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REVIEW

Optogenetics: past, present and future

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ABSTRACT The term 'optogenetics' was introduced into the scientific literature less than a decade ago by Karl Deisseroth, developer of pioneering optogenetic techniques, who defined optogenetics as "the combination of genetic and optical methods to achieve gain or loss of function of well-defined events in specific cells of living tissue". Since then this new field of biology has become a very exciting and rapidly developing area producing hundreds of scientific publications. New methods and tools have been developed and long-sought answers found in these new experimental systems. Discussion and full elaboration of every optogenetic approach and application are beyond the scope of this review, instead, it gives a short insight to (i) how light can be used to manipulate the membrane potential of various cells; (ii) how light-sensitive proteins can be used to regulate targeted gene expression, and (iii) how controlled release or spatio-temporal targeting of certain molecules can be modulated by light. Besides, the most widely used light-sensor proteins, including their structure, working mechanism and their involvement in existing optogenetic applications are also discussed.
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KEY WORDS

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Introduction

The term 'optogenetic' was mentioned in scientific communication for the first time in 2006 (Miller 2006), and some years later the PubMed (pubmed.gov) search engine listed more than 1400 hits for this expression (as of November 2014). The reason of this tremendous emergence is the rapid spreading of this 'cutting edge' technology and its adoption in new fields and applications. There are several different definitions for optogenetics. The first neurobiological applications limited the usage of the term for a relatively narrow subject, "genetic targeting of specific neurons or proteins with optical technology for imaging or control of the targets within intact, living neural circuits" (Deisseroth et al. 2006). When Nature Methods introduced optogenetics as "Method of the Year 2010" (issue January 2011), it was clear that a new definition with much broader meaning has to be announced. Karl Deisseroth, developer of pioneering optogenetics techniques, defined optogenetics as "the combination of genetic and optical methods to achieve gain or loss of function of well-defined events in specific cells of living tissue" (Deisseroth 2011). This definition includes the necessity of genetic intervention together with light control, and also defines the output broadly enough: it can be any detectable change of the living organism.

The possibility to use light for controlling neural activity was proposed for the first time by Francis Crick in 1999 (Crick 1999). Soon the first genetic manipulation which led to photosensitizing selected neurons was reported by the laboratory of Gero Miesenböck (Zemelman et al. 2002; Zemelman et al. 2003). Shortly afterwards modified ion channels were introduced into cell membranes and light was used to alter the ion flow of the cell (Banghart et al. 2004; Volgraf et al. 2006). The next milestone was a study from 2005, which describes photocontrol of animal behaviour by illumination of a genetically modified group of neurons (Lima and Miesenbock 2005). In the same year, the first use of channelrhodopsin as a single-component optogenetic tool was reported in cultured mammalian neuron cells (Boyden et al. 2005) and in *Caenorhabditis elegans* (Nagel et al. 2005). This approach became routinely applied in various cell types/organisms to induce specific responses and alter the behavioural pattern of the various animals. Honouring their pioneering contribution, the 2013 Grete Lundbeck European Brain Research Prize was awarded to Ernst Bamberg, Edward Boyden, Karl Deisseroth, Peter Hegemann, Gero Miesenböck, and Georg Nagel 'for their invention and refinement of optogenetics' (Reiner and Isacoff 2013).

We cannot undertake presenting every existing optogenetic application, thus only some key methods/technology will be demonstrated here. This review gives a short insight

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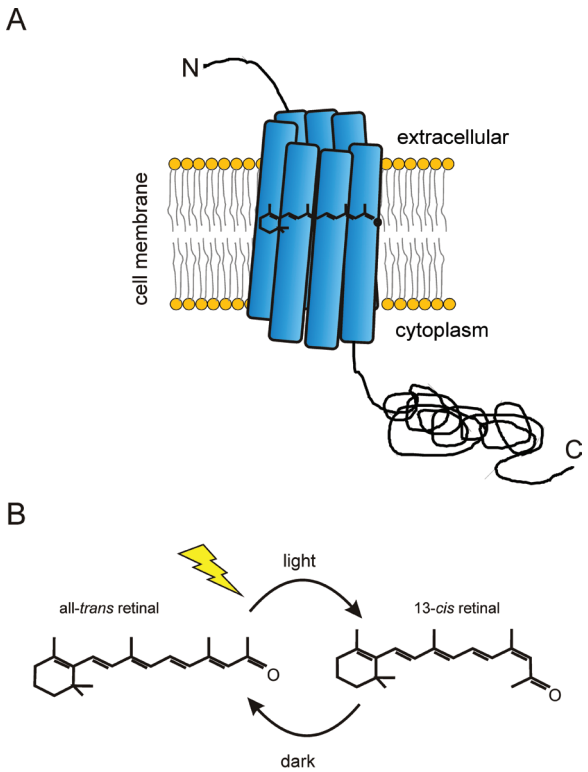

Figure 1. Structural features of microbial opsins. A.) Schematic drawing of microbial opsin holoproteins. Blue bars represent the typical seven transmembrane helixes, black lines mark the N- and C-termini of the polypeptide chain. The cytoplasmic C-terminal part of the molecules is variable in length and structure. The location of retinal molecule hidden between the transmembrane domains is also indicated. B.) Light dependent conformational changes of retinal attached to microbial opsins.

to (i) how light can be used to manipulate the membrane potential of various cells; (ii) how different light-sensitive proteins can be used to regulate targeted gene expression, and (iii) how controlled release or spatio-temporal targeting of certain molecules can be modulated by light.

Opsin proteins used in optogenetic applications

Opsins are a group of light-sensitive 35–55 kDa membrane-bound light receptors divided into two groups. Type I opsins can be found in prokaryotes and algae, whereas Type II opsins are only present in animals (Nagel et al. 2005; Terakita 2005). Although these groups do not show sequence homology to each other, the proteins have similar structure and function. The animal opsins are G-protein-coupled receptors, and mainly function in vision (and regulate circadian rhythm and pigment regulation), but they are not widely used in optogenetic applications, thus are not considered further here (Shichida and Yamashita 2003).

We will introduce three groups of Type I microbial opsins that are able to regulate membrane conductance. The (i) protein structure, (ii) mode of action and (iii) possible optogenetic applications of these opsins will be discussed.

Channelrhodopsin, a light-gated ion channel

Phototaxis is a kind of locomotory movement, which occurs when the direction of the movement of the whole organism depends on the direction of light. The presence of this ability among unicellular green algae is vital in order to optimize their access to light, which can drive efficient photosynthesis. A wide range of algal species owns an organelle named eyespot apparatus. Whereas its existence was observed more than a century ago, its light-sensing function was shown only in 1980 by Foster and Smyth (1980). The structure of the eyespot apparatus was examined extensively (for reviews see: Dieckmann 2003; Kateriya et al. 2004; Kreimer 2009), but the basis of its light sensitivity was not understood for a long time. Because these organisms do not possess nervous systems, their light-controlled phototactic responses must be somehow directly connected to the eyespot, which is responsible for light sensitivity. Patch clamp experiments revealed that light sensing results in depolarization of the cell membrane (cation influx), and leads to cilia- or flagella-driven cell movement (Sineshchekov and Govorunova 1999). Several reports demonstrated that the observed cation (mainly Ca^{2+}) influx requires the presence of vitamin A aldehyde, retinal (Foster et al. 1984; Hegemann et al. 1991). Based on the known structure and dynamics of the animal rhodopsins, it was suspected that similar rhodopsin-type molecules are responsible for phototactic algal responses (Harz and Hegemann 1991). The assumption of this early study was later confirmed when two rhodopsins-like sequences were identified in a *Chlamydomonas* cDNA database and named CHANNELRHODOPSIN 1 and 2 (ChR1 and ChR2, respectively). It turned out that the homologs of these proteins can be found in many algal species, they are located in the eyespot apparatus and responsible both for triggering light-induced membrane depolarization and related phototactic movements (Nagel et al. 2002; Sineshchekov et al. 2002; Nagel et al. 2003).

ChR1 and ChR2 apoproteins consist of about 700 amino acid (aa) residues. Whereas the C-terminal 400 aa stretch forms various structures with unknown function, the N-terminal 300 aa region forms a characteristic conserved transmembrane structure (Fig. 1A). These 7 transmembrane helixes (i) are responsible for the correct positioning of the molecule in the membrane, (ii) bind the chromophore and (iii) are sufficient for light-initiated ion flux (Nagel et al. 2002; Sineshchekov et al. 2002). The helixes are arranged in a ring

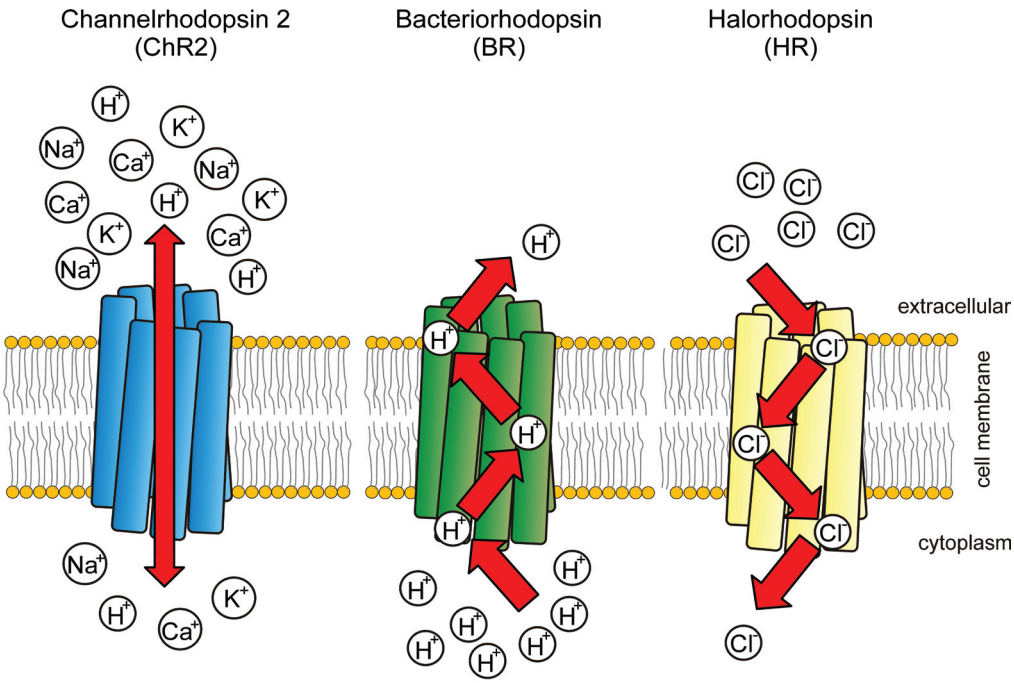

Figure 2. Functional features of microbial opsins. Channelrhodopsin 2 acts as a light-induced cation channel, bacteriorhodopsin functions as a light-induced outward proton pump, whereas halorhodopsin functions as a light-induced inward chloride pump.

shape forming a hydrophobic pocket inside the structure. A conserved lysine residue is positioned here and anchors the retinal chromophore with a covalent bond forming a protonated Schiff-base (Spudich et al. 2000; Muller et al. 2011; Kato et al. 2012). The light sensitivity of retinal provides the basis of channelrhodopsin photoreception. The all-trans to 13-*cis* retinal photo-isomerization (Fig. 1B) takes place around the C13=C14 bond on a ns scale after the onset of blue light (absorption maximum 470 nm) illumination (Bamann et al. 2008). This process results in the channelrhodopsin conformational change opening a 0.6 nm pore between the transmembrane helixes allowing cations to flow into the intracellular space (Richards and Dempski 2012). Whereas natural ChR1 acts as a proton channel, ChR2 is a nonspecific cation channel allowing H^+, Na^+, K^+, and Ca^{2+} passage (Fig. 2). 13-*cis* retinal performs thermal relaxation in darkness (within seconds) leading to closing the pore of the ChR molecule and arresting the ion flux (Bamann et al. 2008). Retinal remains associated with the protein partner during these processes and can maintain fast conformational changes repeatedly. Despite revealing the molecular and mechanistic basis of unicellular algal phototaxis, channelrhodopsins suddenly became interesting molecules in 2003, when functional ChR2 was successfully expressed in *Xenopus* and mammalian cells, and was able to depolarize the cell membrane (Nagel et al. 2003). Boyden and co-workers (2005) developed a lentiviral expression system, which was able to introduce and express ChR2 in mammalian neurons and control hyperpolarization events at millisecond scale by applying brief pulses of light. Within some months after the appearance of this publication, successful applications of ChR2 were documented in several different animal systems: intact brain (Ishizuka et al. 2006), spinal chord (Li et al. 2005), living worm (Nagel et al. 2005) and in the retina (Bi et al. 2006). These pioneering studies used ChR2. It turned out that ChR2 can be expressed at much higher levels than ChR1 and produces higher photocurrents, thus it proved to be a better optogenetic tool. Luckily, the naturally occurring retinal levels are sufficient for ChR function in the examined tissues, thus additional chromophore supplementation is not necessary for optical control of the examined cells (Zhang et al. 2006). Considerable advancements opened up this field for further ChR applications. Numerous ChR variants were cloned and described from different algal species, and more are expected to come as more and more microbial genomes are sequenced (Govorunova et al. 2011; Kianianmomeni et al. 2009; Lin et al. 2009; Zhang et al. 2011; Rein and Deussing 2012; Sineshchekov et al. 2013). Additionally, a vast number of different ChR mutants (mainly molecules carrying amino acid substitutions) were created and functionally characterized. Examining these protein versions help us to understand the essential mechanisms behind the dynamics of ChR-induced ion flux. Additionally,

these mutant ChRs are available as modified optogenetic tools with different photocurrent properties, different light wavelength responsiveness and enhanced kinetic stability (Berndt et al. 2009; Lin et al. 2009; Gunaydin et al. 2010; Lorenz-Fonfria and Heberle 2014). Among many mutant versions of ChR2, ChETA in which the glutamic acid at position 123 is exchanged to a threonine or alanine has many beneficial properties for neurobiologists. This molecule shows reduced desensitization during light exposure, and the active conformation of retinal is destabilized resulting in faster reversion to the inactive state in darkness. Thus ChETA works with reduced extra spikes, reduced plateau potentials at higher frequency excitation, and closes the ion channel faster than its wild-type counterpart in intact mammalian brain tissue (Gunaydin et al. 2010). Nowadays ChR2 is the most widely used optogenetic tool among the available microbial rhodopsins. Besides having already been used in many different tissue cultures, it was successfully introduced into living animals including a broad range of species such as *Caenorhabditis elegans*, *Drosophila*, zebrafish, mouse, rat and non-human primates (for a review, see Fenno et al. 2011).

Despite the tremendous success that ChR-based optogenetic research has achieved so far, ChR engineering has certain limitations. Fundamental changes in ion selectivity of the molecule or its conductance are not expected to be altered in the future. Combination of ion channel proteins with other receptor domains anchoring e.g. flavin-based chromophores, however, could be an interesting future approach to create light-sensitive ion channels with yet unknown properties.

Archaerhodopsins/bacteriorhodopsins, light-driven proton pumps

Bacteriorhodopsin (BR) was identified in the 1960s as "purple pellet" in *Halobacterium salinarum* membrane fraction. Its roles are building up and maintaining a proton gradient between the extra- and intracellular space (Oesterhelt and Stoeckenius 1973). The bacteria use this chemical force to fuel ATP synthesis under anaerobic conditions (Racker and Stoeckenius 1974). BR became the first bacterial opsin with a characterized function and a structure described in detail (Henderson and Unwin 1975; Henderson 1977; Khorana et al. 1979; Stoeckenius et al. 1979). This 248-aa protein possesses seven transmembrane helixes and holds a retinal chromophore molecule attached to a conserved lysine by a protonated Schiff base. Additionally, BR molecules are arranged into trimers (Katre et al. 1981; Luecke et al. 1999). Upon absorbing a photon (yellow light, max = 568 nm), retinal isomerizes from all-*trans* retinal to the 13-*cis* form, and BR pumps a proton from the cytoplasm to the extracellular space (Fig. 2) (Drachev et al. 1974; Lozier et al. 1975). Within 100 ms, retinal relaxes back to all-trans form, and the BR molecule becomes ready for the next photocycle (Varo and Lanyi 1991). The precise details of the proton transfer were revealed and summarized by the end of the 1990s (Lanyi 2004). Bacterio-rhodopsin protein and its activity are stable at room temperature, and this robustness was one of the reasons why BR was the first bacterial opsin to be chosen for expression in animal cells. BR was successfully expressed in *Xenopus* oocytes, and these studies provided further functional characterization of the molecule (Nagel et al. 1995; Chen et al. 2002). Later, when BR was expressed in neurons, it turned out that it can hyperpolarize cells by pumping out protons, thus can act as an optical neural silencer (Chow et al. 2010).

A BR homolog was isolated from *Halorubrum sodomense* called archaerhodopsin-3 (Arch) that works as an outward proton pump when illuminated with yellow light (max = 566 nm). This pump can be expressed at high levels in neurons, is able to maintain near 100% silencing in the targeted cells in the mouse cortex, and shows rapid recovery after prolonged irradiation (Chow et al. 2010). Soon Arch became a more convenient optogenetic tool than BR, supported with conveniently applicable protocols and mutant protein versions with various characteristics (Gong et al. 2013; Flytzanis et al. 2014).

Halorhodopsin, a light-driven chloride pump

Additional to BR, an ion pump with light-dependent chloride import activity was also observed in *Halobacterium halobium* and was named halorhodopsin (HR) (Matsuno-Yagi and Mukohata 1977). Upon illumination, HR builds up the inside negative membrane potential by pumping Cl^- ions into the cell (Fig. 2). This increased potential drives the "A-type" H-ATP synthase resulting in proton uptake, thus alkalization of the cytoplasm (Schobert and Lanyi 1982). HR structure is similar to BR, as the transmembrane domains surround a conserved lysine bound retinal, and HR molecules are also arranged into trimers (Kolbe et al. 2000). The absorption peak of *Halobacter* HR is 578 nm, whereas it is 600 nm in the case of another HR variant isolated from *Natromonas pharaonis* (NpHR) (Scharf and Engelhard 1994). Solving the problem of low expression level and suboptimal intracellular localisation, the codon-optimized version of NpHR became a widely used optogenetic inhibitory tool, and proved to be functional in living worms, brain slices, cultured neurons and even in human retina (Han and Boyden 2007; Zhang et al. 2007; Busskamp et al. 2010). Zhang and colleagues used NpHR to control the movement of *Caenorhabditis elegans* (Zhang et al. 2007). Witten et al. (2010) could inhibit the cocaine-induced activity of cholinergic neurons in living mouse by targeted NpHR introduction and light illumination. Tye and colleagues (Tye

et al. 2011) could control a complex behavioural trait like anxiety of freely moving mice by simultaneous application of NpHR and channelrhodopsin optogenetic tools. The combination of these two molecules is ideal within the same cell due to their different absorption peak wavelengths (470 vs. 600 nm). This experimental setup allows the researchers to excite or inhibit the targeted neurons by illuminating them with different light wavelengths.

Now in 2014 the number of available channels and pumps are still growing. There are fundamental differences between pumps and channels. Pumping always takes place against the electrochemical gradient, and slows down when the gradient is high. Thus for efficient pumping not only sufficient light but also suitable ion concentrations are required, which can be modified within certain limits in a living tissue/organism. These are the main reasons why there are more mutant channels available than modified ion pumps. Yizhar and colleagues summarized and organized many optogenetic tools available for different applications (Yizhar et al. 2011).

It is also important to note that the development of microbial opsin based optogenetic applications does not depend solely on the discovery/creation of new opsin molecules. Together with these achievements, developing new transfection/transformation methods are also necessary. Not only the molecular biological backgrounds of experimental approaches but also finding the best transfection carriers (viruses) are necessary for success. Finally, creation and improvement of state-of-art electronic devices are required for optimal light direction (laser light sources, optical fibers) and observation (wide variety of microscopic techniques) of deeply hidden tissues in living organism in a non-invasive manner. Development of proper signal detection equipments to reach optimal sensitivity is also an indispensable part of these approaches. It is clear that parallel development of the above listed components for successful optogenetic techniques will be necessary in the future.

Optogenetic applications of plant photoreceptors

Light is one of the key environmental signals for plants, being the energy source for photosynthesis and also a signal for photomorphogenic (light-dependent) development. Since plant survival depends on the availability of external light, plants have evolved a wide variety of highly sensitive photoreceptors to monitor the electromagnetic spectrum from UV-B (ultraviolet B) to far-red light (~300-750 nm). Until now 13 different photoreceptor molecules have been identified in the widely used model plant *Arabidopsis thaliana*. They can be assigned into three major classes, namely (i) the red/far-red perceiving phytochromes (phyA-E) (Franklin and Quail 2010); (ii) the blue light-sensing cryptochromes (CRY1-3) (Chaves et al. 2011), phototropins (PHOT1 PHOT2) (Sakai et al. 2001) and Zeitlupe-type proteins (ZTL, FKF1 and LKP2) (Nelson et al. 2000; Somers et al. 2000; Schultz et al. 2001); (iii) and the recently identified and characterized UV-B-specific UVR8 (Rizzini et al. 2011).

Photoreceptor proteins have modular structure; they consist of discrete domains. These protein units have conserved structure and function including sensory function, signal transduction, protein interaction, dimerization, kinase activity etc. The sensory part of the photoreceptor protein cradles a light-sensing non-protein photopigment called chromophore attached to conserved amino acid residue(s). This cofactor molecule absorbs a photon of certain energy (defining the wavelength sensitivity of the receptor), resulting in conformational change of the holoprotein. This leads to altered interaction capacity toward signalling partners, thus initiating/changing specific signal transduction pathways. These photoreceptor-governed pathways are responsible for the photomorphogenic development of plants.

The next section will describe the structure and function of plant photoreceptors and how they can be used in different optogenetic applications controlling diverse cellular processes in heterologous systems.

UV RESISTANCE LOCUS 8 (UVR8), a UV-B-specific receptor

UV-B radiation (λ = 280–315 nm), an integral part of sunlight, has strong impact on terrestrial ecosystems (Ballare et al. 2011). Despite its harmful effects, UV-B radiation also triggers UV-B acclimation and UV-B stress tolerance, and to achieve this the presence of active UV-B sensor molecules are necessary (Jenkins 2009). UV-B perception is facilitated by the UV RESISTANCE LOCUS 8 (UVR8) photoreceptor identified recently in *Arabidopsis.* It was also demonstrated that UVR8 exists as a homodimer, which splits to monomers upon UV-B exposure (Rizzini et al. 2011). After monomerization, UVR8 interacts with the WD40-repeat domain of the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1). These two proteins accumulate in the nucleus, where they regulate the expression of genes necessary to build up protective responses against UV-B (Oravecz et al. 2006; Tilbrook et al. 2013).

Sequence analysis of UVR8 showed that the protein backbone is particularly rich in tryptophan, a UV-absorbing aromatic amino acid. Of the 14 tryptophans identified in UVR8, UV-B perception is mediated by a chromophore made up of at least W285 and W233, which directly absorb the UV-B photons and become excited. In the excited state of W285 and W233 a number of intramolecular cation-π

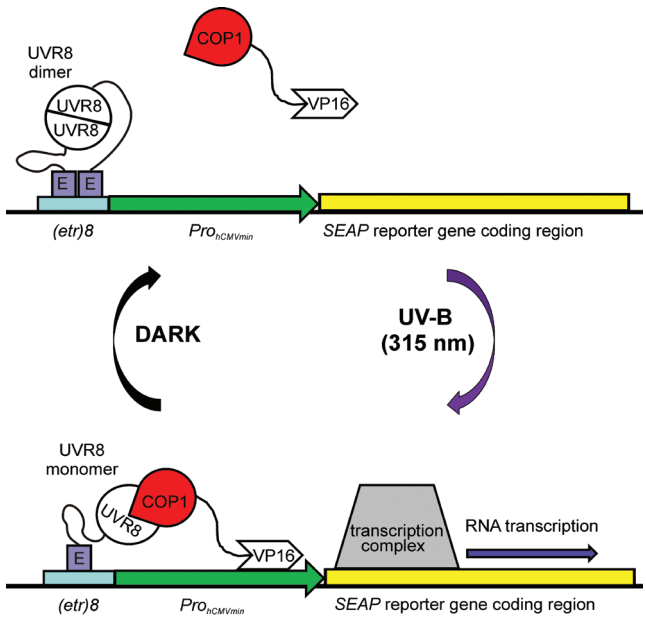

Figure 3. Ultraviolet-B-dependent induction of transcriptional activity. Schematic illustration of the UV-B-induced interaction of the UVR8-macrolide repressor E fusion protein and the COP1 fused to the Herpes simplex-derived transactivation domain VP16. The response construct contains an octameric E-responsive operator motif *(etr)8* upstream the minimal cytomegalovirus promoter (Pro_{CMVmin}) and the reporter *SECRETED ALKALINE PHOSPHATASE (SEAP).* Absorption of UV-B light triggers the dissociation of UVR8 dimers to the monomeric form and the direct binding of COP1, leading to the recruitment of COP1-VP16 to the promoter and the subsequent induction of *SEAP* expression.

interactions with surrounding residues, in particular with R286 and R338 are disturbed. These disrupted interactions in turn destabilize the intermolecular hydrogen bonds of the UVR8 homodimeric interface, leading to homodimer dissociation and the initiation of UV-B signalling (Christie et al. 2012; Wu et al. 2012). As for any photoreceptor, inactivation and ground ("dark") state reversion of UVR8 is of great importance. UVR8 reverts back to its homodimeric ground state by redimerization, which simultaneously stops UV-B signalling and restores UV-B responsiveness (Heijde and Ulm 2013; Heilmann and Jenkins 2013). Regeneration of the UVR8 dimer following UV-B exposure occurs much more rapidly *in vivo* (1-2 hours) than *in vitro* (24-48 hours) (Heijde and Ulm 2013).

UVR8-mediated UV-B perception and the subsequent UVR8-COP1 interaction is a central, primary mechanism for UV-B signalling. The specificity and sensitivity of UVR8 to UV-B predestine that it will be a promising new tool for optogenetics. The first applications of UVR8 in novel optogenetic systems were recently reported, whereby UV-B was used to control nuclear retention, chromatin association, protein secretion and gene expression in mammalian cells. Activation of transcription induced by ultraviolet-B light was achieved by utilizing the protein–protein interactions of UV-B-dependent UVR8–COP1 heterodimerization (Muller et al. 2013b).

Mammals do not possess a UVR8-like UV-B receptor (Rizzini et al. 2011). By using only the WD40 domain of *Arabidopsis* COP1, the ubiquitin ligase activity of COP1 was eliminated to further minimize the risk of cross-talk with the host's signalling pathways. To build up a UV-B-inducible gene expression system, a bipartite UV-B-responsive transcription factor was designed consisting of (i) the UVR8 core domain (amino acids 12–381) fused to the macrolide repressor E (Weber et al. 2002) and (ii) the WD40-domain of COP1 (COP1(WD40)) fused to the Herpes simplex-derived transactivation domain VP16. The response promoter was constructed by fusing an octameric E-responsive operator motif *(etr)8* upstream of the minimal cytomegalovirus promoter $Pro_{hCMVmin}$ (Weber et al. 2002) and the reporter *SECRETED ALKALINE PHOSPHATASE (SEAP)*. In the absence of UV-B, UVR8 is in homodimeric state and does not interact with COP1(WD40). Absorption of UV-B light, however, triggers the transition of UVR8 to the monomeric form, which results in direct binding of UVR8 to COP1(WD40). This process leads to the recruitment of COP1(WD40)-VP16 to the promoter and the subsequent increase of *SEAP* expression. In the dark, spontaneous conversion of UVR8 to the ground state disrupts the interaction between UVR8 and COP1(WD40) and results in termination of gene expression (Fig. 3). This system was functional in various mouse, hamster and human cell lines, and in all cases the illuminated cells showed high reporter induction, demonstrating the broad applicability of this system (Muller et al. 2013b).

Plant blue light receptors

Protein domains of the Light, Oxygen, or Voltage 1 (LOV1) form a subset of the large and diverse Per-ARNT-Sim (PAS) domain superfamily, which has been implicated in cellular signalling processes across all kingdoms of life. LOV domains noncovalently bind flavin mononucleotide (FMN) in the dark. Blue light triggers covalent binding of the FMN chromophore to a conserved cystein residue within each LOV domain. This leads to a conformational change of the protein and results in its enhanced kinase activity (Christie 2007; Tokutomi et al. 2008). The reaction is dark-reversible. Among the numerous LOV-domain photoreceptors of plants, phototropins (PHOT) are the only ones harbouring two LOV domains (Briggs 2007). In *Arabidopsis* a second LOV domain photoreceptor family, comprising ZEITLUPE (ZTL); FLAVIN BINDING, KELCH REPEAT, F-BOX1 (FKF1) and LOV KELCH PROTEIN 2 (LKP2) modulates the circadian clock and the pathway regulating photoperiod-dependent flowering. Members of this family possess an N-terminal LOV domain followed by an F-box and six Kelch repeats, suggesting that they participate in the light-regulated degrada-

tion of proteins. Although the LOV domain of these proteins binds an FMN chromophore and displays photochemical properties analogous to those of phototropin LOV domains, the absence of dark recovery suggests that ZTL family members probably mediate non-reversible light responses (Cheng et al. 2003; Imaizumi et al. 2003). Via its LOV domain, FKF1 binds to a plant-specific protein called GIGANTEA (GI). The combination of circadian expression of GI and FKF1, together with the light-regulated interaction between their gene products, enables expression of CONSTANS (CO), a central element of day-length-regulated flowering, during late afternoon in long day-grown plants (Sawa et al. 2007). This interaction is the basis of an optogenetic gene switch constructed in mammalian cells. For this purpose a split transcription factor was designed, comprising GI fused to the DNA-binding domain of the Gal4 yeast transcription factor on one hand and the nuclear localized N-terminal part of FKF1 fused to the transactivation domain VP16 on the other. Blue light illumination triggers binding of GI to FKF1, which results in the reconstitution of a functional transcription factor from the fused partners, which can initiate transcription of the reporter. 24 h illumination increased the reporter gene expression level five-fold, as compared to non-illuminated control cells (Yazawa et al. 2009). This system has recently been modified by replacing the Gal4 DNA-binding domain with a zinc finger protein and by using three copies of VP16, which resulted in 53 times higher reporter expression levels in the illuminated samples as compared to the controls (Polstein and Gersbach 2012). A major disadvantage of this system is that the kinetics of light-triggered dimerization is slow, requiring tens of minutes. Additionally, FKF1 and GI remain associated for hours following light exposure (Yazawa et al. 2009).

Cryptochromes (CRY) are blue-light receptors that mediate various light responses in plants including de-etiolation, photoperiodic control of flowering, entrainment of the circadian clock, guard cell development and stomatal opening, as well as root growth (Liu et al. 2011) The *Arabidopsis* genome encodes three *CRY* genes, *CRY1*, *CRY2* and *CRY3*. CRY1 and CRY2 act primarily in the nucleus (Wu and Spalding 2007; Yu et al. 2007), whereas CRY3 likely functions in chloroplasts and mitochondria (Kleine et al. 2003). The CRY apoprotein contains two distinct domains: the N-terminal photolyase-homologous region (PHR) and the cryptochrome C-terminal extension (CCE). PHR is the chromophore-binding domain of CRYs that non-covalently binds the flavin adenine dinucleotide (FAD) chromophore and 5,10-methenyltetrahydrofolate (MTHF) as a second chromophore (Lin et al. 1995; Banerjee et al. 2007). MTHF harvests photons and transfers excitation energy to the catalytic chromophore FAD (Cashmore et al. 1999). FAD is a two-electron carrier that can exist in one of the three different redox states. It has been proposed that oxidized flavin is the ground-state chromophore of *Arabidopsis* CRYs, because it absorbs blue light most effectively.

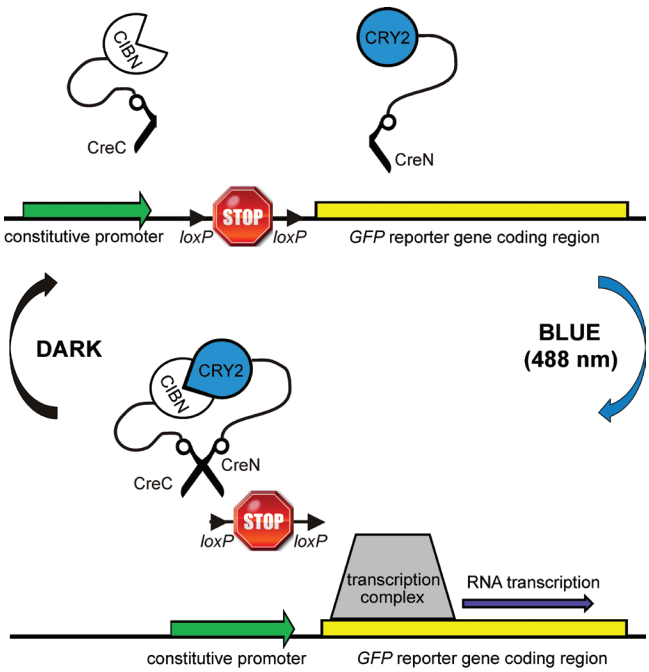

Figure 4. Blue light-induced reconstitution of Cre recombinase. The N-terminal fragment of Cre recombinase (CreN) is fused to CRY2 and the respective C-terminal fragment (CreC) is linked to CIB1 N-terminal region (CIBN). The recombinase is reconstituted through the interaction of CRY2 and CIBN in blue light. Cre activates a reporter construct by removing a stop codon that is flanked by *loxP* sites and is placed between a constitutive promoter and the *GFP* reporter gene.

Blue-light absorption, which leads to reduced FADH2, triggers a conformational change in CRYs and subsequent signal transduction. The reduced flavin is then oxidized to complete the photocycle (Bouly et al. 2007). Similarly to many photoreceptors studied to date, a photo-excited CRY changes its phosphorylation status. *Arabidopsis* CRY1 and CRY2 undergo phosphorylation in etiolated seedlings exposed to blue light, and the phosphorylation of CRY is required for its photoactivation. This blue light-dependent phosphorylation of CRYs causes electrostatic repelling of the CCE domain from the surface of the negatively charged PHR domain of CRY resulting in separation of the two domains, and triggers or alters the interaction between CRYs and their signalling partners (Partch et al. 2005).

A new dimerization module for inducing protein interactions based on *Arabidopsis* CIB1, a basic helix-loop-helix (bHLH) protein, and CRY2, a blue light photoreceptor that binds CIB1 in its photoexcited state, has been described. The ability of the CRY-CIB modules to induce dimerization of a split Cre recombinase was tested, allowing light-dependent control of DNA recombination. Based on a split Cre recombinase (Jullien et al. 2003), CRY2 was fused to amino acids 19–104 of Cre (CRY2-CreN), and CIBN to amino acids

106–343 of Cre (CIBN-CreC). Cre recombinase activity was monitored with a construct containing a transcriptional stop sequence flanked by *loxP* sites preceding the *GREEN FLOUORESCENT PROTEIN* (*GFP*) coding sequence. When CRY2 is activated by blue light, it binds to CIBN resulting in the reconstruction of the Cre recombinase. This protein induces recombination leading to the exclusion of the transcription stop sequence, resulting in reporter transcription induction (Fig. 4). Cells containing both CRY2-CreN and CIBN-CreC incubated in the dark showed equivalent levels of recombination as control cells indicating minimal or no light-independent CRY2-CIBN interaction. But when these cells were exposed to blue light irradiation for 24 hours, they showed a 158-fold increase in the number of EGFP-positive cells as compared with dark treated samples (Kennedy et al. 2010).

Phytochromes, receptors of red and far-red light

Plant phytochromes function as dimers of two apoproteins covalently linked to phytochromobilin, a linear tetrapyrrole bilin compound that acts as chromophore. The inactive Pr form of phytochromes is synthesized in the cytoplasm and is converted most efficiently by red (R) light (max = 660 nm) into the physiologically active Pfr conformer. This can be reverted back into the inactive Pr form upon absorption of far-red (FR) light (max= 720 nm), or by a much slower thermal relaxation (dark reversion). In the ground state of the phytochrome (Pr), phytochromobilin is in the C15-Z, anti conformation and is ready to absorb red light. Upon the absorption of red light, the C15-Z,anti conformation is converted to the C15-E,anti conformation (Nagatani 2010). These structural changes cause conformational changes of the holoprotein, triggering its translocation to the nucleus where it initiates signal transduction pathways leading to changes in various biological responses. The Pfr form interacts with other proteins; among the most important partners are members of the PHYTOCHROME INTERACTING FACTOR (PIF) family, acting as negative regulators of photomorphogenesis. The ability to phototransform repeatedly between Pr and Pfr enables phytochromes to act as long-lived photoswitches in various signalling cascades (Bae and Choi 2008).

The phytochrome multigene family consists of 5 members in *Arabidopsis*. Among them, PHYB is the most abundant phytochrome in R light. The PHYB apoprotein consists of two major domains, of the chromophore bearing light sensor N-terminal, and the C-terminal responsible for the dimerization and nuclear localization of the molecule (Nagatani 2010). A truncated PHYB comprising the N-terminal 651 (PHYB651) amino acids proved to be functional when fused to a dimerization motif and a nuclear localization signal. This chimeric phyB651 shows nuclear import and controls most of the R light-induced photomorphogenic responses and setting of the circadian clock (Matsushita et al. 2003; Palagyi et al. 2010). The active Pfr form of the phyB N-terminal domain interacts with PIFs and, as a consequence, PIFs are released from their DNA targets (Park et al. 2012).

Blue light-regulated gene switches raised the problem of slow reversibility, since those systems cannot be actively switched off after having been switched on. Furthermore, the relatively high-energy blue light might affect the stability of culture medium components (Wang 1976), or can cause cytotoxicity that may limit the applicability of blue light in optogenetics (Cadet et al. 2012; Pattison et al. 2012; Crefcoeur et al. 2013). These drawbacks could be overcome by a phyB-based gene switch. Photons of R light have lower energy and can penetrate tissues deeper than blue light. The phyB–PIF interaction has already been used to control gene expression or light-induced protein splicing in yeast (Shimizu-Sato et al. 2002; Tyszkiewicz and Muir 2008) and to induce actin polymerization in *Escherichia coli* (Leung et al. 2008).

A recently published red/far-red light regulated gene switch for mammalian cells was aimed to gain full control of gene expression in time and space. The red light-inducible expression system was based on the concept of split transcription factors and utilized the red/far-red light-reversible interaction of phyB and the PHYTOCHROME INTERACTING FACTOR 6 (PIF6) from *Arabidopsis*. The tetracycline repressor TetR (Gossen and Bujard 1992) was fused to the N-terminal half of PIF6 (amino acids 1–100) that had been shown to be sufficient for selective binding to the Pfr form of PhyB (Khanna et al. 2004; Levskaya et al. 2009). The photosensory N-terminal domain of PhyB (amino acids 1–650) was fused to the Herpes simplex-derived VP16 transactivation domain and to a nuclear localization sequence. The split transcription factor components were expressed from a bicistronic expression vector. The response construct contained multiple repeats of the TetR-specific *tetO* operator to the minimal human cytomegalovirus immediate early promoter ($Pro_{hCMVmin}$) (Fig. 5A). This chimeric promoter controlled the expression of different reporter genes of interest, for example *SEAP* or human vascular endothelial growth factor (*hVEGF*). Co-transformation yielded a rapidly reversible gene switch with 60-fold induction levels in red light, as compared to far-red light-illuminated CHO-K1 cells. Transfection of the red light-inducible expression system into different human-, mouse-, hamster- and monkey-derived cell lines or human primary cells all resulted in up to 65-fold induction levels, suggesting a cross-species applicability of this expression control strategy. Repeated high expression levels under inducing conditions and background levels under repression suggest full reversibility of transgene expression, meaning that spontaneous dark reversion does not have a significant impact on transgene activity (Muller et al. 2013a). The phyB chromophore phytochromobilin is not available in mammalian cells, but it can be substituted by phycocyanobilin purified

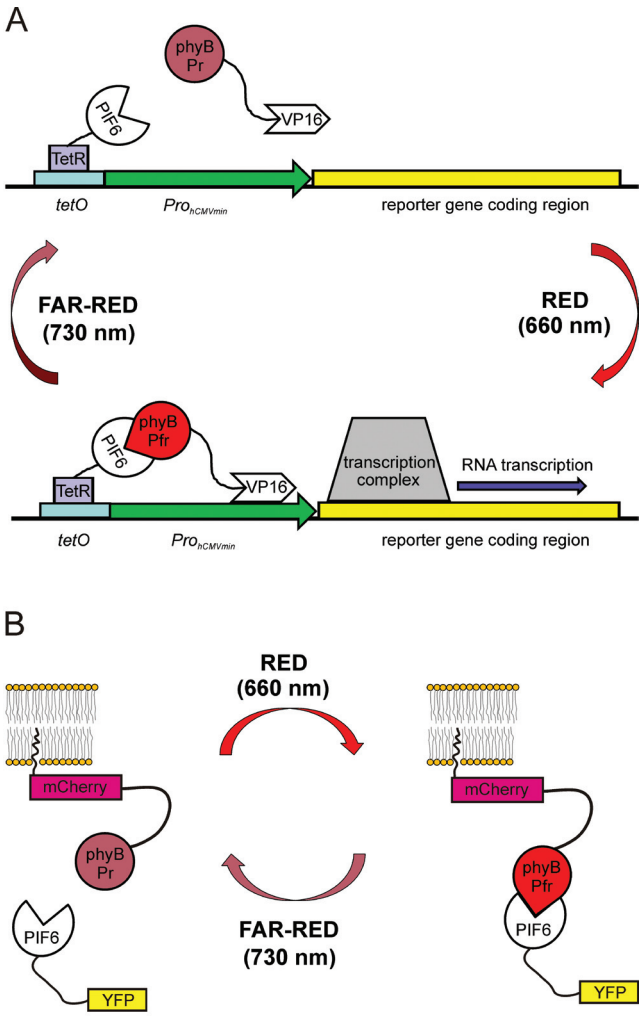

Figure 5. Phytochrome-based optogenetic systems. A) Red light-inducible gene expression switch. The split transcription factor components are expressed as phyB fused to the Herpes simplex-derived VP16 transactivation domain and the PIF6-tetracycline repressor (TetR) fusion protein. The response vector comprises of the TetR-specific operator *tetO* fused to the minimal human cytomegalovirus immediate early promoter ($Pro_{hCMVmin}$) followed by a reporter gene. Red light illumination converts phyB into the Pfr form and induces heterodimerization with PIF6 tethered via TetR to the *tetO* operator site. The phyB-fused VP16 domain recruits the transcription initiation complex and triggers activation of the minimal promoter. Far-red light converts phyB back into the Pr form and triggers dissociation from PIF6, thereby resulting in de-activation of the target promoter and transcriptional silence. B) Red-light controlled reversible translocation of proteins to the plasma membrane. PIF6-YFP fusion protein is recruited to membrane anchored phyB-mCherry upon red light illumination that converts phyB from the inactive Pr form to the active Pfr form. The PIF6-YFP fusion can actively be released by switching phyB back to the Pr form using far-red light.

from the cyanobacterium *Spirulina*. The exogenously applied phycocyanobilin penetrates into the cells and autoligates to phyB. Transgenic expression of two cyanobacterial enzymes in mammalian cells overcomes the chromophore limitation by endogenously producing the compound from heme. This metabolic engineering approach opened the possibility to operate the red/far-red light-switchable expression system in an entirely genetically encoded manner (Muller et al. 2013c).

The need for developing similarly inducible gene expression systems in plants emerged when researchers wanted to analyse genes or complete regulatory systems that cause severe developmental effects when expressed constitutively. For this purpose the above described split transcription factor-based R/FR light-switchable transgene expression system was adapted for plants (Muller et al. 2014). To optimize the system for plant cells, first the TetR DNA-binding domain was replaced by the plant-compatible macrolide repressor protein from *E. coli* (E DNA binding protein), and the components of the red light-responsive transcription factor were placed under the control of the constitutive *35S* promoter of the cauliflower mosaic virus (Odell et al. 1985). Analogous to mammalian cell lines, plant protoplasts can be used as transient expression system. Among other favourable characteristics, protoplasts retain the identity of the tissue they originate from, and have been successfully applied to dissect various plant pathways (Yoo et al. 2007). Protoplasts from *Nicotiana tabacum* were transformed with the red light-responsive split transcription factor along with a firefly luciferase reporter plasmid. After illumination with red light for 24 h, quantification of luciferase luminescence revealed high expression levels, while expression in the dark-incubated protoplasts remained at basal level. For application in whole plants, it is essential to grow plants in white light without inducing transgene expression. It has been shown in *Nicotiana*-derived protoplasts that transgene expression induced by white light can be repressed to levels comparable to that of dark-incubated samples by using supplemental FR light.

To demonstrate the applicability of red light-controlled gene expression in the analysis of plant signalling and for the production of biopharmaceuticals, red light was used successfully to manipulate auxin signalling in tobacco protoplasts and to produce a therapeutic protein, the human vascular endothelial growth factor in the moss *Physcomitrella patens* (Muller et al. 2014).

Another interesting application using the interaction between phyB and PIF6 is the light-controlled reversible translocation of proteins to the plasma membrane. In an experimental system described by Levskaya et al. (2009) the phyB-mCherry fusion protein was bound to the plasma membrane by the C-terminal CAAX motif of KRAS GTPase. Upon red light illumination phyB is converted from the inactive Pr form to the active Pfr form, thus it can bind the PIF6-YFP fusion protein and recruit it to the plasma membrane. Far-red light illumination actively releases the PIF6-fusion protein by switching phyB back to the Pr form. These processes can be followed by confocal laser scanning microscopy

monitoring the intracellular localization of the mCherry and YFP fluorescent proteins (Fig. 5B). The possibility to change the intracellular localisation of proteins in a light-dependent manner opens up new opportunities, for example the targeted application of therapeutic agents or drugs.

Light-responsive gene switches generally allow the expression of a single gene with spatiotemporal precision. Biological processes, however, are usually controlled by concerted action of multiple genes. By combining red/far-red and blue light responsive gene switches with a UV-B-controlled expression system, three genes in a single cell culture have been successfully activated. Multi-chromatic multi-gene control was also used in an *in-vitro* model, to initiate new blood vessel formation by light-triggered sequential expression of growth factors. However, this kind of approach raises the problem of overlapping absorbance spectra of the applied photoreceptors, making their orthogonal operation within a single cell challenging (Muller et al. 2013b).

Light-activated fluorescent proteins as optogenetic tools

Fluorescent proteins (FPs), as fusion tags, are widely used reporters for monitoring the stability or subcellular localization of proteins. Several variants of FPs are available with various excitation/emission spectral properties allowing simultaneous *in vivo* detection of different fusion proteins (Fernandez-Suarez and Ting 2008). Since most FPs function as simple 'labels', they can be used as qualitative or quantitative outputs, but not as regulatory switches of optogenetic processes.

One of the exceptions, Dronpa, is a fluorescent protein variant that was derived from the coral *Pectiniidae* (Ando et al. 2004). The original protein is tetrameric and shows low-level fluorescence with excitation/emission peaks at 503 and 518 nm, respectively. In order to be usable as other FPs, dimeric and monomeric variants with enhanced fluorescence properties have also been engineered (Andresen et al. 2008). However, the most prominent characteristic of Dronpa is that the fluorescent state of the protein can be switched on and off with illumination by 390 or 490 nm light, respectively. Hence the name: dron is a Ninja term for vanishing, whereas pa stands for photoactivation. The molecular base for photoswitching is the *cis-trans* isomerization of the side chain of the tyrosine residue functioning as chromophore. Importantly, isomerization drives a reversible conformational change of the protein, which is accompanied by a change in capacity for oligomerization. In other words, activated (or fluorescence-capable) Dronpa tends to form dimers, whereas in the switched-off state the protein exists as a monomer. This feature of Dronpa was utilized by Zhou and co-workers (Zhou et al. 2012), when employing the protein as a light-regulated optogenetic control tool in two different experimental setups. First they showed that Dronpa anchored in the plasma membrane via an added farnesylation motif was able to reversibly bind or release a soluble fusion protein consisting of another copy of Dronpa and mNeptune, a different FP. This intermolecular interaction was effectively controlled by short 390 or 490 nm light pulses. Next, the authors fused Dronpa molecules to both the amino and carboxyl termini of a guanine nucleotide exchange factor (GEF) and a protease. The function of these target proteins was monitored by appropriate molecular, physiological and biochemical assays. The results clearly demonstrated that upon activation by 390 nm light the two copies of Dronpa bound to each other, forcing the GEF and protease molecules into a particular quaternary structure, which inhibited their function. Irradiation by 490 nm light induced dissociation of Dronpas, leading to the relaxation of the GEF and protease molecules, regaining their function. This work demonstrated that light-regulated reversible inter- or intramolecular dimerization of Dronpa proteins can be effectively used for controlling subcellular localization or activity/function of proteins of interest.

Dronpa, similarly to other fluorescent proteins does not require any co-factors (e.g. exogenously applied chromophore) for light absorption, thus the assembly of the functional switch is largely independent of the metabolic state of the cell. The protein is controlled by visible light that is harmless for general cellular processes. The construction of Dronpa fusion proteins (or switches) is simple, because folding of Dronpa is not severely affected by the fusion protein partner that makes testing series of linkers unnecessary. The dimer/monomer state of the switch can be easily monitored by Dronpa's own fluorescence.

Dronpa has been intensively engineered in order to optimize its structural and functional properties. One can speculate that further modifications of the protein could shift the spectrum of photoswitching, opening the way to simultaneous control of Dronpa-based systems within the same cell.

Future directions

Optogenetics is a fast developing interdisciplinary scientific field with huge future potential. Excitation and inhibition of nerves with such high accuracy in a non-invasive and reversible manner have never been available before. The living brain (and also other tissues examined) tolerate the expression of different microbial rhodopsins and contain retinal at the necessary level for full functionality. Additionally, introduction of plant photoreceptors to animal tissues established easily controllable systems for regulating diverse cellular processes including gene expression and protein distribution. Besides the obviously interesting advances of optogenetics in fundamental scientific approaches, this technique has also

the potential to become a powerful "new therapy" providing answers to medical problems. The following list is a collection of possible future developmental directions for optogenetics.

1. New optogenetic tools (channel proteins, receptor-controlled gene expression modulation, different expression levels, speed, etc.) will be developed and tested in various host cells/organisms.

2. Development of increasingly sophisticated opto-electric devices, with higher spatial and temporal precision and for better readout is expected.

3. New opportunities for network analysis of cultured nerve cells *in vitro* will be established.

4. Examining/mapping of different brain functions and their direct linking to animal behaviour (*in vivo* studies on living animals, even on *Primates*) will be extended by optogenetic approaches.

5. Development of new gene therapies are expected (e.g. reconstruction of missing neural functions; curing certain neural disorders including epilepsy and Parkinson disease; recovering of sensual functions including hearing and vision etc.)

6. Biomedical applications under optogenetic control could be innovated (e.g.: synthesis and/or redistribution of therapeutic proteins inside the patient in response to light.)

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