependent activation of retinoic acid (RA) signaling, using diethylaminobenzaldehyde (DEAB) to block endogenous RA production and exogenous *cis*-13 RA. Chemical treatments and 365-nm illumination were conducted at the designated times. (b) Photo-control of ionotropic glutamate receptors with the tethered azobenzene ligand MAG-0. (c) Photofentany-2, a photochromic agonist for μ -opioid GCPRs. (d) Optovin, a photoactivatable agonist of the TRPA1 channel.

Figure 6. Optogenetic probes of protein function

(a) Light-dependent plasma membrane recruitment of the OCRL phosphatase domain using the CRY2 PHR domain and the first 170 amino acids of CIB1 (CIBN). The resulting loss of PI(4,5)P₂ disrupts cortical actin polymerization. (b) Spatially controlled OCRL recruitment to the plasma membrane in *Drosophila* embryos, leading to localized arrest of ventral furrow formation. Blue-light illumination was initiated just prior to the onset of gastrulation. Scale bar: 10 μm. Adapted from ref. 70 (CC BY 4.0). (c) A PHOT1 LOV2 domain-based system for peptide masking that has been used to regulate protein localization and degradation. (d) LOV2 domain-dependent control of Rac1 function. (e) Photoactivatable Nodal receptors composed of myristoylated type I and II receptor kinase domains fused to dimerizing AUREO1 LOV modules. The presumptive light-state heterodimer is shown, and other active complexes are possible. (f) Light-inducible expression of the Nodal target genes *goosecoid* (*gsc*) and *sex-determining region Y-box 17* (*sox17*) using the photoactivatable Nodal

receptor system. 6-hpf embryos are shown. Scale bar: 200 μ m. Reproduced from ref. 87 (CC BY 4.0).

Figure 7. Optochemical and optogenetic studies of embryonic patterning

(a) Optical profiling of the Tbx16-regulated transcriptome using caged fluorescein dextran (cFD) and a *tbx16* cMO. Ventral mesodermal progenitor cells targeted by UV light at 6 hpf are colored green. The embryos were then dissociated into single cells at 9 hpf, and the targeted cells were isolated by fluorescence-activated cell sorting (FACS) for transcriptional profiling. Adapted with permission from ref. 60, (copyright 2015, Nature Publishing Group).

(b) Optical control of Ras/Erk signaling using a PHOT1 LOV2 domain-based system to recruit the SOS catalytic domain to the plasma membrane. (c) Spatially restricted activation of Ras/Erk signaling in *Drosophila* embryos reveals regiospecific consequences of Ras/Erk hyperactivation. Endogenous Ras/Erk pathway activity is restricted to the embryonic poles.