Clinical applicability of optogenetic gene regulation

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Abstract

The field of optogenetics is rapidly growing in relevance and number of developed tools. Amongst other things, the optogenetic repertoire includes light-responsive ion channels and methods for gene regulation. This review will be confined to the optogenetic control of gene expression in mammalian cells as suitable models for clinical applications. Here optogenetic gene regulation might offer an excellent method for spatially and timely regulated gene and protein expression in cell therapeutic approaches.
Well-known systems for gene regulation, such as the LOV-, CRY2/CIB-, PhyB/PIF-systems, as well as other, in mammalian cells not yet fully established systems will be described. Advantages and disadvantages with regard to clinical applications are outlined in detail. Among the many unanswered questions concerning the application of optogenetics, we discuss items such as the use of exogenous chromophores and their effects on the biology of the cells and methods for a gentle, but effective gene transfection method for optogenetic tools for *in vivo* applications.

Keywords

Optogenetics, gene and protein regulation, mammalian cells, clinical applications

1 Introduction

The term optogenetics is defined as an experimental approach, where cells are genetically manipulated in order to become light-sensitive. There is a huge variety in optogenetic tools available depending on the application. Amongst other things, the optogenetic repertoire includes, amongst other things, light-responsive ion channels, protein-protein interactions and a switching function for gene expression. It is possible to interfere and analyze neural networks and functions, control gene and thus protein expression and enzyme activity (Deisseroth, 2011). The usage of light for the activation or deactivation of cell function yields several benefits for its application like non-invasiveness and a high temporal and spatial resolution. Different wavelengths can be applied to enable multichannel control of responsive elements to further enhance the specificity (Häusser, 2014).
In order to cure diseases and build up optogenetic implants for patients, the requirements for the optogenetic tools are significantly more demanding than for *in vitro* experiments. *In vitro* cell cultures are mostly 2-dimensional and usually based upon a single cell type, which are easy to handle, immortalized and have a substantially altered and non-physiological, cancer-cell-like function. Living organisms on the other hand are very complex, harbor various cell types and regulatory pathways. Thus *in vitro* experiments are not directly transferable to *in vivo* or to clinical settings and even animal models can have different bio-kinetics as compared to humans (Saeidnia et al., 2015).

Channelrhodopsins (ChRs) are a membrane protein family well-known in optogenetics. These light-activatable ion channels isolated from algae are typically used to depolarize membranes and trigger action potentials in neuronal cells (Lin, 2011), therefore manipulating nervous transmission and neuronal activity. Since this family of optogenetic tools is not the specific interest of this review, we will refer the well-disposed reader to other publications, which focus on optogenetic applications in a neuronal (Mahmoudi et al., 2017) and cardiovascular context (Joshi et al., 2020). Instead, we will focus on optogenetic tools relevant for the gene regulation of mammalian cells and discuss advantages and disadvantages for their clinical applications.

2 Main Text

2.1 Short overview of optogenetic systems for gene expression in mammalian cells

Generally, an optogenetic system for gene expression consists of two components – a photosensor and an interaction partner. Both interact with one another after light-induction, in the presence of a chromophore (see Figure 1). The interaction/binding characteristics of the two components can comprise four different categories: (i) split proteins, (ii) dimerization & DNA-binding, (iii) compartmentalization/localization and (iv) steric/allosteric effects (Q. Liu & Tucker, 2017). Typically, one of the partners is fused to a DNA binding domain (DBD) with a distinctive binding motive, while the other partner harbors a transcription factor (TF), also called activation domain, which induces gene expression of the target gene through the binding process.

All optogenetic systems for gene expression require the presence of a chromophore, which is bound to an intramolecular binding site of the photosensor. Typically these chromophores are covalently bound to a cysteine residue from the photosensor (Scheerer et al., 2010). Upon absorption of a photon, the electron density changes within the chromophore, leading to a conformational change of the chromophore and the respective photosensor (von Horsten et al., 2016). Phycocyanobilin (PCB), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and biliverdin (BV) are the most common chromophores for molecular light sensing. Two more uncommon chromophores are 5'-deoxyadenosylcobalamin (AdoB12) and *p*-coumaric acid.
Figure 2 gives an overview of the optogenetic tools for modulation of gene expression in mammalian cells. They are explained more detailed in the next chapter.

2.2 Detailed view on the optogenetic tools

The most important optogenetic tools for this field of activity are the CRY2/CIB system (Kennedy et al., 2010), the PhyB/PIF system (Müller et al., 2013) and the LOV system (Crosson et al., 2003).
The three frequently used optogenetic systems for gene expression in mammalian cells are: PhyB/PIF, CRY2 and LOV2. Their general working principle will be explained in the following.

2.2.1 PhyB/PIF

The two major components of the PhyB/PIF-system are the photoreceptor phytochrome B (PhyB) and its interaction partner the phytochrome-interacting factor (PIF) (Baaske et al., 2019; Beyer et al., 2015; Gomez et al., 2016; Müller et al., 2013; Noda & Ozawa, 2018), both initially derived from the plant *arabidopsis thaliana* (Khanna et al., 2004). PhyB consists of two major domains, whereas only the N-terminal domain is used for optogenetics. It constitutes the photosensory domain and binds the exogenous chromophore (PCB). Until chromophore absorption, PhyB remains in its inactive state (named as $PhyB_R$). After chromophore binding, PhyB is able to absorb a red photon (660 nm) and isomerizes, which leads to a conformational change of $PhyB_R$ to its active state $PhyB_{FR}$. $PhyB_{FR}$ is able to bind to PIF and therefore initiates gene transcription, hence the transcription factor is fused to PhyB. By absorbing a far-red photon (740 nm), the conformation of $PhyB_{FR}$ changes back to the conformation of $PhyB_R$. As a consequence, the PhyB-PIF complex dissociates and the gene transcription of PIF is terminated (Müller et al., 2013). The working principle is depicted in Figure 3.
The PhyB/PIF system therefore is an optogenetic toggle switch, which can be activated and deactivated using light of two different wavelengths. If the system is not deactivated by far-red light, it will slowly (about 24 hours) revert back to its thermally more stable dark state, also known as thermal or dark revision (Rockwell & Lagarias, 2010).
In addition to gene expression the PhyB/PIF system is also used to translocate a variety of proteins in order to reshape and direct cell morphology in mammalian cells. Levskaya et al., 2009 and Leung et al., 2008 focused in their work on the actin cytoskeleton and actin polymerization, while Toettcher et al., 2011 worked with phosphoinositide 3-kinase activity and the signaling protein RAS (Toettcher et al., 2013). Also other cell functions can be controlled such as intracellular transport (Adrian et al., 2017) and protein localization (Buckley et al., 2016).

2.2.2 CRY2 (CRY2/CIB)

The CRY2/CIB-system consists of the photoreceptor cryptochrome circadian regulator 2 (CRY2), its interaction partner, the protein CIB and the chromophores flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) (Baaske et al., 2018; Kennedy et al., 2010; Konermann et al., 2013; Lee et al., 2017; Nihongaki, Yamamoto, et al., 2015; Pathak et al., 2017; Polstein & Gersbach, 2015; Quejada et al., 2017a; Yamada et al., 2018). At the N-terminal domain of CRY2 is a photolyase-homologous region (PHR), that binds to the chromophore. CRY2, from the plant *arabidopsis thaliana,* is an unusual photosensory protein, because it is able to interact in two different ways after blue light illumination (450 nm). The first interaction pathway is a homo-oligomerization without contribution of another interaction partner leading to the formation of clusters of different CRY2 molecules upon blue light stimulation (Figure 4).

In the last couple of years, the application of CRY2 homo-oligomerization has become more and more popular, in order to regulate cell functions and protein-protein interactions (Bugaj et al., 2013). Since this review is focused on gene expression, this may be considered an unwanted side effect and will be discussed regarding its impact on biosafety of optogenetics.

After light induction, CRY2 can also interact with cryptochrome-interacting basic-helix-loop-helix (CIB) (Figure 5).

The exact binding mechanism of CRY2 and CIB is still not fully understood. However Kennedy et al., 2010 utilized the CRY2/CIB system to control protein-protein interactions. More precisely they induced protein translocation, transcription and Cre recombinase-mediated DNA recombination. Idevall-Hagren et al., 2012 controlled the phosphoinositide metabolism in mammalian cells using CRY2/CIB. Phosphoinositides are lipid components of cell membranes regulating a variety of cellular functions. Duan et al., 2015 utilized CyRY2/CIB in order to control the transport and distribution of organelles by light. They achieved it by optically recruiting molecular motors onto organelles through the heterodimerization of CRY2 and CIB.

2.2.3 LOV

The **l**ight-**o**xygen-**v**oltage (LOV) photoreceptor is one of the most versatile optogenetic photoreceptors, because there are many different possibilities for genetic engineering and various mutants, resulting in a huge variety of applications. LOV domains can be found in several plant, fungal und bacterial proteins (Baaske et al., 2018; Bubeck et al., 2018; Crosson et al., 2003; Müller, Engesser, et al., 2014; Niopek et al., 2016; Reade et al., 2017; Wenjing Wang et al., 2017; Yumerefendi et al., 2016). The typical LOV photoreceptor has no interaction partner, since it is working by conformational change of a helix, since the main mechanism of action is the regulation of activity via steric hinderance modulated by a conformational change of a helix. However, there are few LOV variants using different interaction partners, which are mentioned later.

LOV belongs to the **P**er-**A**RNT-**S**im (PAS: **p**eriod circadian protein-**a**cryl hydrocarbon receptor nuclear translocator protein-**s**ingle-minded protein) domain family and senses blue light (450 nm - 500 nm) with the help of the chromophore flavin mononucleotide (FMN) or flavin-adenine dinucleotide (FAD). Both chromophores are endogenous in mammalian cells and interact non-covalently with LOV in the dark (Möglich et al., 2010). Upon absorption of a photon, a covalent bond between the chromophore and the PAS core is formed, which leads to a conformational change and a rearrangement of the non-covalent hydrogen bonds (Crosson & Moffat, 2002). This conformational change (seconds to minutes) leads to a

dislocation of the Aα and Jα helices from the C- and N-terminal domain (Harper et al., 2003), exposing the caged DBD (de Mena et al., 2018). For the regulation of gene expression, a transcription factor is fused to the Jα helix (Figure 6).
In the dark, a spontaneous mechanism eliminates the bond between LOV and the chromophore, which deactivates the system within a half-life time of 50 seconds. Applications, which rely on this light-induced conformational change, are for example, protein-protein interactions (hetero-, homodimerization and dissociation based) (Crosson et al., 2003; Duan et al., 2015; Kennedy et al., 2010), light-dependent allostery (masking/unmasking of effector sites, photocaged peptides and light induced disorder) (Baarlink et al., 2013; Dagliyan et al., 2016; Strickland et al., 2008) and gene expression.

Various variants of LOV are known, which can help to enhance the optogenetic performance of the system, dependent on the application. Common LOV systems with a different optogenetic principle mechanism are: FKF1 (*A. thaliana* flavin-binding), asLOV2 (*avena sativa* phototropin1), EL222 (*erythrobacter litoralis* LOV) and VVD (*neurosporea crassa* Vivid).

The photoreceptor **FKF1** undergoes a heterodimerization with the interaction partner GI (gigantea) subsequent to a non-covalent chromophore binding (FAD/FMN) and blue light illumination. A transcription factor fused to FKF1 activates gene transcription after heterodimerization (Polstein & Gersbach, 2012; Quejada et al., 2017a; Yazawa et al., 2009). GI on the other hand is fused to the DNA binding domain (zinc finger protein), which locates the initiation complex at the target gene (Figure 1).

The working principle of photoreceptors **as LOV2** and **EL222** is similar to that of the typical LOV domain (Figure 6). The photoreceptor is bound to a helix-turn-helix domain (HTH) in the dark state, which blocks the α helix essential for DNA binding. Upon chromophore binding and blue light illumination, the photoreceptor changes its conformation. As a consequence, the α helix of HTH is no longer blocked and the DBD-element can bind to the DNA binding motive. A transcription factor, that is fused to the photoreceptor, starts the gene expression. Back in the dark, thermal revision happens rapidly (Motta-Mena et al., 2014).

The **VVD** (vivid) photoreceptor is characterized by its dimerization to the interaction partner Gal4 upon chromophore binding and illumination (Figure 1). Gal4 has a distinctive DNA binding motive, therefore the transcription factor fused to VVD can initiate gene expression. In the dark, the heterodimer dissociates back to its initial form and gene transcription will be terminated (X. Wang et al., 2012).

2.2.4 Other optogenetic systems for gene expression

One of the rarely applied systems for gene expression in mammalian cells is the **UVR8-COP1** system, where UVR8 is the photoreceptor and COP1 its interaction partner. The UV resistance 8 locus (UVR8) uses intrinsic amino acids (Trp) as chromophores, which differentiates this optogenetic system from the others (Rizzini et al., 2011). In this inactive state, UVR8 forms homo-dimers, which dissociate upon ultra violet B (UV-B) radiation (280-315 nm). The UVR8 monomers are now able to bind to the constitutively expressed factor photomorphogenic 1 (COP1). For gene expression, each partner is fused to a DNA binding domain or a transcription factor respectively (Figure 1). UVR8 monomers re-dimerize within

48 hours in the dark, although in the presence of UV-B photomorphogenesis 1 and 2 (RUP1/ RUP2) the revision happens rapidly (Rizzini et al., 2011).

The **BphP1-PpsR2** system consists of the photosensory core (BphP1), that binds to the endogenous chromophore biliverdin (BV). After near infra-red (NIR) light (740 - 780 nm) absorption, BphP1 changes its conformation, which leads to a heterodimerization with the interaction partner PpsR2 (*rhodopseudomonas palustris* bacteria) (Kaberniuk et al., 2016; Redchuk et al., 2017; Rottwinkel et al., 2010). The dissociation of the heterodimer is triggered by white light (390 - 700 nm) illumination or through thermal relaxation in the dark (Bellini & Papiz, 2012).

The **CarH/CarO** system is a green light activated (525 nm) optogenetic system. CarH is a light-sensitive bacterial transcription factor, harboring the chromophore AdoB12 (5`deoxyadenosylcobalmin), an endogenously produced chromophore in mammalian cells. Unlike most of the other optogenetic systems the CarH/CarO system is deactivated by light. In the dark, CarH forms tetramers, which bind to the CarO (DNA operator sequence form *thermus thermophilus*) and drive gene expression. After green light irradiation, photolysis of AdoB12 is triggered leading to the dissociation of the CarH tetramers and the termination of gene expression (Figure 1). The half-life time of AdoB12 is about 24 hours, which is relatively slow (Chatelle et al., 2018). Since this system originally comes from plant cells, it is mainly used in plant cells. In mammalian cells CarH alone is used for switching integrin-mediated cell adhesion to extracellular matrix on and off (D. Xu et al., 2020) and gene expression (Chatelle et al., 2018).

Another photoinducible system for gene expression is named **magnet**, as its photosensitive components are named pMag (positive magnet) and nMag (negative magnet). Upon blue light illumination, these two proteins heterodimerize through electrostatic interactions (Kawano et al., 2015). Each of them is coupled with a C- or N- terminal fragment of Cas9, from *streptococcus pyogenes*. The dimerization leads to a reassembly of the Cas9 fragments and forms a functional Cas9 nuclease. Typically, Cas9 binds and cleaves a target DNA sequence complimentary to its sgRNA (single- guide DNA). In order to activate gene expression, a transcription factor is fused to Cas9, which enables gene transcription after DNA binding (Figure 1). In the dark, pMag and nMag dissociate back to monomers (Kawano et al., 2016; Nihongaki, Kawano, et al., 2015).

In the **PYP/CREB** system, the photoactive yellow protein (PYP) binds the chromophore p-coumaric acid and therefore becomes responsive to blue light. Upon photon absorption, PYP changes its conformation and binds to the interaction partner CREB (cyclic AMP response element binding protein), which is a transcription factor that regulates gene expression (Figure 1). In the dark, the conformation of PYP spontaneously reverts back to its inactive state (Ali et al., 2015).

2.3 Considerations for clinical applications

Considering clinical applications for optogenetic gene expression systems, there are several factors which have to be considered. Subsequently, the main obstacles will be discussed.

(i) Construct size: Optogenetic systems for gene expression are typically composed of different genes for the optogenetic proteins. The expression of the light responsive elements needs to be driven from a promoter and the corresponding RNA should include a terminator sequence. The gene of interest should be flanked by an upstream binding motive to initiate gene expression and a terminator sequence to terminate the generation of RNA. Typically, the optogenetic systems span around 5 to 6 kb of genomic information.

(ii) Regulatory elements: A very important component of optogenetic systems is the chosen promoter. Not all promoters show high gene expression rates in each cell type (Xia et al., 2006). In addition, promoters can also be silenced by methylation of the transfected DNA, when being used in the wrong cell type (Qin et al., 2010), thus limiting the protein productivity. In clinical applications, a slowly progressing silencing can be utilized selectively in order to deactivate an optogenetic system over time. This might be advantageous for cell therapies where temporarily regulated gene and protein expression is crucial with cells being optogenetically activated over a limited period (e.g., in clinical applications with cells surrounding implants optimizing the healing phase). On the other hand, the promoter choice also allows targeting of specific cells while omitting others. Therefore, the promoter should be adapted to the used cell type and the application. Equal care should be taken, when selecting the transcription factor. It is well documented, that excessive gene expression rates should be avoided, since high levels of protein expression can affect cell health and even result in cell death (H. S. Liu et al., 1999).

(iii) Transfection method: One of the most important considerations for *in vivo* application is the way the optogenetic system is inserted into the host cells. Almost all optogenetic systems for gene expression were originally composed of multiple plasmids. On the one hand this is due to difficulties which arise from larger plasmids, while on the other hand this design is facilitates rapid changes of genetic cassettes and tight control over the stoichiometry of these cassettes. On the downside however, co-transfection of multiple plasmids is more demanding than transfecting a single plasmid.
While choosing a suitable the transfection method, cell type and clinical aim must be considered. An ideal transfection method has a high transfection efficiency, low cell toxicity, minimal effects on the cell physiology and is easy and reproducible (Kim & Eberwine, 2010).
Thinking about clinical applications, not all transfection methods are suitable. One of the most common transfection methods *in vivo* is the viral transfection, which stands out due to its high transfection efficiency. Depending on the used type of virus, the transfection can be transient or stable and can specifically be targeted at a certain cell type. On the other hand, mutagenesis and immune reactions are known side effects of viral transfection (Pfeifer & Verma, 2001). A detailed discussion of this topic would go beyond the scope of this review, Anguela & High, 2019, as well as (Kim & Eberwine, 2010) however have given extensive reviews on the topics of (viral) gene therapy.

(iv) Leakage: All optogenetic systems are in an equilibrium between their on and off state. By activating or deactivating the system with light, the equilibrium is shifted to one of those sides. Consequently, the background expression, also named leakage, is never zero. On the other hand there is no full activation either (Möglich & Moffat, 2010). Leakage and activation strength should be taken into consideration, when selecting an optogenetic system for an application. Dependent on the application a high leakage can be negligible or even harmful depended on the application.

(v) Optical properties of tissue and light delivery: When choosing an optogenetic system, the tissue penetration of light plays a key role and greatly depends on the wavelength (see Figure 7). In the visible spectrum red light has the highest tissue penetration with up to 5 mm, while blue light only penetrates tissue up to 2 mm (Barolet, 2008). Overall, the tissue penetration of light is too low to reach deep tissues, in addition, light scattering can become problematic, if a high spatial accuracy is needed.
There are four possible ways to overcome the poor tissue penetration of blue and green light. The first possible method is using 2-photon-techniques as activation source, allowing precise 3D cell targeting in tissue. 2-photon techniques utilize the absorption of two lower energy photons for excitation. These photons are typically from near infrared (NIR) light, which is able to penetrate tissue deeper. Furthermore, the excitation is spatially localized in the targeted volume and produces only relatively low heat in comparison to 1-photon methods (Benninger & Piston, 2013). Therefore, the usage of NIR light reduces tissue scattering and phototoxicity, while minimally interference with the body, which is advantageous for *in vivo* applications. Additionally, the so produced photons are robust enough to activate several optogenetic constructs, e.g. LOV, CRY2 and ChR2 (Zhang et al., 2016). A second option to overcome poor tissue penetration are implantable µLED devices controlled by radio frequencies, which can be utilized in close proximity to the target site. They effectively deliver light to a specific place, limiting the usage of applications involving more than one specific area of the body (Park et al., 2015). The third (experimental) way is the application of upconverting lanthanide nanoparticles, which absorb NIR light in deeper tissues and emit upconverted blue light. The usage of NIR light brings the above-mentioned advantages. In addition, it is possible to create cell-specific targeting associated with spatial control through surface modifications of the nanoparticles, e.g., with anti-bodies or ligands. However, safety of these upconverting nanoparticles has to be evaluated. A fourth method to generate photons even in deep tissue is bioluminescence. Here, an enzymatic reaction between *gaussian* luciferase and coelenterazine results in the formation of photons. It is the simplest and most non-invasive of the four strategies, but the production of a sufficient light intensity is still challenging. Additionally, it lacks spatial control and rapid reversibility (Berglund et al., 2016).

(vi) irradiation/light effects: The applied irradiation also has side effects depending on the wavelength of the light. While having the deepest tissue penetration, the tissue heating from red light is only moderate. For shorter wavelengths however, the tissue penetration is reduced while the light harbors more energy leading to significant tissue heating depending on the irradiance. Furthermore, energy rich light induces the formation of reactive oxygen species (ROS), which inflict mutations in the genetic material by oxidating nucleotides and thus promoting mismatches (Meyskens et al., 2001). Blue light and UV light have the most mutagen and non-specific damaging effects. UV-B irradiation induces a cascade of neuroactive and vasoactive mediators and cytokines, resulting in an inflammatory response (Sarasin, 1999). In higher doses apoptotic pathways are activated by keratinocytes, resulting in cell death (Rizzini

et al., 2011). Also, UV radiation can be absorbed by pyrimidines in the genetic material, resulting in a cleavage of double bonds and formation of abnormal bonds, which are highly mutagenic (Sarasin, 1999).
The optogenetic systems differ in activation wavelength, chromophore, reversibility and the deactivation time of the gene expression. Since every system has its own advantages and disadvantages, there is not the perfect optogenetic system for all kinds of clinical applications. Therefore, it has to be chosen carefully in dependence on the desired application. The pros and cons of the main optogenetic systems are summarized in Table 2 and will subsequently be discussed in more detail.

PhyB/PIF: The PhyB/PIF system is dependent on the chromophore PCB, which is not endogenous in mammalian cells. Apart from safety concerns, this is a severe **disadvantage** since PCB therefore has to be delivered to the optogenetic system, which requires injections and perfusion of the targeted tissue (Müller, Zurbriggen, et al., 2014). Repetitive addition of PCB is mandatory since the half-life time of PCB is only approximately 1 hour. One option to overcome this disadvantage is to co-transfect the cell with the genes for PCB synthesis from heme (Müller et al., 2013). However, it has been demonstrated that introducing PCB synthesis alone was not sufficient, instead other pathways for the heme metabolism had to be suppressed to reach adequate concentrations of PCB (Uda et al., 2017). Additionally, heme is also crucial for other vital body functions, like oxygen transport, so greatest caution is advised when perturbing heme metabolism.
The PhyB/PIF system is activated and deactivated with (far) red light, which is **advantageous** for *in vivo* usage, because it penetrates tissue deeper than light with a shorter wavelength. In addition, red light contains a lower energy as compared to light of a shorter wavelength, so possible tissue damaging effects are decreased, which is a vast advantage for *in vivo* use (Müller et al., 2013). The PhyB/PIF system generally has a moderate to high leakage (about 5 to 10% of the activation) (Müller et al., 2013).

CRY2: The CRY2 system is dependent on blue light activation with an activation half-life time of 5.5 min. Therefore, and although the high temporal resolution appears attractive, a more elaborate constant or repetitive blue light illumination must be provided to keep the system in the activated state, which could represent a **disadvantage**. Continuous blue light illumination is not recommended in order to limit cell damage. Furthermore, the homo-dimerization of CRY2 can lead to undesired side effects and lower the overall performance of the gene expression. It has not been shown yet, if an unwanted CRY2 homodimerization causes any further side effects in the cells, resulting in a risk for biosafety. However, (Duan et al., 2017)were able to show that homodimerization takes place at the C-terminal domain while CRY2/CIB heterodimerization occur at the N-terminal domain. By engineering the charges at the C- and N-terminal domains, they were able to elevate or suppress one of the reactions. On the other hand, FMN and FAD are endogenous in mammalian cells, which is **advantageous**. Furthermore, the short half-life time of the activated state leads to a good temporal resolution and the leakage of the system is low (1-2%) (Quejada et al., 2017a).

LOV: LOV systems inherit variable properties depending on the utilized variants. The leakiness of the FKF/GI system can be as low as 1-2% (Quejada et al., 2017b), as well as LOV2 with 2% (Yao et al., 2008), VVD/Gal4 with 0.8-0.9% (X. Wang et al., 2012). and EL222 about 1% (Motta-Mena et al., 2014). However, the leakiness of asLOV is about 9% (Lee et al., 2017). Similarly, to the leakiness, the deactivation time of the LOV systems greatly depends on the used distinct

variant ranging from 17 s to 62 h. Thus, similarly to systems utilizing CRY/CIB, continuous or pulsed blue light illumination is required to activate LOV systems. As previously discussed, blue light has undesirable optical properties and can inflict undesired damage on the irradiated tissue (Baaske et al., 2018). A big **advantage** of the LOV system is that LOV elements are small and the chromophore FMN is endogenous in mammals. With their great versatility regarding their application, LOV systems are an important optogenetic tool (Kennedy et al., 2010).

Other optogenetic systems: The **UVR8-COP1** does not require an exogenous chromophore, which is **advantageous**. The spontaneous revision time of the activated state is 24 hours; however, a revision can be induced utilizing RUP 1 and 2. The biggest **disadvantage** of UVR8 systems is the high energy UV-B radiation, which, besides its low tissue penetration, inflicts most severe damage to the cells and can even cause cell death.
Advantageous NIR light activates the **BphP1-PpsR2** system, which therefore reaches the maximum tissue penetration (Weissleder & Ntziachristos, 2003). In addition, this system depends on an endogenous chromophore and is deactivatable with white light. However, the deactivation with white light is also a **disadvantage**, since the system has to be protected from visible light.
The **CarH/CarO** system is a green light system with a good tissue penetration, an endogenous chromophore (AdoB12) and very low leakiness of 0,65% (Chatelle et al., 2018). The half-life time of the system is approximately 24 hours.

A big **advantage** of the **pMag/nMag and the PYP/CREB** systems is that no exogenous chromophore is needed. The deactivation time varies depending on the specific variant. **Disadvantageous** is the dependence on blue light with its low tissue penetration and inflicted tissue damage.

2.4 Clinical applications for optogenetic systems

Historically, optogenetic originated from light activated ion channels, which logically have been applied on neurons and have enabled scientists to make significant progress. Since then the optogenetic repertoire has been significantly expanded. Here we will give a short overview over the achievements of classic and modern optogenetics; however, we will refer the well-disposed reader to other publications with the respective focal points.

In the field of neurodegenerative diseases such as Parkinson's and Alzheimer's disease, optogenetics were successfully utilized to identify affected neurons and their role in the neuronal circuits (Chen et al., 2015; Nowak et al., 2010; Ordaz et al., 2017; Richner et al., 2014). Other studies shed light on the mechanisms of psychiatric diseases such as depression and drug addiction (Albert, 2014; Belzung et al., 2014; Jarvis & Schultz, 2015; Lobo et al., 2012; Richner et al., 2014) obsessive-compulsive disorders (Montagni et al., 2019) and post-traumatic stress disorders (Jarvis & Schultz, 2015). Optogenetic neuromodulation has also been demonstrated to enhance regeneration of damaged neuronal circuits e.g. after myelin degeneration (Montagni et al., 2019; Ordaz et al., 2017), stroke (Cheng et al., 2014; T. Kushibiki et al., 2015; Pendharkar et al., 2016) or spinal cord injury (Ahmad et al., 2015; Ordaz et al., 2017) and to improve memory function (Roy et al., 2016). Optogenetic pacemakers have also been studied in epilepsy (Jarvis & Schultz, 2015; Ji & Wang, 2015; Ordaz et al., 2017), for the treatment of chronic pain (Mickle & Gereau, 2018; Nowak et al., 2010), to substitute electrical deep brain stimulation (Delbeke et al., 2017), or to resynchronize the heartbeat (Bingen et al.,

2014; Crocini et al., 2016; Nussinovitch & Gepstein, 2015). More direct approaches have been made to control muscle functions (Bruegmann et al., 2015), restore vision via optogenetic retinal gene therapy (Mirzapour Delavar et al., 2016), or to restore hearing with an optogenetic cochlear implant (Dieter et al., 2020).

Clinical applications in non-neuronal contexts are less frequent. One example is the optogenetic induced flux of calcium ions, which has been utilized to secrete insulin from transgene mouse cells (T. Kushibiki et al., 2015; Toshihiro Kushibiki & Ishihara, 2018; Mirzapour Delavar et al., 2016) and could become an option in treating diabetes in combination with an implant regulating insulin secretion depending on the blood glucose level. Another application focusses on light activated chemokine receptors for localized immunomodulation e.g. in tumors for cancer treatment (Y. Xu et al., 2014). However, the repertoire of these optogenetic tools is much more inhomogeneous.

Optically activated adenylate cyclase (Fomicheva et al., 2019) and guanylate cyclase (Kyung et al., 2015) grant control over the intracellular levels of the second messengers cAMP and cGMP, while light-driven G-protein-coupled receptors (Opto-XRs) directly address G protein-mediated signaling cascades (Spangler & Bruchas, 2017). Other functions of optogenetic systems include modulation of kinase activity (Bubeck et al., 2018), induction of apoptosis (Bubeck et al., 2018; Hughes et al., 2015), and epigenetic editing (Bubeck et al., 2018). As shown previously optogenetic tools can also be utilized for gene expression, protein localization (Bubeck et al., 2018) and protein degradation (Baaske et al., 2018).

In theory, these diverse possibilities to modulate or enhance cellular functions should be reflected in an adequate number of applications. However, beside neurons, optogenetic has just begun to progress beyond the proof of concept stage in animals and the repertoire is still expanding. Konermann et al., 2013 presented an experimental approach in which the CRY2 system was established in the brain of mice *in vivo* inducing a reversible endogenous gene expression in neuronal cells. Reade et al., 2017 showed succesfull optogenetic gene activation in zebra fish using the EL222 system while optimizing toxic side effects. In addition, first attempts in stem human cells were performed. Klapper et al., 2017 developed a method generating a conditional and stable optogenetic human stem-cell line, which can easily be differentiated into functional neurons. Despite the fact that this advanced and user-friendly system is still an *in vitro* development, it allows a more widespread application of optogenetics in stem-cell-derived neurons and is an important step in the direction of *in vivo* applications. In order to study activity-dependent neurogenesis and to regulate the differentiation of transplanted neural stem cells, Teh et al., 2020 used transformed neural stem cells, which stably express channelrhodopsins. He used a non-viral transfection method with a lower carcinogenicity. Hörner et al., 2019 utilized the cyanobacterial phytochrome 1 in a poly(ethylenglycol) matrix in order to change its mechanical properties. Therefore, he constructed a hydrogel to control human mesenchymal stem cell migration in 3D cell culture approaches. Weicai Wang et al., 2019 showed an optogenetic model for optimization of mesenchymal stem cell fate towards precise bone regeneration *in vivo* in rats and *in vitro* based upon the FKF1/GI (LOV variant) system. These newly approaches are a huge step in the direction of *in vivo* applications.
On the other hand, detailed mechanistic knowledge is still lacking for most diseases and many issues inherent to gene therapy and optogenetic systems are still to be addressed as discussed

in the previous chapter. Nevertheless, optogenetics is a promising technique for applications which require precise inputs on specific cells or with a high temporal and spatial resolution.

3 Conclusion & Outlook (Concluding Remarks)

The optogenetic repertoire is steadily expanding and optogenetics are getting more refined and adapted to the specific application. Today optogenetics are able to modulate neuronal activity, gene expression, intracellular transport, protein-protein interactions, cell morphology and cell metabolism.
In this work, however, we focus on regulation of gene expression for mammalian cells. The three frequently used optogenetic systems for gene expression in mammalian cells are: PhyB/PIF, CRY2 and LOV2, which can be further divided into the different LOV2 variants FKF1, asLOV2, EL222 and VVD. Other not so well known optogenetic systems for gene expression in mammalian cells are UVR8/COP1, BphP1/PpsR2, CarH/CarO, pMag/nMag and PYP/CREB.
Each of the mentioned systems has distinctive properties in terms of dependent chromophore, reversibility, kinetics of gene expression. Furthermore, the optical characteristics such as tissue penetration, light scattering and tissue damage due to light exposure mostly depend on the utilized wavelength for activation/deactivation.

The application of light stimuli grants optogenetic an extraordinary spatial and temporal resolution, which can be further enhanced by selective cell targeting. Multiple optogenetic systems can be operated in parallel due to selective usage of different wavelengths. However, most of the optogenetic systems presented here are *in vitro* proof of principle applications in animal cell lines or easy to handle human cell lines.
Application of these optogenetic systems *in vivo* requires constitutive expression of optogenetic components by additional regulatory elements, thus corresponding constructs have to be delivered via preceding gene transfer. In order to increase the gene and protein expression rates within an optogenetic system, several factors are important: besides general factors regarding gene manipulation such as the distinctive promoter or the transfection method used, in optogenetics these are the illumination strength and length of cell exposure. The effect of these factors on expression efficiency is multiplex since they interact with each other, and optimization must be performed with regard to all these interdependencies. Another important factor for a successful transfer of optogenetics to *in vivo* applications is to deliver light effectively with minimized invasiveness to the target cells. Most optogenetic systems are dependent on blue light (450 nm), which has a low tissue penetration, thus limiting *in vivo* applications. We presented four different strategies to overcome this obstacle. The most promising ones are the implantation of µLEDs or the usage of near infrared light (NIR, wave length 800 to 2500 nm) to reach deep tissues in combination with 2-photon-microscopy or upconverting lanthanide nanoparticles. These methods enhance the effectiveness of optogenetic systems to be applied in living organisms.
In a clinical setting, optogenetics represent a specialized form of gene therapy inheriting the distinctive concerns of gene manipulations regarding safety risks. Furthermore, optogenetic systems and exogenous co-factors themselves are of xenogeneic origin to humans. Thus, potential harmful consequences like cell toxicity or immune reactions need to be excluded to ensure long term safety of patients.
Moving towards clinical applications, these optogenetic tools need to be explored and studied in *in vivo* animal models, before suitable medical tools can be developed with their help.

Beside neurons, which has a huge variety in *in vivo* applications, optogenetic has just begun to progress beyond the proof-of-concept stage in animals and the repertoire is still expanding. First attempts with diverse optogenetic systems in animals were made in rats, mice and zebrafish in order to regulate gene expression. In addition, there are also a few examples for optogenetics used in human stem cells. The first steps towards a transfer to *in vivo* applications for clinical applications have already been made.

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Tables

Table 1: *Advantages, neutral facts and disadvantages of the main optogenetic systems (PhyB/PIF, CRY2/CIB, LOV).*

Optogenetic system	**advantage**	**neutral**	**disadvantage**
PhyB/PIF (red light)	- high tissue penetration with negligible cell damage - Light switchable - Stable activation for 12 hours	- Leakage (5-10%)	- Exogenous chromophore - Short half life time of the chromophore (about 1 hour)
CRY2/CIB (blue light)	- Endogenous chromophore - Time resolution (activation for 5.5 min) - Low leakage (1-2%)	- Low tissue penetration (but possible enhancement via 2-photon microscopy) - Blue light may cause cell damage, but this can be overcome by pulsed illumination	- Homodimerization of CRY2 as side reaction
LOV (blue light, properties dependent on distinct variant)	- Endogenous chromophore - Huge variety in applications and modifications	- Low tissue penetration (but possible enhancement via 2-photon microscopy) - Blue light may cause cell damage, but this can be overcome by pulsed illumination - Wide range for time resolution (activation for 17 s up to 62 h dependent on the distinctive variant) - Wide range of leakage (1-2% FKF/GI; 2% LOV2; 0.8-0.9% VVD/Gal4; 1% EL222; 9% as LOV)	

Figures with legends

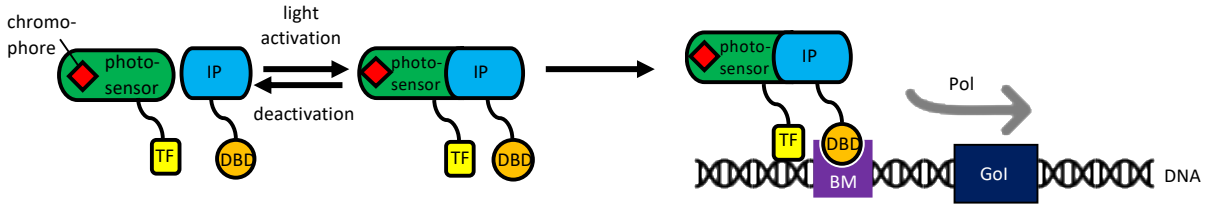
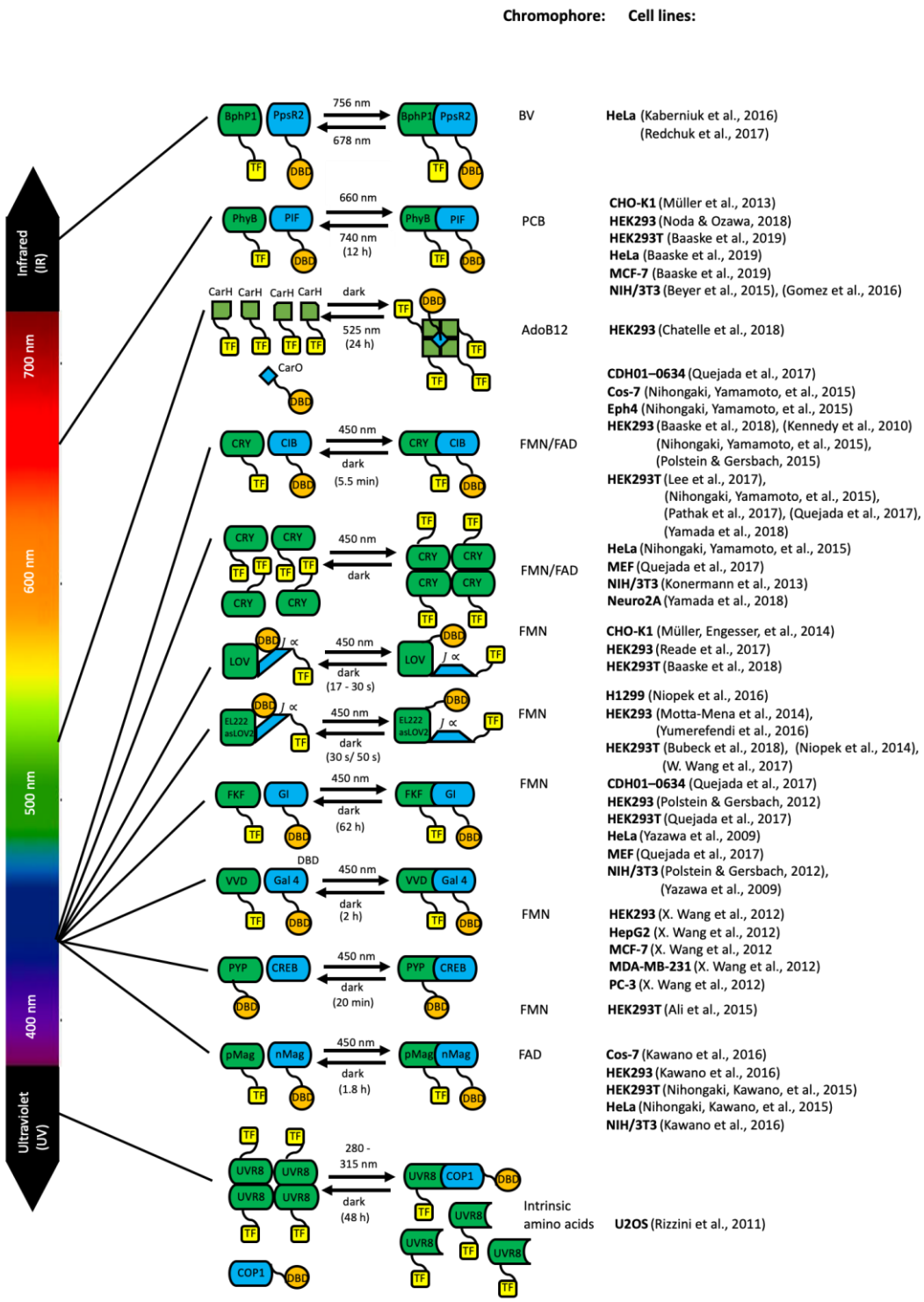


Figure 1: General function of the optogenetic systems: *A photosensor (green) and an interaction partner (IP: blue) interact in the presence of a chromophore (red). For gene induction, one of the partners is fused to a DNA binding domain (DBD: orange), while the other partner harbors a transcription factor (TF: yellow), also called activation domain. After light-induction, the resulting protein complex is bound to the binding motive (BM: purple) of the DNA and the TF recruits the RNA-Polymerase in close proximity in order to activate gene expression of the gene of interest (GoI: dark blue).*



BphP1 - bacterial phytochrome P1 ***PpsR2*** *- transcriptional regulator* ***TF*** *- transcription factor* ***DBD*** *- DNA binding domain* ***BV*** *– biliverdin* ***PhyB*** *- phytochrome B* ***PIF*** *- phytochrome interacting factor* ***PCB*** *- phycocyanobilin* ***CarH*** *- carotenogenic transcription factor* ***CarO*** *- carotenogenic operator* ***AdoB12*** *- 5'-deoxyadenosylcobalamin* ***Cry2*** *- cryptochrome circadian regulator 2* ***CIB*** *- cryptochrome-interacting basic-helix-loop-helix 1* ***FMN*** *- flavin mononucleotide* ***FAD*** *- flavin adenine dinucleotide* ***LOV*** *- light oxygen voltage* J_α *- alpha-helix at C-terminus of LOV* ***EL222*** *- erythrobacter litoralis* ***asLOV2*** *- light oxygen voltage domain of avena sativa* ***FKF*** *- flavin-binding kelch repeat F-box1* ***GI*** *- gigantea* ***VVD*** *- vivid* ***Gal4*** *- galactose-responsive transcription factor* ***PYP*** *- photoactive yellow protein* ***CREB*** *- cyclic AMP response element binding protein* ***pMag*** *- positive magnet* ***nMag*** *- negative magnet* ***UVR8*** *- ultra violet resistance 8* ***COP1*** *- constitutive photomorphogenic 1*

Figure 2: Overview of the different optogenetic systems used for gene and protein expression in mammalian cells, their working principles, chromophores and the cell lines they were already used in: *BphP1/PpsR2, PhyB/PIF, CarH/CarO, Cry/CIB, LOV, EL222, asLOV, FKF/GI, VVD/Gal4, PYP/CREB, pMag/nMag, UVR8/COP1. Chromophores are not displayed for the sake of clarity.*

Figure 3: Working principle of the PhyB/PIF system: *The optogenetic system consists of two components – a photosensor (green: PhyB) and its interaction partner (blue: PIF)–, which interact with one another after light-induction, in the presence of a chromophore (PCB- not shown).*

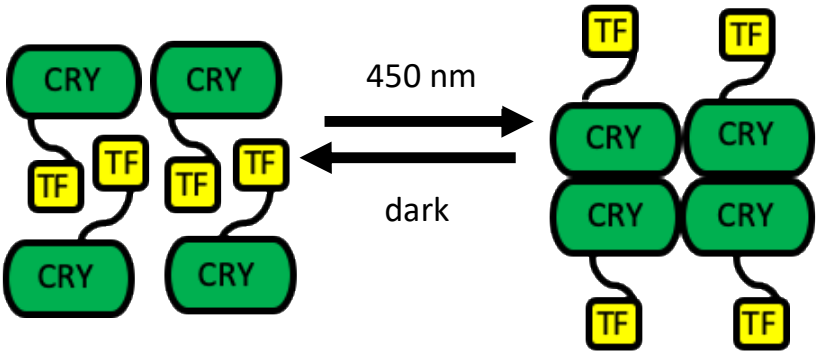


Figure 4: Working principle of the CRY2 homo-oligomerization *after light-induction, in the presence of a chromophore (FAD/FMN- not shown).*

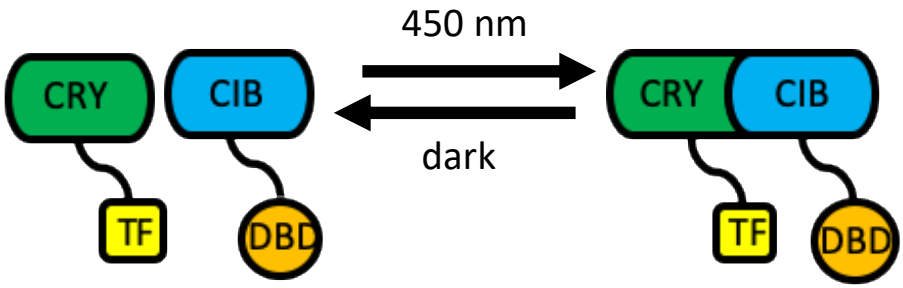


Figure 5: Working principle of the CRY2/CIB system *after light-induction, in the presence of a chromophore (FAD/FMN not shown).*

Figure 6: Working principle of the LOV system (green): *Upon chromophore (FAD/FMN- not shown) binding and light-induction, the Jα helix (blue) unfolds and the transcription factor (TF: yellow) is no longer sterically hindered, which induces gene expression of the target gene.*

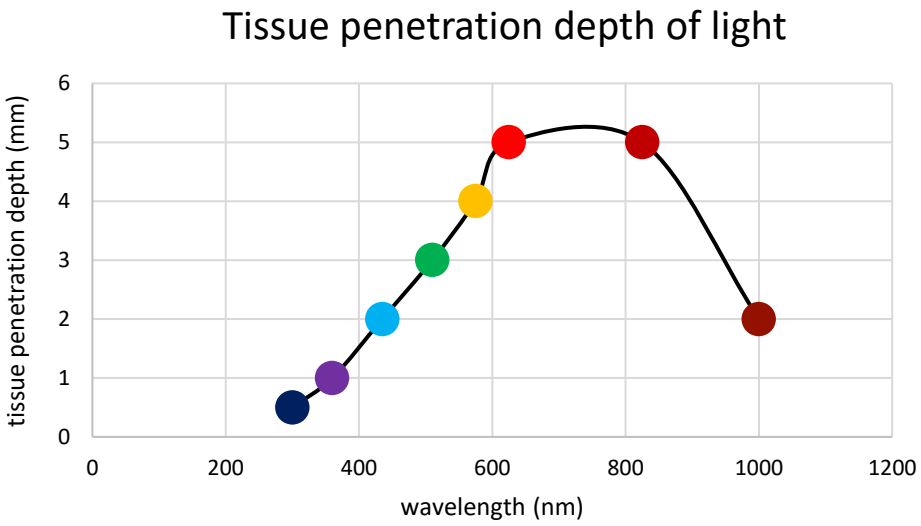


Figure 7: *Tissue penetration depth of light dependent on the wavelength (adapted after Ruggiero et al., 2016).*