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# Synthetic Biological Approaches for Optogenetics and Tools for Transcriptional Light-Control in Bacteria

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**Light has become established as a tool not only to visualize and investigate but also to steer biological systems. This review starts by discussing the unique features that make light such an effective control input in biology. It then gives an overview of how light-control came to progress, starting with photoactivatable compounds and leading up to current genetic implementations using optogenetic approaches. The review then zooms in on optogenetics, focusing on photosensitive proteins, which form the basis for optogenetic engineering using synthetic biological approaches. As the regulation of transcription provides a highly versatile means for steering diverse biological functions, the focus of this review then shifts to transcriptional light regulators, which are presented in the biotechnologically highly relevant model organism *Escherichia coli*.**

## 1. Introduction

Light is a crucial source of life and an inspiration for the mind. Organisms in all domains of life have evolved to sense and utilize the energy contained in the electromagnetic radiation of sunlight, through photosynthetic processes. But light can be more than a form of energy. It is used by organisms to gain information about their surroundings through various processes, the most familiar of which is vision. Along with other senses, vision provides information for perception and through circadian rhythms, life adapts to and aligns itself with light. Although providing the basis for life, light can also have detrimental effects on organisms such as photo-oxidative cellular damage. Organisms have evolved a variety of photosensitive proteins, called photoreceptors or photosensors, to initiate photoprotective mechanisms to counteract light-related damage, while in other contexts, such photoreceptors are used to align with and harvest light. Often, these photoreceptors have distinct "dark/ground" and "light-activated" states which confer different functions to adapt to a light stimulus.

Optogenetics is a biological technique that exploits the information carrying property of light by using such photosensors for bioengineering. Light can then be used to control genetically encoded light-sensitive proteins, which in turn can influence diverse functions of cells. The word "optogenetics" was first used in the context of light-gated ion channels in 2006.[1] The use of these channels has revolutionized neuroscience as well as other biological disciplines. The first genetically engineered light-sensitive proteins date back to 2002 when a yeast-two-hybrid system was coupled with photosensitive domains to create a light-regulated transcription system in yeast.[2] That same year, the discoveries of light-gated ion channels were published.[3,4] Since then, light has been used to perturb and control a variety of cellular functions using different approaches. These approaches, some of which will later be discussed, are either made possible through the use of light as an input, or are used as alternatives for small molecule inputs, such as chemical inducers (for gene expression) or hormones (to elicit cellular responses). In this regard, light fulfills a similar function to small molecule signals which have been used extensively in biological research and biotechnology. For example, small-molecule inducible gene expression systems are key components in synthetic biology[5] and biotechnological applications.[6] However, in contrast to small molecules, which usually bind to specific sensors, light transfers information through photons, which provides unique properties: precise spatiotemporal and orthogonal inputs.

1. Temporal control: Sensing of small molecules can be achieved for example through chemical binding events that lead to cellular signaling. Depending on the experimental setup, it can be challenging or might not be feasible to remove such inducing chemicals, once they were added, especially as small molecules are often relatively stable. As an alternative, sophisticated cellular mechanisms are required to stay responsive to changes of the chemical signal. An example for this is bacterial chemotaxis, in which the cellular regulation adapts to pre-induced levels once the chemical was sensed and an appropriate response initiated.[7] In contrast, photons, originating from the Greek word phōs for "light," are the quantum of light and do not show this limitation. The electromagnetic radiation is effectively being absorbed and transformed into internal energy, especially

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adbi.202000256.



**DOI: 10.1002/adbi.202000256**

chemical and thermal energy. This property is advantageous if a system needs to adapt to varying inputs over time, since this absorption happens practically instantaneously when compared to timescales of cellular processes such as transcription. This, however, is not the case for chemicals in environments which are either diffusion or degradation limited, and therefore they need to be removed by other means. But such removal of inducers is not practical in certain experimental or biotechnological settings, for example in industrial batch cultures. This temporal controllability property of light enables fast and easy changes in the light intensity through amplitude modulation or regulation through pulsed illumination in such settings (**Figure 1**A).[8–10]

2. Spatial control: Electromagnetic radiation spans a large range of wavelengths from $10^{-12}$ m of gamma radiation to 3–3000 m of long radio wavelengths.[11] As the wavelength along with the numerical aperture are the defining properties for the resolution of a light input, the most familiar and optogenetically used class of electromagnetic radiation is visible light, which ranges from 400 to 700 nm. This allows for high spatial resolution of light-inputs at a single cell (Figure 1B left) or even subcellular scale (Figure 1B right), depending on optical aberrations, reflection, refraction, and diffraction limitations. On the other hand, small-molecule-induced systems are limited in their spatial resolution due to difficulty in the precise spatial application of the small molecule and its subsequent diffusion in the medium. This spatiotemporal resolution of a light-input compared to a chemical input can be enabling for applications such as spatial patterning.[12]
3. Orthogonal input: Small chemical inducers often require uptake and/or conversion to an active form for signaling which can be involved in complex feedback regulation (Figure 1C left). Two prominent examples for this are the lactose and arabinose operons in *Escherichia coli*. In addition, these interconnections can be influenced by different factors, such as growth phase, the available carbon source(s), and environmental conditions, such as temperature. Such factors can lead to unpredictable behavior and to increased heterogeneity. In contrast, light is a noninvasive orthogonal input for non-photoresponsive organisms such as *E. coli* (Figure 1C right). This allows for the possibility to introduce perturbations using a light-input with little to no cross-talk, which is difficult to achieve with small molecules or global perturbations, such as temperature shift. Altogether, light-inputs hold the promise of better predictability, robustness, and homogeneous and rapid control. Depending on the organisms studied, side-effects that can be caused by high intensity light such as phototoxicity have to be considered and circumvented through appropriate light intensity and duration inputs.[13]

These three features of light (orthogonality, spatial, and temporal controllability) distinguish it from chemical inducers and hold the promise for more precise perturbation studies, which will help decipher dynamic and interactive cellular networks. This includes the involved and active proteins of such networks, their timing, and spatial location. Also, it enables regulation strategies for biotechnological applications that require fast changeable or spatially defined inputs (Figure 1D).

## 2. Photoactivatable Compounds and Optogenetic Proteins

Light-activation in biological systems can be either achieved through chemical modification with photosensitive groups and chemical effectors (chelators, isomers), or through genetically encoded photosensitive domains. The latter approach is referred to as optogenetic. This section discusses both approaches in general, and the properties of the individual components in particular as these lay the basis and set the limitations of engineering approaches for light-controllable systems. Chemical approaches such as photocaged molecules have been used decades before the first optogenetic methods were developed. However due to their flexibility and their unique dynamic properties, optogenetic regulators have quickly caught up as they are highly versatile for implementation in diverse cellular functions, and offer unique spatiotemporal control opportunities. The most important classes of light-sensitive protein modules for synthetic biology form the foundation for the discussion of potential engineering strategies for optogenetic regulators, and will be the main focus of this section.

### 2.1. Photoactivatable Compounds

Long before the term optogenetics was defined in 2006, optochemical approaches to measure and influence biological responses had been developed as early as the 1960s. This section gives an overview on photocageing groups, photosensitive chelators and cis-trans isomerization of azobenzenes. These serve as examples of some of the biologically relevant approaches, and this subsection is by no means intended to be comprehensive.

#### *2.1.1. Photolabile Protecting Groups (PPGs)*

Probably the most versatile of the three approaches is the use of PPGs (or photocaging groups) (**Figure 2**A). Although they have been used in different ways, the overall principle is that photocaged compounds contain a photolabile group, which renders a biomolecule inactive. Light induces the cleavage of the photolabile group which releases the biomolecule to its native function.[14] In 1962, Barltrop and Schofield[15] described the principle of PPGs. In 1977, Engels and Schlaeger described the synthesis and photolysis of an o-nitrobenzyl-caged cAMP and tested their activity using cAMP-dependent protein kinase.[16] One year later, Kaplan et al.[17] showed photolytic release of "caged ATP." Since then, photocaging has been applied to numerous molecules from proteins to nucleic acids using a variety of photocleavable groups. For example, light can be used to remove protective groups in DNA synthesis, or fluorescently tagged photocleavable nucleotides in next generation sequencing approaches, such as sequencing by synthesis, or sequencing by ligation and microarray synthesis using photolithography and solid-phase synthesis.[14,18–20]

The caged molecule varies depending on the cellular function that needs to be controlled with light. This molecule is

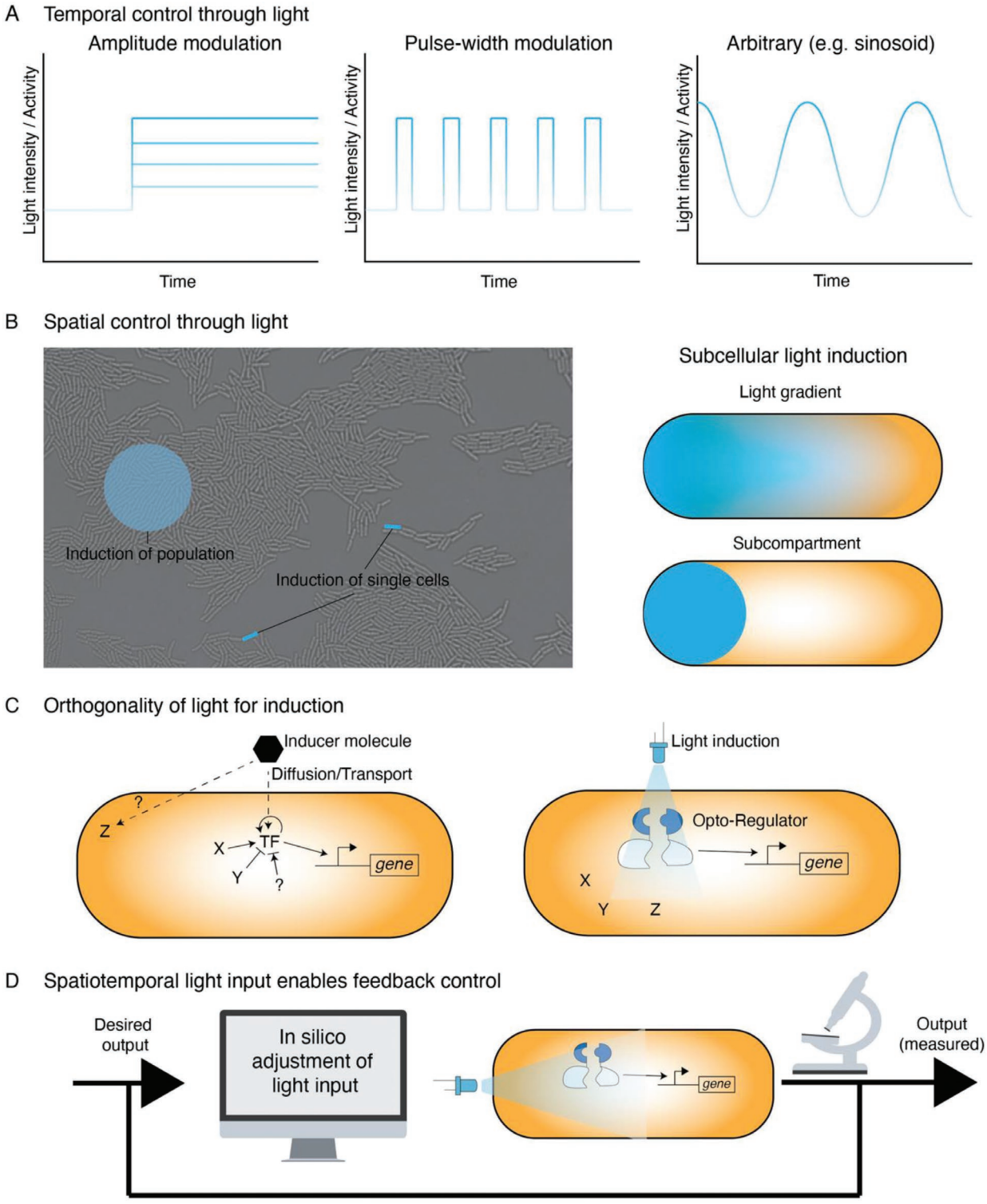


**Figure 1.** Spatiotemporal control through light. A) Light can be modulated precisely in time, enabling adjustment of optogenetic protein activity levels through amplitude or pulse-width modulation and allows application of desired arbitrary inputs. B) Spatial application of light can be used to induce populations or single cells (left) or even at the subcellular level (right). C) Endogenous transcription factors (TFs) steered by small molecule inducers (e.g., sugars) can be involved in complex feedback regulation with potentially unknown interconnections and potential other recognizing factors for the inducer which might lead to unpredictable behavior. Light inputs in contrast are orthogonal input signals for non-photoresponsive organisms such as *E. coli*. D) Spatiotemporal induction enables application of computer control of biological systems, in which light controls a specific cellular function which can be measured (e.g., transcription of a gene of interest). The light input is adjusted automatically depending on the measured output levels, through an in silico feedback control system, to reach desired predefined values.

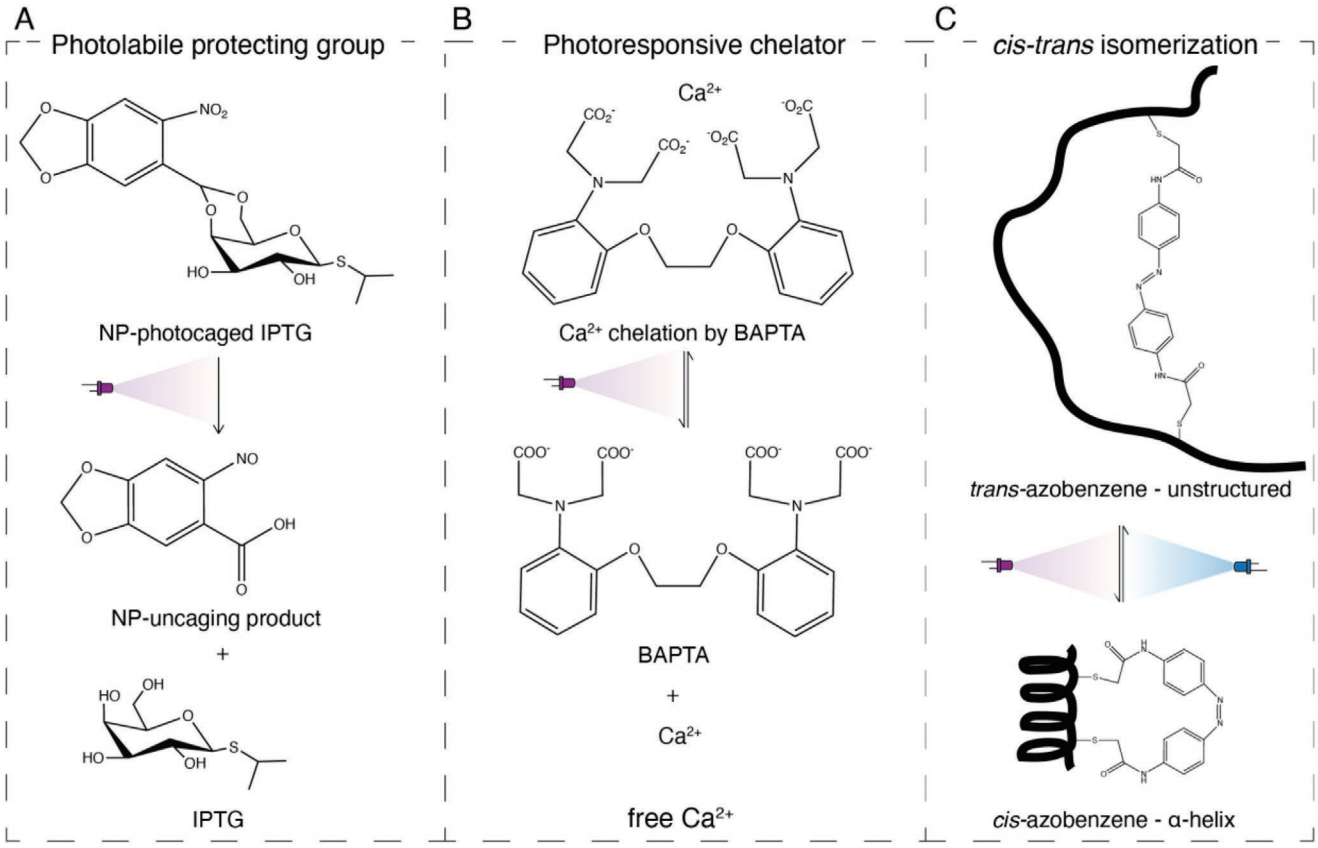


**Figure 2.** Photoactivatable compounds. Examples for A) photolabile protecting groups, B) photoresponsive chelators, and C) *cis-trans* isomerization of azobenzene. A) UV light cleaves nitropiperonal (NP)-photocaged IPTG, which releases the photocaging group and active IPTG.[200] B) Chelation by BAPTA. Carboxylic acid functional groups reversibly bind $Ca^{2+}$ ions.[201] C) *Cis-trans* isomerization of diiodoacetamide azobenzene photoswitch covalently linked to peptides can alter secondary structure elements.[202] Illustrations were drawn based on mentioned publications using ChemDraw Professional 17.0 (PerkinElmer).

rendered inactive through the caging group which contains a conjugated $\pi$ system that can be cleaved-off upon photon stimulation. Two widely used photocaging groups are o-nitrobenzyl- and coumarin-based, which, depending on the substituents, can show absorption maxima from the UV to the green light spectrum.[21] Although many other caging groups exist, more than 80% of published photocaging approaches incorporate a nitrobenzyl group, which can be released in a well characterized photolysis mechanism.[14] Nitrobenzyl groups can be photocleaved using an excess of UVA light, even though the absorption maximum of most compounds lies in the UVB–UVC range, as UVA is considered far less damaging to cells than UVB–UVC.[14]

While numerous biofunctional chemicals have been caged (e.g., DOX, IPTG, arabinose, theophylline) with photosensitive groups (Figure 2A), the caging of proteins might show advantageous features: 1) the activity of cellular functions is precisely targeted through protein key players 2) only low concentrations compared to photocaged chemicals might avoid problems with photolysis byproducts. However, it needs to be considered that absorption of tryptophans could aid in the energy transfer and uncaging, and could also quench photolysis. In addition, pH and the local dielectric constant play an important role in the ground state absorption properties of a photolabile group, and one needs to take into account that for example the "apparent pKa" of a group can be different in the active site of an enzyme compared to other environments. Another difficulty can be the size, structure, and complexity of proteins, as photoreactive groups also need to be released after uncaging from an active site.[14] Overall, the advantages of photocaged proteins come with increased complexity in their synthesis.

Photosensitive groups can be introduced to proteins in vitro, for example through random modification with the oxycarbonylchloride of 1-(2-nitrophenyl)ethanol. The PPG primarily reacts with lysine residues, which was used to create light-activatable antibodies.[22] Another strategy targets cysteine residues as their nucleophilic groups allow selective modification by an electrophilic caging reagent.[14]

These and other strategies for in vitro synthesis of photoactivatable proteins can either be used in extracellular systems or need to be introduced into the cell using techniques such as microinjection, which can be prohibitive for many studies and applications. Therefore, in vivo synthesis of photoactivatable proteins can be enabling. An interesting approach is to expand the genetic code through the use of tRNA/aminoacyl-tRNA synthetase pairs, allowing one to include amino acids with photosensitive groups such as o-nitrobenzyl-caged tyrosine at amber sites. These caged amino acids can be located at functional sites

of proteins such as the active site of an enzyme or the binding site of a protein.[23–25]

#### 2.1.2. Photoresponsive Chelators

A different approach exploits chelators for light-control of cells by influencing the intracellular concentration of free metal ions, which fulfill numerous functions and are important cofactors for enzymes. This approach was very successful for buffers and optical indicators for $Ca^{2+}$, which were synthesized based on BAPTA (l,2-bis(o-aminophenoxy)ethane-A,M-A/A'-tetraacetic acid).[26] This enabled experimenters to nondestructively measure intracellular $Ca^{2+}$ levels through shifts in the absorption spectrum of unbound to $Ca^{2+}$-bound chelator (Figure 2B).[26] $Ca^{2+}$ ions have been of great interest for biological and biomedical studies, as it is an important component of cell signaling, and concentration changes are involved in diverse effects such as neuronal activity, cell motility, muscle contraction, apoptosis or transcription.[27] Although being able to measure intracellular $Ca^{2+}$ concentrations was effectively used for gaining biological insights, control of intracellular concentrations was highly desirable for perturbation studies. It was later realized that not only could concentrations be measured, but the affinity of the $Ca^{2+}$ chelators also changes with light.[28,29] Chelator molecules, such as BAPTA, EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) form a "cavity" through the steric disposition of the carboxylate groups.[30] UV light is absorbed (maximum at 350 nm) by the caged compound and can lead to photolysis of the chelator to products that show a lower $Ca^{2+}$ affinity. Chelators for other divalent metal ions have also been developed.[31]

#### 2.1.3. Cis–Trans Isomerization of Azobenzene

*Cis–trans* isomerization of azobenzene in response to light was described by Hartley in 1937.[32] This light-induced change in the structure of the chemical was later exploited to implement light control. It was shown that both forward and backward reactions are activated by light, and that the thermal reaction is slow. While the trans configuration, which is produced by 410–450 nm light, is planar, the benzene rings in the *cis* configuration are skewed at 53°, which can be triggered with 300–350 nm light.[29] Although the discovery of this phenomenon dates back more than 80 years, the underlying photochemistry and isomerization mechanism is still under investigation, with four proposed mechanisms: rotation, inversion, concerted inversion, and inversion-assisted rotation.[33] The isomerization can be repeated numerous times, as azobenzene shows high photostability, making it interesting for repeated photoswitching in dynamic control. The change in geometry can be used to modulate accessibility or activity of biomolecules. For example, it was shown that a 16 amino acid peptide tethered to an azobenzene can switch between a more helical structure in the *trans*, and a reduced helical content in the *cis* configuration (Figure 2C).[34] Tethered azobenzene was also used to controllably block and release the pore of a $K^+$ channel. Light with 380 nm wavelength, which creates the *cis* isomer, shortens the azobenzene and deblocks the pore, which allows $K^+$ ions to pass the channel, while 500 nm light creates the trans isomer which blocks the pore.[35]

### 2.2. Photoactivatable Proteins

As photocaged compounds show limited reversibility and spatial control once their protecting group is released, and as photoresponsive chelators and azobenzenes can be applied only in specific cases, new ways for light-control and the genetic implementations needed to achieve it, have been explored. Photoactivatable proteins or domains are essential components for optogenetic protein engineering. All photoactivatable domains used to-date in synthetic biology are derived from natural photoreceptors. Just like in other sensory signaling proteins,[36] modularity is common also in photosensors. In general, the input sensing domains or motifs are physically and functionally separable from the catalytic activity or the output domains.[36] This modularity feature of photosensory domains, which enables evolution through recombination, deletion, or insertion in the natural context, is also an enabling feature for bioengineering of novel light-inducible regulators. Indeed, such biological parts or modules with defined functionality are the fundamental basis for synthetic biology approaches. Through re-designing, combination with other modules, and engineering of the parts themselves, new functions can be created in organisms.[37] A prerequisite for the utilization of modules is their functional characterization, ideally with an understanding of the mechanism, the underlying structural basis, and the minimal requirements for the individual modules to work (e.g., availability of a specific cofactor or chromophore). As the understanding of photodomain properties is essential for development of functional optogenetic proteins, these properties are discussed in the following section.

The most widely used photoactivatable protein family in biology and medicine are light-sensitive transmembrane proteins. These proteins contain the chromophore retinal, or a variant, which in response to light isomerizes between an 11- or 13-*cis* and all-*trans* retinal. The chromophore isomerization translates to a structural change in the apoprotein. Depending on their origin, these so-called opsins are divided into microbial (Type I) and animal (Type II) opsins. The natural function of these proteins ranges from vision in animals, osmotic regulation in halobacteria, to photoperiodism in plants and animals. Although opsins have been used extensively in neurobiology and regenerative medicine for light-control of ion-fluxes and cell signaling, other non-opsin photoactivatable proteins lay the foundations for most protein engineering strategies in optogenetics.[38] Therefore, we present an overview of non-opsin photoactivatable proteins from nature, which have been adopted for synthetic biological approaches.

### 2.3. Chromophore and Photocycle

Photoactivatable proteins absorb light of specific wavelengths through an organic chromophore or cofactor which contains

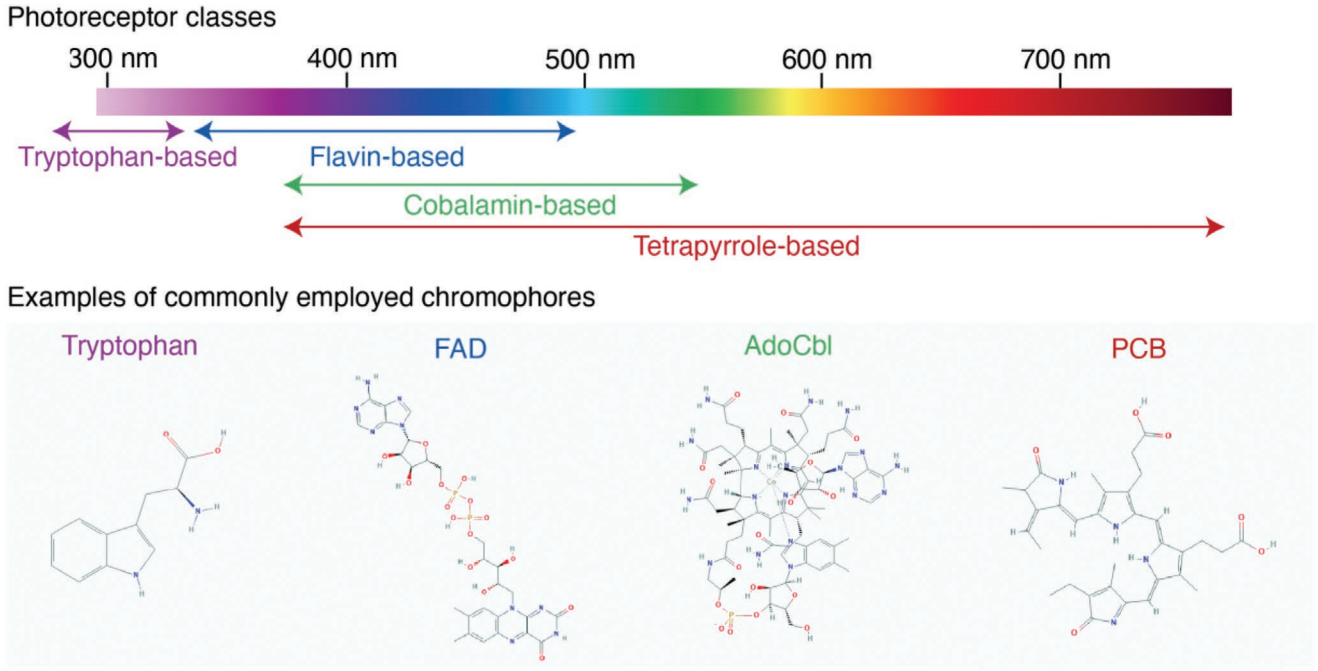


**Figure 3.** Schematic overview of photosensor classes and their approximate activation light wavelength ranges. Below: Examples of chromophores showing one representative example for each class (indicated by color) to illustrate the structural differences. The illustration is based on information and figures shown in refs. [39,101,203] and chemical structures from PubChem (Tryptophan: CID 6305; Flavin adenne dinucleotide, FAD: CID 643975; 5′-Deoxyadenosylcobalamin, AdoCbl: CID 70678541; Phycocyanobilin, PCB: CID 5460417).

a conjugated $\pi$ electron system.[39] Chromophores are molecules or chemical moieties that absorb light in the UV–vis spectrum.[40] The absorbed light leads to electron jumps from a lower to a higher energy molecular orbital, which in double-bonded molecules causes $\pi$–$\pi^*$ transitions. Conjugated $\pi$ systems with conjugated electrons show a lower $\pi$–$\pi^*$ energy gap than single double-bonds and therefore absorb longer wavelengths and favor light absorption.[40] This chromophore excitation in turn leads to a structural change of the chromophore (e.g., *cis–trans* isomerization) and/or its interactions with the apoprotein, which leads to changes in the protein structure from a dark to a light activated state in a process called "photocycle." This photocycle is closed once the activated photoactivatable protein reverts to the dark state. This dark state reversion is thermally driven, and its timescale can range from milliseconds to hours, depending on the photoreceptor.

### 2.4. Photosensor Classification

For the purpose of synthetic biology, we adopt an intuitive classification of photoactivatable proteins that is based on the protein's incorporated chromophore or the photoresponsive domain structure, which in part also determines the range of activation/inactivation wavelength peaks. Accordingly, four classes of photoactivatable proteins are described in this subsection: 1) light-activation via an intrinsic tryprophane; 2) chromophores which are based on flavin, 3) cobalamin or 4) tetrapyrroles (**Figure 3**). Fluorescent proteins such as PhoCl, PYP or Dronpa, which were also used to implement light control are not discussed further. Our focus will be on the structural changes that light activation induces in the photoreceptor, as this is the basis for how they can be used in protein engineering approaches.

#### *2.4.1. Intrinsic Tryptophan Regulated UVR8: UV Receptor*

This photoreceptor class uses intrinsic tryptophanes for light absorption, with UVR8 as a prominent example. The photoregulator UVR8 was discovered in *Arabidopsis thaliana* as a mechanism to optimize growth and survival in the presence of UV-B.[41] UVR8 occurs as a homodimer in the dark state.[41] UV-B light is absorbed by tryptophane amino acid residues (W233, W285, and W337) which leads to monomerization of UVR8, and in turn allows for heterodimerization with UVR8-binding partner COP1.[41] W233 and W285, which act as UV-B chromophore, show cation–$\pi$ interactions with R286 and R338 and stabilize the protein structure. Excitation of the tryptophan indole rings through UV-B light disrupts these interactions, which leads to the release of arginine-mediated intermolecular hydrogen bonds between the homodimers and UVR8 monomerization.[42] The photocycle is closed as the indole rings dissipate energy and return to their ground state over time, leading to homodimerization of UVR8.[42]

#### *2.4.2. Flavin-Based Cryptochromes, BLUF, and LOV Domains: Blue Light Receptors*

Cryptochromes, LOV, and BLUF domains contain a flavin chromophore, either flavin mononucleotide (FMN) or flavin

adenine dinucleotide (FAD), which can be covalently or noncovalently bound to the apoprotein.[43] FMN and FAD are present in most organisms. Flavin shows maximum absorption in the blue light range which causes a photochemical reduction of the oxidized form to a semiquinone or the fully reduced form.[44,45] The isoalloxazine ring system allows for one- or two electron transfer processes.[43] Also multi-photon excitation with near-infrared light was shown, allowing for 3D activation of flavin-based systems and deep tissue penetration.[46–48]

The evolutionarily highly conserved cryptochromes CRY1 and CRY2 belong to the family of flavoproteins that exist in all kingdoms of life, where they are involved in developmental and circadian responses.[49] Cryptochromes evolved from photolyases and contain an *N*-terminal photolyase homology (PHR) domain, which binds FAD as chromophore.[49] In a proposed light-activation mechanism in plant cryptochrome, oxidized FAD in the dark state is reduced to a neutral semiquinone through light, which induces a negative charge in the vicinity of the flavin. This might lead to the release of ATP from its binding pocket, and the subsequent C-terminus unfolding of the protein. Through the conformational change and release of the C-terminus, amino acid residues are accessible for phosphorylation, which then allows for binding of other proteins.[50] A widely used cryptochrome is CRY2 from *Arabidopsis thaliana* (*At*CRY2). CRY2 is monomeric in the dark state, and oligomerizes upon blue light activation.[51] In the photoexcited state, it can also form a heterodimer with cryptochrome-interacting basic-helix-loop-helix protein (CIB1).[52] The half-life of this interaction is in the minute-scale, and it can be tuned through modulating mutations in the PHR domain.[53]

The second class of sensors that absorb blue light using FAD (BLUF domain) was discovered in *Rhodobacter sphaeroides*[54,55] and *Euglena gracilis*,[56] where they aid in adaptation of photosystem synthesis depending on oxygen and light conditions.[57] Although protein structures of BLUF domain containing proteins have been solved, the mechanism of photo-activation is still under debate.[57,58] Through blue light-illumination, an electron and then a proton is transferred from a conserved tyrosine to the flavin, which leads to formation of flavin and tyrosine radicals.[48,59] The bi-radical then might induce a hydrogen bond rearrangement in the flavin binding pocket.[57] PixD is an example of a pentameric BLUF domain that, together with PixE, form large molecular weight aggregates of two pentameric PixD and 5 PixE subunits.[60] Through illumination, this complex can be destabilized, resulting in monomeric PixE, and two pentameric PixD.[60]

Another widely used class of blue light-sensitive proteins are flavin mononucleotide (FMN)-binding light oxygen and voltage (LOV) domains. In contrast to cryptochromes, where light causes electrostatic changes in the apoprotein and subsequent conformational changes, the FMN C(4a) in LOV domains usually forms a covalent adduct with an adjacent conserved cysteine, which in turn also causes conformational changes in the Per-ARNT-Sim (period clock protein, aromatic hydrocarbon receptor nuclear translocator, and single minded; short PAS) core.[61–63] Apart from FMN, FAD functions also as a chromophore in LOV domains, as for example in the photoregulator Vivid.[64] PAS domains are found in all kingdoms of life and commonly act as molecular sensors and transducers.[65,66] The generated structural changes through cysteinyl-(C4a) formation propagate to N- or C-terminally attached effector domains via amphipathic $\alpha$-helical and coiled-coil linkers. This conformational change can initiate diverse mechanisms such as dimerization of LOV domains in Vivid,[62,64,67] the unfolding and displacement of a J$\alpha$ helix in the case of *As*LOV2,[66] or the rearrangement and release of a helix-turn-helix (HTH) domain for EL222[68] from their respective LOV core. Based on these characteristics, LOV1 domains usually involve a PAS core in some cases with an additional N-terminal cap (NCap) through which the proteins associate.[69–71] LOV2 domains, on the other hand, contain a C-terminal J$\alpha$ helix which displaces after light-stimulation.[72,73]

#### *2.4.3. Cobalamin-Based Binding Domains: Green Light Receptors*

Cobalamin-binding domains (CBDs) are green light photoreceptors that utilize cobalamin as chromophore for photosensing. CBDs were found to play a photoprotecting role in diverse bacteria[74–76] and were identified through their role in light-dependent carotenoid synthesis which quenches reactive oxygen species (ROS).[77–79] One of the CBDs that were used so far in synthetic biology is CarH. The CarH photoreceptor dimer binds 5′deoxyadenosylcobalamin (AdoCbl) as its chromophore to form a tetramer. AdoCbl and methylcobalamin (MeCbl) are the two major biological forms of Vitamin $B_{12}$ which are produced by microorganisms.[80] Mammalian cells are capable of AdoCbl import and its conversion from Vitamin B12.[81,82] AdoCbl and MeCbl differ in the 5′deoxyadenosyl and methyl group that is covalently bound to cobalt, which in both cases has low bond dissociation energies. The low dissociation energies allow for the cleavage of the respective 5′deoxyadenosyl or methyl groups with wavelengths ranging from near-UV light up to wavelengths of 530 nm in the green light spectrum.[80] In the case of CarH, the proteins form head-to-tail tetramers as a dimer-of-dimers in the presence of AdoCbl which involves numerous hydrogen bonds of the apoprotein with cobalamin and hydrogen bonds and ionic interactions with the 5′deoxyadenosyl group.[83] Light exposure triggers dissociation of the 5′deoxyadenosyl group, which leads to reorientation of the four-helix bundle of the protein disrupting the head-to-tail interface, resulting in CarH monomerization.[83] Since the cobalamin forms a covalent adduct with CarH through bis-His ligation, this reaction is irreversible, and photolyzed cobalamin cannot be exchanged with photosensitive AdoCbl.[83] This limits applicability of CarH for fast dynamic optogenetic control.

#### *2.4.4. Tetrapyrrole-Based Phytochromes: From UV to Far-Red Receptors*

Apart from flavin-based photoreceptors, phytochromes are the second widely used class of light-inducible domains which were used in the first optogenetic regulators in eukaryotic[2] and bacterial[84] cells. Phytochromes (Phy) were discovered through their role in promoting development in plants such as germination and flowering in response to red-light.[85–87] However, phytochromes are not just present in plants, but also in

bacteria[88,89] (BphP) and fungi[90] (Fph). Phytochromes are classified depending on their activation light wavelength to type I, which is activated by far-red light (730 nm absorption peak), and type II, which is activated by red light (660 nm absorption peak). The two types therefore differ in their thermal ground states and can reversibly switch between a red-absorbing (Pr) and a far-red-absorbing (Pfr) state. However, algal phytochromes[91] and cyanobacteriochromes[92] were described and shown to cover the full visible spectrum and even reach into the UV range. For example, phytochromes with blue(Pb)–green(Pg)[93] and green(Pg)–red(Pr)[94]-activating wavelengths were discovered. Even though they absorb a wide range of wavelengths, all phytochromes use a tetrapyrrole chromophore, either in the reduced form as phycocyanobilin (i.e., plant phytochromes and cyanobacteriochromes), or in the oxidized form as biliverdin as used by bacterial and fungal phytochromes.[95] Biliverdin (BV), the chromophore for BphP and Fph, is synthesized from heme in one step through heme oxygenase HO1. In cyanobacteria and green algae, BV is further reduced to phycocyanobilin (PCB) by a ferredoxin-dependent bilin reductase PcyA. In plants however, an enzyme of the same family called HY2 reduces BV to phytochromobilin (PΦB).[95–97] The different bilins are bound in a GAF domain, which is highly conserved. Typical phytochromes show a PAS-GAF-PHY domain photosensory module structure, although variations in this structure exist.[95] In a suggested photoactivation mechanism, light induces a Z–E isomerization in the C15-C16 double bond of the tetrapyrrole,[98] inducing a rotation on the D-ring of the molecule, which in turn generates a rearrangement of the hydrogen bonds of the GAF domain that propagates to the PHY domain.[95,99] These structural changes can have diverse effects in different phytochromes and, for example, allow for heterodimerization of light-induced PhyB with phytochrome-interacting factor (PIF3), or homodimerization in the case of cyanobacterial phytochrome Cph1.[88] Furthermore, phytochromes do not just rely on dark state reversion over time through energy dissipation, but they can be induced specifically with deactivating light at a wavelength different from the induction wavelength (e.g., far-red light for PhyB[100] or Cph1[88]). This gives phytochromes a superior temporal resolution.

## 3. Design of Optogenetic Proteins

Protein engineering strategies for optogenetic proteins involve a photosensory domain (e.g., the ones previously discussed) as well as an actuating module. Their covalent connection is usually mediated with protein linkers that need to be optimized in length and structure, depending on the engineering strategy and the requirements for coupling between the modules.[101–103] The choice of the photosensory domain can be based on structural considerations of the protein itself or unique properties that different photosensory domains contain.

Such structural considerations can involve the homology to sensory domains that are naturally linked to the actuating module, which make protein engineering easier through "domain swapping" of, for example, a small molecule or hormone sensing domain with a photoactivatable domain. In other cases, the actuation module might be incorporated into the structure of a photosensory protein, which sets special structural and sequence requirements. Also, it may be of interest to reduce the size of a photosensory domain, or in other cases, to provoke sterical hindering. Similarly, certain properties of photosensory domains might be crucial for specific applications. For example, if highly dynamic optoproteins are desired, fast $k_{off}$ rates or light sensors that have an inactivating wavelength should be considered. However, if light-toxicity or light-delivery is problematic, high light-sensitivity and slow dark-reversion might be preferable. Also dark to light state fold-inductions of different photoregulators can be important for certain applications, although such a comparison might be case-specific and cannot always be taken from other studies in the literature. Availability of the chromophore in the used organism or medium could be another consideration.

Usually, the choice of the actuating module is very case-specific and depends on the application that the optoprotein needs to fulfill and the output it should produce, and will therefore not be further discussed. However, this section will extract general principles from successful previous optoprotein engineering efforts. Another important aspect is the screening of the functionality of optoproteins: As the protein engineering efforts might require large libraries, high-throughput methods[104] for functional screening, such as selection systems or fluorescence readouts, are preferred.

A general aspect of optogenetic proteins is that light-regulation is always based on conformational changes of the photosensory domain. Different classifications for photoactivatable proteins were previously used (e.g.,[99,105–108]), however, we chose to classify light-regulated proteins slightly differently: To control the activity of the protein of interest, two general concepts were applied, which are either proximity- or protein-structure-based, and in some cases combined approaches are needed.

### 3.1. Proximity-Based Activity Control: Intermolecular Light-Control

The distance between proteins is an ubiquitous regulation factor in biology.[109] For example, assembly of multiple subcomponents is required to initiate transcription at the location of a promoter, and several factors are required to interact for protein transport or degradation. Both soluble and membrane-bound proteins are mostly symmetrical oligomeric complexes with two or more subunits. This makes complexes more stable due to reduced solvent area, provides a form of error control in protein synthesis and regulation, and allows cooperative function such as allosteric regulation and multivalent binding.[110] Chemicals and external signals can induce oligomerization of proteins, such as receptors, that subsequently initiate signaling and cellular responses.[111] The underlying regulation mechanism is based on proximity and distance of specific proteins.[109] Such proximity-based regulation is also the basis of many natural light-sensing modules in which the interaction, and therefore the distance of the interaction partners, is controlled through light. Due to allosteric changes of the photosensitive proteins upon light-induction, an interaction surface is exposed, which either allows for interaction with other identical photosensors

(homodimerization or oligomerization), or for interaction with other proteins (heterodimerization). Both concepts can be utilized for proximity-based control. Therefore, proximity-based light control involves at least two fusion proteins.

Proximity can induce function in two ways:

1. Activation: inactive protein domains fused to photoactivatable domains which upon a light-input are assembled into an active protein
2. Recruitment: protein-recruitment of an active protein to a specific location of action

#### *3.1.1. Activation*

For the first case, light regulates assembly or release of domains necessary for the function of the protein. Prominent targets for proximity-based protein activation are signaling kinases. These kinases react to a plethora of environmental and cellular cues and control diverse functions of proteins from changing their expression, activity or localization, through altering their phosphorylation with serine, threonine, and tyrosine as their main targets. Such kinases usually show multidomain structures, containing a sensing domain that reacts to external cues often through induced oligomerization and autoactivation.[111] Light-controlled homodimerization domains were used instead of the sensing domains to implement optogenetic control usually through homodimerization.[112–118]

Heterodimerization, however, can be used if two different interaction partners are needed for the function of a protein. This is for example the case for synthetic split proteins which hold great potential for implementation of optogenetic regulation. In split protein approaches, a functional enzyme is artificially fragmented into inactive subunits. These inactive subunits are fused to light-inducible dimerization domains which reconstitute the enzyme to the active protein.[108,119–121] An example for this is the light-inducible T7 RNA polymerase,[120] which consists of two inactive split parts that are reconstituted upon light-induced dimerization of photosensory domains (**Figure 4**A left).

#### *3.1.2. Recruitment*

In the second case of proximity-based control, light initiates the subcellular localization of a constitutive active protein to a location of action. For example, light-inducible heterodimerization domains were used to recruit transcription activation domains to specific sites of a promoter through promoter-bound DNA-binding proteins to initiate transcription (Figure 4A right).[2,13,122] In a highly similar strategy, DNA and histone modification enzymes such as DNA methyltransferases, histone deacetylases, methyltransferases, and acetyl–transferase inhibitors were used instead of transcription regulators for epigenetic regulation.[123,124] Another widely used example is light-induced recruitment of proteins to membranes[125] where they exhibit their function, such as cAMP-dependent protein kinases which phosphorylate membrane bound substrates.[125,126]

In both proximity-based activation and recruitment, the photosensitive domain and the effector domain(s) function independently. Engineering of such regulators mainly requires structural considerations to avoid interfering with the function of the actuator domain and to allow for correct assembly of split proteins[120]—factors that have to be considered in the choice of the light-inducible domain as well as the type of linker, its length, and its structure.[65,102,120]

### 3.2. Protein-Conformation-Based-Light Control: Intramolecular Light-Control

While proximity-based light control always involves two separate fusion proteins, allosteric conformation-based approaches only involve one protein which consists of a fusion of the effector domain and the light-inducible domain(s). The transition from the dark- to the light-induced state of photoactivatable proteins initiates a structural rearrangement that is transmitted to the effector domain leading to allosteric protein activity change or steric effects, for example, blocking and release of the active site of the effector domain.

The photoreceptors LOV2 from *Avena sativa* or from *Arabidopsis thaliana* are two prominent examples that have been used for such intramolecular light-control. The structural rearrangement upon blue-light stimulation leads to the release of a C-terminal helix (J$\alpha$-helix) from the PAS core.[127] Although this mechanism of LOV2 was also utilized for intermolecular light control,[128,129] the dramatic conformational change makes this photosensor attractive for intramolecular light-control approaches.

#### *3.2.1. Allosteric Regulation*

Building upon studies that show that domain insertion into enzymes can be used for allosteric regulation,[130] in a pioneering example, LOV domains were used to implement light-control in a similar manner.[131] The light-induced conformational change of LOV2 is transmitted to the protein into which it is inserted, changing its activity and/or function. Such an engineering approach requires X-ray or NMR protein structures or homology models and detailed structure–function information to preselect candidate sites for insertion positions, which are usually located in surface exposed flexible loops at not conserved residues of the target protein.[132] An example are OptoNBs,[133] in which light-induced conformational changes of photosensory domains are transmitted to nanobodies leading to changes in their binding affinities (Figure 4B left).

#### *3.2.2. Steric Regulation*

In contrast to allosteric regulation, steric approaches utilize the light-induced conformational changes of a photosensory domain to change the accessibility of functionally important sites of the regulated protein (e.g., active site, regulator binding, or recognition sites). For example, the J$\alpha$-helix of LOV2 was successfully modified to incorporate signal peptides of different functions, which upon light-induction are released from the PAS core and only then fulfil their function, for example,

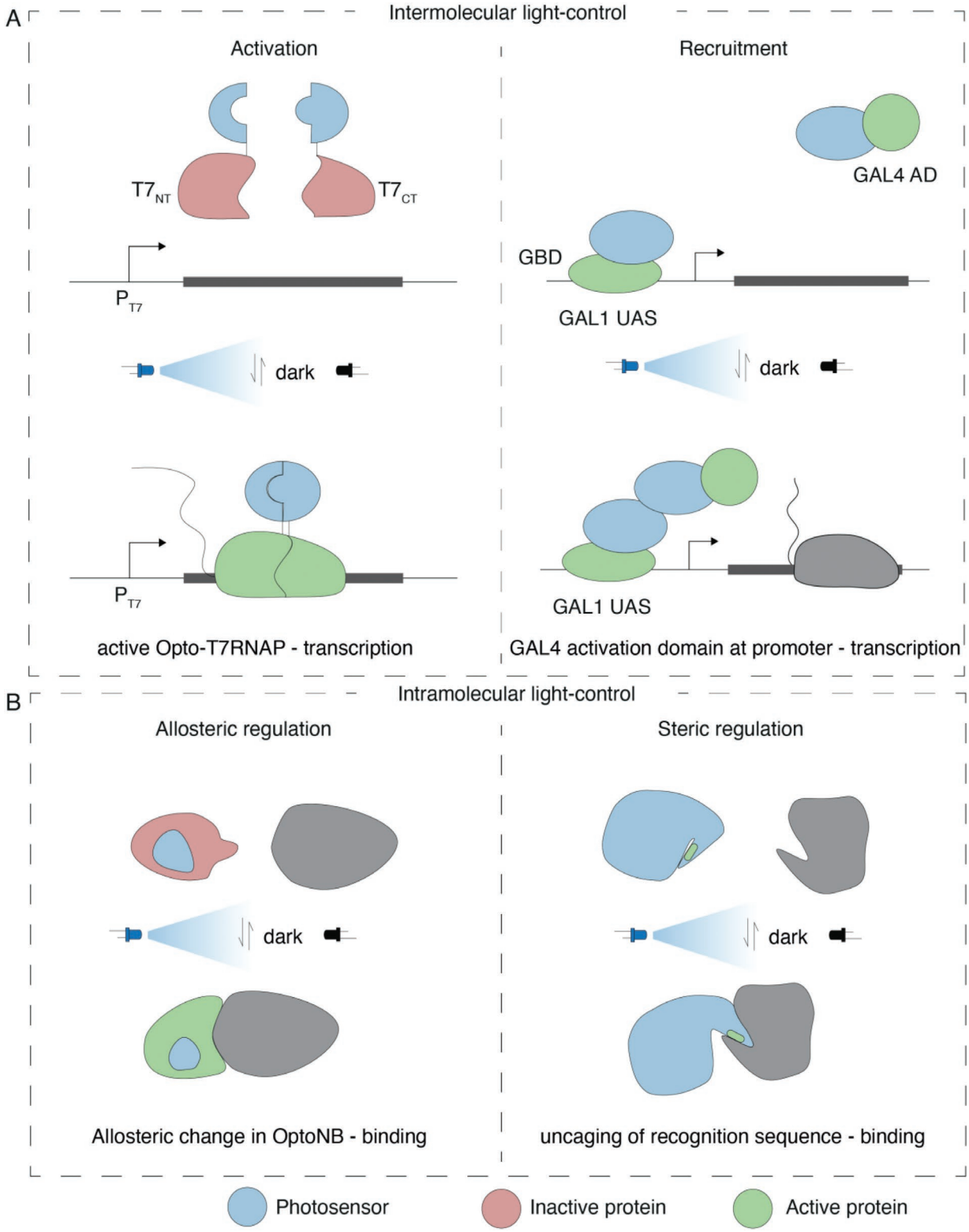


**Figure 4.** Conceptually different designs for implementation of light-control in proteins. A) Proximity-based intermolecular light control. Light-induced binding of photosensory domains causes either activation of inactive proteins or protein fragments (left, example based on[120]), or recruitment of active proteins to locations at which they perform a specific function (right, example based on[2]). B) Protein conformation-based intermolecular light control. A single photosensory domain is fused to an active protein or peptide whose conformational change upon light-induction causes either an allosteric change which alters the activity of the effector protein (left, example based on[133]) or the accessibility of an active site is sterically affected (right, example based on[129,204]).

nuclear localization,[134] protein degradation,[135] or other functional domains[136] (Figure 4B right).

Although LOV2 is a prominent example for protein conformation based light control, other photoregulators were also used for this type of control. For example Dronpa mutants were employed for light-inducible steric hindering of active sites.[137,138] These mutants are dimeric or tetrameric, monomerize with cyan light and revert by either homodimerizing (Dronpa145K/N) or homotetramerizing (Dronpa145N) with UV light.[139] N- and C-terminal fusion of Dronpa to a protease was used for steric blockage of interaction sites, which is removed with the light-input and monomerization of the photosensor.[139]

## 4. Optogenetic Transcription Regulation in the Bacterium *E. coli*

Microbes are renewable factories for the production of proteins and chemicals. *E. coli* has been one of the main workhorses of industrial biotechnology and synthetic biology because, amongst other advantageous features, 1) it grows quickly, 2) has low demands for its growth medium and as a facultative anaerobic organism also allows for cultivation without oxygen, 3) it is well studied—a lot of information exists about its regulatory and metabolic networks and the proteins involved, and 4) a large toolset is available for its genetic manipulation.[140] Apart from protein production for which *E. coli* accounts for about 30% of approved therapeutics,[141,142] the versatile metabolism of this bacterium and its relatively easy expansion through genetic manipulation allows production of a vast variety of metabolites through metabolic engineering.[140] Such metabolic engineering usually involves pathway and host engineering. While pathway engineering involves adjustments in the expression of endogenous or heterologous enzymes needed for conversion of a substrate to the desired product, host engineering is mostly focused on competing or regulatory pathways.[143] Both aim toward increased flux to the desired product, increased productivity and product titers, and fewer byproducts. Dynamic pathway regulation is already established for adjustment between cell growth and production for which inducible promoter systems are well suited.[143] Also dynamic regulation of pathway enzymes, used to direct flux into the production pathway and to down-regulate competing enzymes of the native cell metabolism, is becoming a promising approach.[143–147] More complex pathways and products increase the requirements of such dynamic control. For example, toxic intermediates or depletion of essential metabolites requires continuous adjustment of the enzyme concentrations in the metabolic pathway to maximize productivity.[148] Such dynamic adjustment requirements cannot be fulfilled by traditional small molecule transcriptional inducers, a limitation that does not hold for optogenetic regulators as previously discussed. This requires new dynamic regulation schemes.[149–152] If a toxic intermediate or other important process variables are measurable during the process, such dynamic regulation can be automated, as in the case of in-silico feedback systems.[153–157] (Figure 2D) Using such measurements and either a proportional controller or, if a model of the controlled system is available, also model predictive controller (MPC), the process can be precisely controlled via light-inputs.[154] A versatile knob for such regulation can be implemented through light-induced expression systems, as such a system is not linked to a particular pathway, but can be applied ubiquitously. Of course, the application of these systems is not limited to metabolic engineering, which served as a highly relevant example, but are also enabling for numerous other fields such as dynamic perturbation for the study of organisms, specific genes, spatial patterning, and so on. In general, probably the most versatile regulation is on the transcriptional level, as it can be implemented for expression of any metabolic enzyme or gene of interest. The currently available light-inducible transcription systems that can be used for such dynamic regulation are described in this section.

### 4.1. Two Component Systems (TCSs)

The first optogenetic transcription regulator in *E. coli* was an engineered two-component system, consisting of a light-sensitive sensor kinase and an intracellular response regulator.[84] In general, TCSs are present in all kingdoms of life and are widespread in bacteria.[158,159] Diverse environmental signals are detected through sensory domains which initiate conformational changes in the sensor that are transmitted to its C-terminal histidine kinase domain, changing its activity[159,160] which ultimately regulates the phosphorylation state of a response regulator. The majority of response regulators are DNA binding domains and involved in transcriptional regulation.[159]

#### *4.1.1. Red/Far-Red Light TCS*

This is also the function of the EnvZ/OmpR TCS, in which OmpR differentially regulates the expression of *ompF* and *ompC* porins for osmoregulation in *E. coli*.[161] Osmolytes (e.g., NaCl) lead to conformational changes in EnvZ and its subsequent autophosphorylation.[162,163] To create a light-inducible gene expression system using the components of this endogenous system, Levskaya et al.[84] created chimeras called Cph8 based on the sensor kinase EnvZ with the *Synechocystis* phytochrome photosensor Cph1 instead of the native sensory domain. This changed the sensing property of the TCS from osmotic pressure to red light (maximal excitation 650 nm), while leaving the transcriptional regulation properties of OmpR unaltered (**Figure 5**A). Light-inactivation with Far-Red light (705 nm) allows for fast dynamic regulation. Expression of a reporter gene was used to demonstrate light-defined spatially confined expression. The chromophore PCB, which is required by photosensor Cph1, is not naturally produced in *E. coli*. Therefore, they also introduced the previously described genes *ho1* and *pcyA*[96] for PCB production in *E. coli*.

#### *4.1.2. Green/Red Light TCS*

To engineer a second light-inducible transcription system with a different absorption spectrum to the Red/Far-Red system, the previously discovered cyanobacterial two-component system CcaS/CcaR[94] was transferred into *E. coli* where its

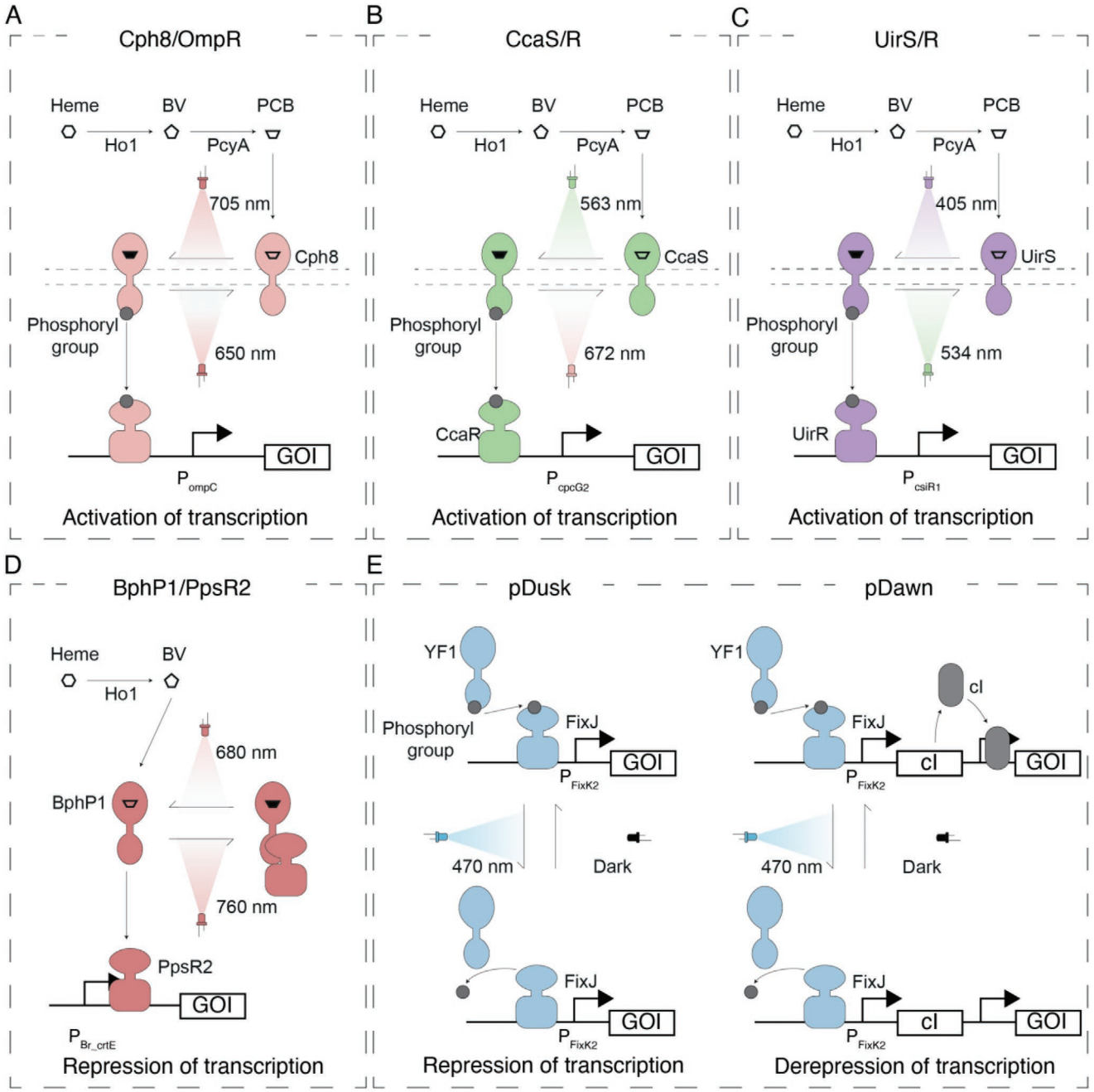


**Figure 5.** Light-inducible two component systems. A) Red/Far-Red light TCS Cph8 activates expression from $P_{ompC}$ upon excitation with 650 nm light and requires synthesis of the chomophore PCB from heme through enzmatic conversion by Ho1 and PcyA.[84,164] B) Green/Red light TCS CcaS/R activates expression from $P_{cpcG2}$ through excitation with 562 nm light and requires synthesis of the chomophore PCB as described in (A).[164] C) UV/Green light TCS UirS/R activates expression from $P_{csiR1}$ via excitation with 405 nm light and also requires synthesis of the chomophore PCB as described in (A). D) Red/Near-Infrared light TCS BphP1/PpsR2 regulates expression from $P_{Br\_crtE}$ via derepression upon excitation with 760 nm light and requires synthesis of the chomophore BV through Ho1-catalzyed conversion of heme. E) pDusk and pDawn are optogenetic systems based on the blue light TCS YF1/FixJ. YF1 uses endogenous FMN as chomophore and regulates FixJ via controlling its phosphorlyation state.[65] pDawn is an extention of pDusk which inverts the light signal through expression of the $\lambda$ cI repressor, which in turn regulates a gene of interest.[176]

functionality was demonstrated.[164] The TCS consists of the sensor histidine kinase CcaS and its response regulator CcaR. Green light induces CcaS (maximal excitation 535 nm) autophosphorylation and subsequent phosphotransfer to CcaR, which initiates transcription from the promoter of the phycobilisome linker protein cpcG2. Red light (672 nm) leads to its inactivation (Figure 5B). CcaS also uses PCB as its chromophore. The activation and inactivation wavelength of green and red light made it compatible with the red/far-red Cph8-based system.[164] Combining the two systems also

allowed for independent control of expression of two genes with different wavelengths of light.[164] These proof of concept systems showed limitations in the dynamic range (<10-fold) which were addressed in subsequent studies through optimization of the expression levels of the sensor and response regulators as well as the output promoter, thereby increasing the dynamic range to 72-fold for the red/far-red, and 117-fold for the red/green system.[165] Nakajima et al.[166] later identified that a miniaturized CcaS, lacking two PAS domains and consisting only of the light-responsive GAF and the histidine kinase domain, improved the dynamic range further, allowing it to reach 593-fold.[167]

#### 4.1.3. UV/Green Light TCS

UirS/UirR,[168] a TCS from the cyanobacterium *Synechocystis* sp. PCC 6803 which is activated with UV (382–405 nm) and inactivated with green light, could successfully be transferred to *E. coli* as well.[169] The light sensor UirS employs PCB as chromophore and the response regulator UirR has a REC domain and an AraC-family DNA binding domain. The *csiR1* promoter, which was proposed to be a target of UirR in *Synechocystis*, was also used.[170] Membrane bound UirS binds UirR and sequesters it to the membrane in the dark state, which is liberated and phosphorylated by UV light. This enables binding to the *csiR1* promoter and transcription initiation with a dynamic range of about 4-fold (Figure 5C).[39]

#### 4.1.4. Red/Near-Infrared Light TCS

A synthetic TCS, which involves the creation of the second messenger cyclic dimeric GMP (c-di-GMP), was engineered using a bacteriochrome diguanylate cyclase (DGC) and was named BphS. Cyclic mono and di-nucleotides are second messengers that regulate diverse processes including transcription, translation, and protein activities and interactions.[171] BphS was engineered from *Rhodobacter sphaeroides* protein BphG1 to enhance DGC activity.[172] Light-induction led to an increase of c-di-GMP levels of 11-fold.[172] The increased DGC activity also led to higher dark-state activity of BphS which required the expression of an additional c-di-GMP phosphodiesterase (PDE, YhjH) to decrease c-di-GMP levels in the dark state. Furthermore, *Klebsiella pneumoniae* MrkH, a c-di-GMP responsive transcription factor, and the corresponding *mrkA* promoter were used to transform the c-di-GMP output from the light sensor into a signal for transcriptional regulation.[172] The system reached a 10-fold activation after a 4 h light input, and increased to 40-fold after an 8.5 h light input.

While c-di-GMP is a common regulator for diverse bacterial functions (e.g., metabolism, cell-wall modification, biofilm formation, cell division, etc.[173]), this system shows shortcomings that limit its use as an orthogonal system for transferring a light-input into a biological effect. However, it could have potential in higher eukaryotes that mostly do not utilize c-di-GMP as second messenger.[172] Further, the relatively slow dynamics limit the applicability of this system,[172,174] especially for fast regulation. To tackle these limitations, Ong et al.[172] transferred the light-sensory BphP1 from *Rhodopseudomonas palustris* CGA009[175] together with PpsR2 into *E. coli*. This system does not function via a second messenger, but BphP1 binds and inactivates the transcriptional repressor PpsR2 when activated with red light (760 nm) and is inactive with far-red light (Figure 5D). The final system showed a 2.5-fold difference in the expression level when comparing dark and light state.[172]

#### 4.1.5. Blue Light TCS

While all of the above TCSs require a tetrapyrrole chromophore which needs to be either added to the medium or synthesized through a heterologous pathway in *E. coli*, Möglich et al.[65] used a LOV photosensor that uses FMN as chromophore, which is natively available in the cell, to create a blue-light activatable TCS. As the basis of their engineering, they used the TCS FixL/FixJ from *Bradyrhizobium japonicum* and replaced the sensory domain of the sensor histidine kinase with the light responsive LOV domain of YtvA from *Bacillus subtilis* due to the structural similarity of their PAS domains. The blue light-induced conformational change and rotational movement of the newly created YF1 light-sensor regulate the kinase/phosphatase activity of FixL, which then regulates phosphorylation of the response regulator FixJ, thus enabling the control of the FixK2 promoter (Figure 5E left).[65] In the dark state, YF1 exhibits net kinase activity, while in the light state it exhibits net phosphatase activity.[65]This system shows a 12-fold difference in the light/dark expression levels of the fluorescent protein DsRED[176] through light-induced repression of the TCS. To achieve light-induced activation, they used the YF1/FixJ expression system to control expression of the $\lambda$ phage repressor cI (Figure 5E right), similar to the inversion of multichromatic applications of the red/far-red Cph8 with the green/red CcaS/R.[164] This inverts the light signal for genes expressed from the $\lambda$ promoter and showed a dynamic range of 430-fold.[176] Lalwani et al.[177] used the pDawn system for optogenetic expression of lacI, hence transforming the lac operon independent from the common inducer IPTG, making its transcriptional activity dependent on light through the concentration of LacI.

### 4.2. One Component Systems

"One component" light systems are defined in this section as those with one or more proteins that become active through light-induced dimerization. They differ from the TCSs of the previous section in that they do not comprise of separate light-inducible sensor and response regulator proteins, but include light-sensitive and effector-domain in the same protein.

Conceptually, four such systems have been published to date: a synthetic light-inducible repressor based on the endogenous repressor LexA, called LEVI and LexRO,[102,178] a light-inducible transcription factor called EL222 that was transferred from *Erythrobacter litoralis* into *E. coli* to function as an activator or repressor,[179] a synthetic light-inducible activator based on the endogenous transcription factor AraC, called BLADE,[180] and light-inducible T7 RNA polymerases named

Opto-T7RNAPs[120,181] which function as heterologous transcription systems. All of these systems employ a LOV domain. LEVI and Opto-T7RNAPs employ the FAD-bound photosensor VIVID (VVD) and its derivative called "Magnets." EL222 and LexRO use FMN as cofactor. In addition to these four concepts for light-inducible transcriptional regulation, the cobalamin binding domain CarH will be discussed, which was not developed for transcriptional control in *E. coli*, but generally could be used as such.

#### 4.2.1. Light-Inducible Repressor Based on an Endogenous Repressor: LEVI/LexRO

Chen et al.[178] engineered a blue light-inducible repressor through fusion of the photosensor VVD to the DNA-binding domain of LexA ($LexA_{DBD}$) which they named "LEVI" (**Figure 6**A left). The repressor shows a high dynamic range of 10 000-fold and similar to Ohlendorf et al.[176] they used the $\lambda$ phage repressor cI and the corresponding promoter to invert the signal, which led to a reduction in the fold-induction to 1000-fold. While showing good fold-induction, it has to be considered that LexA regulates the SOS regulon in *E. coli*, which initiates DNA repair and mutagenesis through error-prone DNA polymerases. LexA represses at least 20 genes and is proteolytically cleaved upon DNA damage. RecA initiates this by sensing DNA damage at stalled replication forks which in the native context leads to binding and activation of autoproteolysis of LexA.[182] Targeting of endogenous LexA-responsive genes by LEVI was very recently addressed by Li et al.[102] through the use of a mutant promoter and LexA variant.[183,184] Li et al.[102] also used $LexA_{DBD}$ and a LOV domain from *Rhodobacter sphaeroides* (RsLOV). In contrast to VVD which was used in LEVI, it monomerizes with blue light and dimerizes in the dark to create LexRO (Figure 6A right). This changed the property of the regulator from light-induced repression to light-induced transcription through release of the repressor. By using the LexA408 repressor domain which recognizes an operator mutant in promoter $colE_{408}$, the authors decouple the repressor from the endogenous network.

#### 4.2.2. Light-Inducible Activator Based on an Endogenous Activator: BLADE

To make an endogenous transcriptional activator in *E. coli* light-inducible, the DNA-binding and transcription activation domain of the regulator AraC ($AraC_{DBD}$) was fused to light-inducible LOV homodimerization domains VVD and VfAu1.[180] These blue light-induced AraC dimers in *Escherichia coli* (BLADE) enable activation from the $P_{BAD}$ promoter through binding of light-inducible homodimerization and binding of the $AraC_{DBD}$ to I1-I2 operator sequences (Figure 6B). Light-induced expression from a synthetic promoter containing two I1 binding sites was also shown. Both N- and C-terminal fusion of photosensors to the AraC domain created functional constructs through applying a general optimization strategy for optogenetic regulators and development of a high-throughput 96-well light induction device.

#### 4.2.3. Light-Inducible Heterologous Transcription System: Opto-T7RNAPs

To exploit a commonly used orthogonal transcription system, light-inducible T7 RNA polymerases (Opto-T7RNAPs)[120,181] were created by splitting the polymerase into two nonfunctional parts, which were fused to light-inducible Magnet dimerization domains. This strategy was based on previous studies that showed that the T7RNAP can be split into nonfunctional parts and activity can be restored with SYNZIP domains[185,186] or through trans splicing.[187] Through light induction, the two nonfunctional T7 split parts are aligned and come in close spatial proximity which reconstitutes their function, leading to expression from a T7 promoter (Figure 6C). This was demonstrated by reaching a light-induced fold-induction of 300-fold and dynamic expression profiles.[120]

#### 4.2.4. Light-Inducible Orthogonal Repressor/Transcription Factor: EL222

Motta Mena et al.[188] created a light-inducible mammalian gene expression system by fusing the trans-activator from *Herpes simplex* VP16 that induces transcription with the light-inducible DNA-binding protein EL222 from the bacterium *Erythrobacter litoralis* HTCC2594. EL222 contains a LOV domain and a helix-turn-helix (HTH) DNA-binding domain, which is characteristic of LuxR-type DNA-binding proteins.[68] Light leads to the release of the HTH 4$\alpha$ helix essential for dimerization and DNA binding, which in the dark is bound to the LOV domain.[68] This allows for light-induced binding to promoter sequences engineered to contain the corresponding binding sequence, and initiate transcription via the transactivation domain. Jayaraman et al.[179] used unmodified EL222 also for transcriptional activation and repression in *E. coli*. For this, they exploited the similarities of EL222 with LuxR and replaced the lux box from the luxI promoter, which is usually bound by LuxR, with the EL222 binding sequence. Blue light-induction initiates EL222 binding to the promoter and recruits RNA polymerase for transcription initiation (Figure 6D left) similar to LuxR-type transcriptional activators with a fold change of up to 5-fold. To also create a light-induced repression system, they expanded previous work that demonstrated that LuxR can be converted into a transcriptional repressor by placing the lux box in between consensus −35 and −10 regions.[189] For this, they again replaced the lux box with EL222 binding sites to block initiation of transcription through steric hindrance of RNA polymerase binding from the promoter (Figure 6D right), which showed a 3-fold reduction of fluorescence with blue light-induction.[179]

#### 4.2.5. Light-Inducible Adenylate Cyclase: CarH

To study the interaction of cobalamin binding domains, Ortiz-Guerrero et al.[190] used a previously described system for the study of protein interactions in *E. coli* as a model organism. This system consists of two complementary fragments T25 and T18 of *Bordetella pertussis* adenylate cyclase. Dimerization of these domains leads to functional reconstitution of the

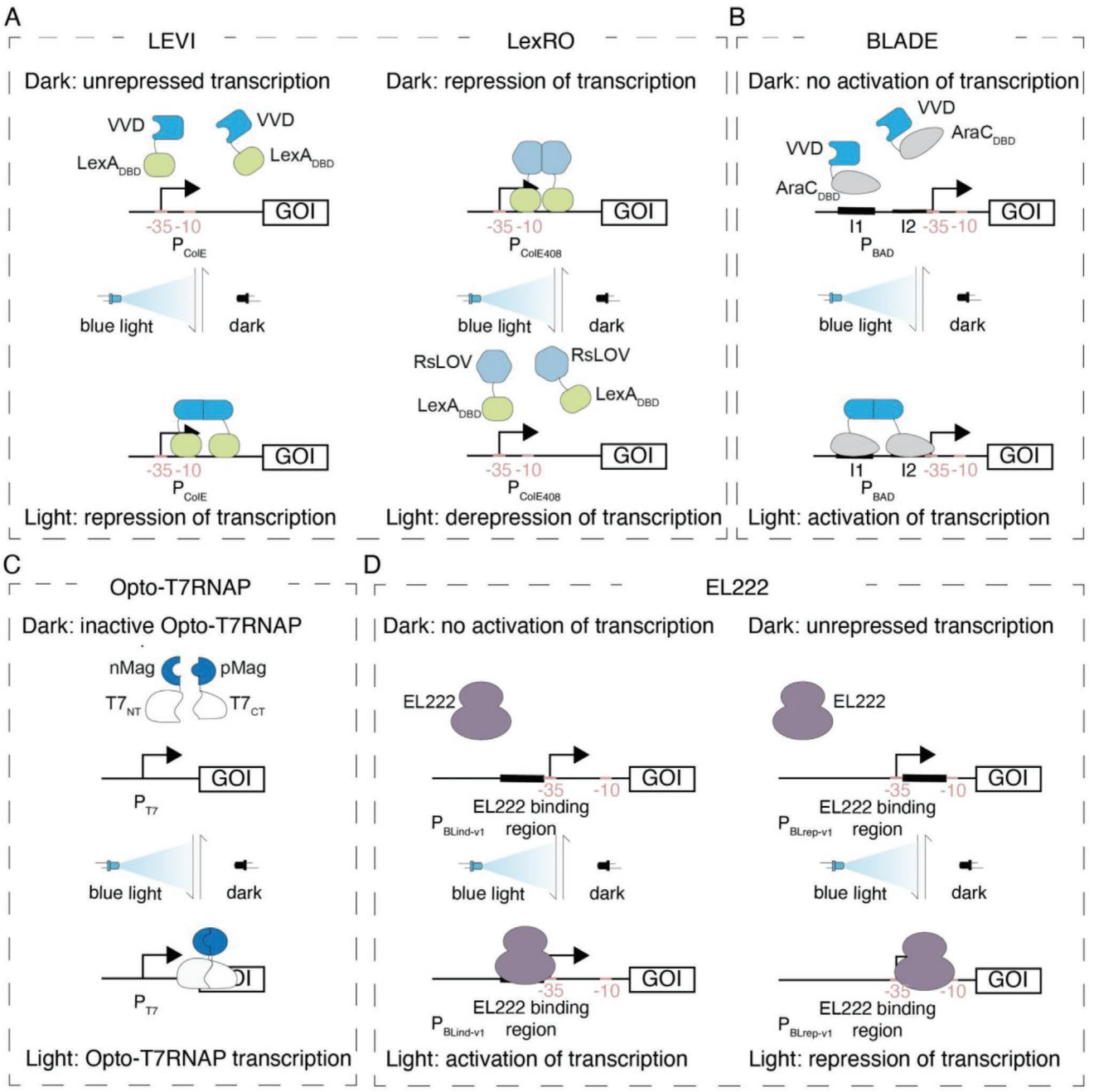


**Figure 6.** Blue light-inducible one component systems. A) LEVI is a light inducible repressor created through fusion of the photosensory protein VVD and the DNA binding domain of the *E. coli* repressor LexA allowing for regulation of the cognate $P_{ColE}$ promoter through light-induced dimerization.[178] Exchanging the photosensor VVD with the photosensor RsLOV inversed the light signal to create the light-inducible derepression system LexRO.[102] B) Blue Light-inducable AraC Dimers in *E. coli* (BLADE) are light-inducible activators which employ the DNA-binding and transcriptional activation domain of the *E. coli* transcription factor AraC and enable light-controlled expression from the $P_{BAD}$ promoter through light-induced dimerization and binding of I1-I2 operator half-sites.[180] As photosensory domains, two different LOV domains, VVD and VfAu1, were used. C) Opto-T7RNAPs are engineered light-inducible variants of the T7 DNA-dependent RNA polymerase that was split into nonfunctional parts and fused to light-inducible Magnet domains. Light initiates specific binding of the T7 polymerase split fragments, which reconstitutes the function of the enzme and initiates transcription from the $P_{T7}$ promoter.[120] D) EL222, which contains a LOV domain and a helix-turn-helix (HTH) DNA-binding domain, was successfully transferred from the bacterium *Erythrobacter litoralis* to *E. coli*. By replacing the lux box from the *luxI* promoter with the EL222 binding sequence, the promoter $P_{BLind-v1}$ was created which allows for activation of transcription. Through placing the EL222 binding sequence in between the –35 and –10 promoter regions, the light-repressable $P_{BLrep-v1}$ promoter was created.[179]

enzyme and cyclic adenosine monophosphate (cAMP) synthesis, which induces expression of lacZ through transcriptional activation by cAMP-CAP. LacZ concentration in turn can be colorimetrically measured with 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-Gal) or *o*-Nitrophenyl-$\beta$-galactoside (ONPG). Their in vivo analysis revealed that CarH or its C-terminal domain self-interact only in the presence of B12.[190] While this system was used to verify functionality of CBDs in vivo in general and in *E. coli* in particular, it holds promise for future development of light-regulated systems using CBDs. The use of the light-activated adenylate cyclase for gene expression in *E. coli,* however, is extremely limited, as cAMP induces expression of a large number of endogenous genes. cAMP is a second messenger found in all three domains of life.[191] In *E. coli*, it is involved in carbon catabolite repression, which describes the order that different carbon sources are catabolized involving regulatory positive and negative feedback networks. As one major component of this regulation, cAMP binds to the pleiotropic transcriptional activator catabolite activator protein (CAP, or catabolite/cAMP receptor protein CRP) which activates expression of proteins for secondary carbon source metabolization.[191,192]

Although promising, CBD-based systems will show limitations regarding dynamic regulation with light, as light-induced inactivation of the chromophore leads to a covalent adduct with the apoprotein, therefore rendering it insensitive to newly synthesized or added chromophore, as discussed in the previous section.

### 4.3. Photocaged Molecules

Photocaged molecules implement light-control through photolytic release of photosensitive groups, or isomerization of chemicals into biological processes. Although these approaches are very sophisticated and useful for certain applications, most of the current methods show limitations in making use of the spatiotemporal features of optogenetic strategies.

Two of the biotechnologically very relevant inducers, IPTG and arabinose, have been photocaged in recent years. IPTG was photocaged with 6-nitropiperonal (NP), which sterically hinders binding to LacI (Figure 2A).[193] UV light with 365 nm wavelength was used to release the caging group in a time and concentration dependent manner. The half-life for the conversion was 11 s at a concentration of 0.1 mM, 5.1 min at 0.5 mM, or 11.8 min at 1.0 mM. Uncaging achieved a 10-fold activation of expression from a lac promoter. It was further shown that caged IPTG is nontoxic and it is taken up by the cell.[193] Also, arabinose was photocaged with 6-nitropiperonylalcohol (NP) and 1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethanol (NBE) to inhibit binding to AraC with reported half-lives at 8.1 mM of 19.1 min for NB caged and 13.7 min for 6.9 mM NBP caged arabinose.[194] It was hypothesized that passive diffusion of the caged arabinose into the cell leads to a more homogeneous gene expression compared to uncaged arabinose.[194] The tetracycline analogue doxycycline was also previously photocaged,[195–197] but it has not yet been shown if it can also be applied for gene expression control in bacteria. Along with in vivo experiments that demonstrated that caged doxycycline can be used to induce cells, DMNPE-caged doxycycline was shown to quickly cross the membranes of unilamellar vesicles assessed by isothermal titration calorimetry.[197] Also, control of translation through NP-caging of theophylline was shown, which has been used as an inducer for hammerhead riboswitches.[198]

The photocaged T7RNAP from Chou et al.[24] represents a photocaged protein for control of gene expression in bacteria. The general strategy to implement a photocaged amino acid was described in a previous section. In this work, NB-photocaged tyrosine was introduced into the active site of the polymerase, inhibiting its function. Upon UV-induced photocleavage, the caging group is released and the polymerase is able to transcribe from the cognate T7 promoter. Functionality was shown in both mammalian and bacterial cells. Application of this approach requires extension of the biosynthetic machinery of the cells and cognate tRNA for incorporation of the caged amino acid at positions of an amber stop codon.

### 4.4. Exploiting Light Sensitivity of Inducer Molecules: aTc

Another approach for transcriptional regulation using light in *E. coli* is conceptually different from the previously described photoactivatable compounds or optogenetic proteins and relies on the photosensitivity of bioactive chemicals. Photosensitive molecules contain light absorbing moieties similar to photoactivatable compounds. However, in photoactivatable compounds, photosensitive protecting groups usually render a molecule inactive until the protecting group is released via light, which "activates" the compound. In contrast to these concepts, photosensitive molecules do not contain protecting groups, but are chemically transformed through light, which in some cases can inactivate their specific biological function. Such light-inactivation was shown for the transcriptional inducer anhydrotetracycline (aTc), which binds to the repressor TetR.[199] The addition of aTc activates gene expression from TetR controlled promoters, which was shown to be reversible by applying UVA light (**Figure 7**A). This method shows the unique feature compared to other light-regulation approaches, namely "dynamic and setpoint chemo-optogenetic control." This means, that the concentration of the molecule can be changed by either adding more of the molecule or inactivating it with light (Figure 7B). Without addition or inactivation, the concentration of the molecule, and therefore its activity, stays constant. This is inherently different to photoactivatable proteins, which inactivate after a certain time depending on their dark-reversion rates, and to photoactivatable molecules which can only be activated and not inactivated. As aTc was not modified, uptake into cells is not altered compared to the previous use of the inducer.

## 5. Conclusion

Numerous approaches for implementation of light-control into cells via chemical and genetic approaches have led to generalizable principles, many of which are discussed in this review. These principles, together with an understanding of the properties that the different approaches for light-control possess, will allow for faster and precisely tailored engineering of

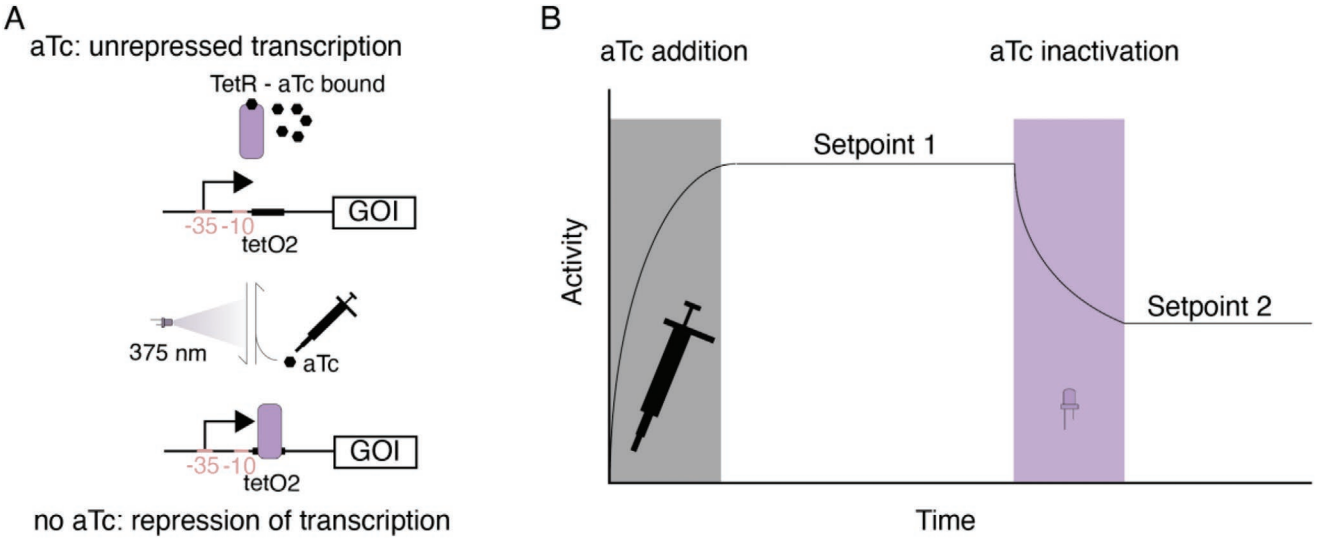


**Figure 7.** Photosensitivity of aTc used for light-control. A) The transcriptional repressor protein TetR binds tetO operator sequnences in the absence of aTc. In the precense of the transcriptional inducer aTc, TetR unbinds tetO leading to derepression and initiation of gene expression. aTc can be added or inactivated through light to modulate TetR activity.[199] B) aTc addition and its light-inactivation changes the corresponding TetR binding activity. When no such input is applied, the concentration and thus the activity of TetR remains constant at this setpoint.

light-sensitivity into biological systems to fulfil specific tasks. The resulting systems hold significant promise for engineering advanced biosystems with novel applications.

## Acknowledgements

The authors wish to thank Dr. Stephanie Aoki for proof reading the manuscript. This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (CyberGenetics; grant agreement 743269), and FET-Open research and innovation actions grant under the European Union's Horizon 2020 research and innovation programme (CyGenTiG; grant agreement 801041). The authors sincerely apologize to all colleagues whose work was not cited due to space limitations.

## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

bacterial optogenetics, cybergenetics, light control, light-inducible transcription, optogenetics, photoactivation

Received: September 4, 2020
Revised: January 11, 2021
Published online: February 9, 2021

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