Published in final edited form as:

Methods. 2019 July 15; 164-165: 81–90. doi:10.1016/j.ymeth.2019.03.011.

Advances in optogenetic regulation of gene expression in mammalian cells using cryptochrome 2 (CRY2)

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Abstract

Synthetic regulation of gene expression provides a powerful approach to reprogram molecular and cellular processes and test the function of specific genes and gene products. In the last decade, optogenetic systems that allow light-dependent gene regulation have become valuable tools, providing tight spatiotemporal control of protein levels. Here we discuss and build on recent optogenetic approaches for regulating gene expression in mammalian cells using cryptochrome 2 (CRY2), a photoreceptor protein from *Arabidopsis*. We provide detailed protocols for using light to manipulate activity of a CRY2-based engineered photoactivatable Cre DNA recombinase, and to induce or disrupt transcription factor function. In addition, we provide instructions and software for building an inexpensive Raspberry-Pi-based programmable LED device for optogenetic experiments, delivering pulsed light with customized control of illumination duration, frequency, and intensity.

Keywords

optogenetics; cryptochrome; light; transcription; Cre recombinase; LED

1. Introduction

Gene regulation, involving the transduction of a stimulus resulting in increased or decreased production of a specific gene product, is a fundamental process that occurs within all biological systems. Tools that provide synthetic control over gene expression, allowing induction or repression of synthesis of protein products, are powerful reagents for dissecting the biological role of specific genes and gene products. The most widely used inducible methods to regulate gene expression involve chemical approaches, including chemical-responsive transcriptional activators such as engineered Tet-ON and Tet-OFF systems [1,2], hormone-responsive transcription factors [3], and hormone-responsive DNA recombinases[4] [5]. Chemical inducers of dimerization (CIDs), chemicals that mediate

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Declaration of interest: None

dimerization of two protein domains within cells [6] [7], have also been used to control gene expression by inducing reassembly of split versions of transcription factors [8], T7 RNA polymerase [9], Cre recombinase [10] and CRISPR/dCas9 systems [11]. While chemically-regulated systems are rapid and inducible, such approaches cannot provide spatial resolution, lack tissue specificity, may have off-target effects, and can show delayed or limited reversibility.

To overcome the limitations of chemical systems and provide additional benefits such as spatial resolution, many groups have explored the use of optogenetic approaches to regulate gene expression. In optogenetic systems, engineered photoreceptor proteins are used to transduce a light stimulus, ultimately resulting in enhanced or reduced gene expression [12, 13]. The photoreceptors are typically bound to a chromophore, a small molecule ligand that undergoes a chemical change upon light absorption. The chemical change in the chromophore induces a conformational change in the photoreceptor as it adopts a light-excited state. This conformational change can allosterically regulate adjacent domains or alter affinity of interaction with other proteins. The most common photoreceptors used for engineering include flavin-based UV/blue-light receptors such as cryptochromes, BLUF, and LOV-domain-containing photoreceptors, retinal-based green-light receptors, and linear tetrapyrrole-based red-light receptors [13–15].

Within mammalian cells, optogenetic regulation of gene expression has been achieved through a number of different approaches (Fig. 1). The most common approach uses photosensory domain protein interactions to reconstitute activity of split transcription factors or other enzymes that impact gene expression, such as Cre DNA recombinase or Cas9 (Fig. 1A). A second approach involves controlling nuclear access of an enzyme that affects gene expression, utilizing photosensory proteins that have been engineered to show light-dependent nuclear localization or exclusion (Fig. 1B). A third approach uses a photoreceptor protein that dimerizes and binds or dissociates from DNA when illuminated (Fig. 1C). Finally, a fourth approach uses photosensory domain insertions to modulate activity by steric and allosteric mechanisms (Fig. 1D).

Table 1 summarizes novel optogenetic approaches reported in the literature that have been used to control gene expression in mammalian cells. Each of these different systems have different properties, thus the selection of an ideal optogenetic tool for manipulating gene expression requires consideration of the properties of each system within the context of the experimental system to be regulated. The different approaches vary with regard to their protein targets (eg., transcription, recombination), background or uninduced activity, inducible range, light sensitivity, kinetics, ability to induce or block gene expression, reversibility, domain size, and ability to be multiplexed.

In this article, we focus on optogenetic approaches developed by our group, which use the plant photoreceptor cryptochrome 2 (CRY2) to regulate gene expression in mammalian cells. We provide detailed methods and new data related to three different approaches: 1) direct DNA manipulation using the light dependent interaction of CRY2 and a partner, CIB1, to reconstitute activity of a split Cre DNA recombinase [16], 2) use of CRY2-CRY2 self-association to disrupt transcription factor activity with light [17], and 3) a CRY2-CIB1

split Gal4-VP64 transcriptional activator that can be induced with light [17]. While these systems can be actuated by any type of blue light (or white light containing blue wavelengths), for more precise control of gene expression we use pulsed illumination that allows control of light duration, intensity, and frequency. Here we also provide instructions for constructing a Raspberry-Pi-controlled LED array to deliver pulsed light illumination to cells when performing optogenetic gene expression experiments.

2. Materials

2.1. Constructs

Optogenetic constructs described in this article are available at Addgene (www.addgene.org). Individual split Cre constructs, mCh-IRES-CRY2(L348F)-CreN (p688, Addgene #75368) and mCherry-IRES-CIB1-CreC (p645) are described in Taslimi et al., 2016 [16], CRY2(+NLS, FL)-Gal4BD(1-147)-VP16AD(short) (Addgene #92031), mCherry-IRES-CRY2(+NLS,FL)-Gal4BD(1-65) (Addgene #92035) and CIB1(+NLS, FL)-VP64 (Addgene #92037) are described in previously [17]. The loxP-STOP-loxP-EGFP reporter of Cre recombinase activity contains a *loxP*-flanked transcriptional stop cassette (PGK-Neo-polyA and 3x-polyA), followed by EGFP [18]. For luciferase reporter experiments, we used pGL2-GAL4UAS-Luc (Addgene #33020), and RSV- β Gal (Addgene #24058). In some experiments, a pcDNA3.1+ empty vector was used to normalize the total amount of DNA transfected.

The mCherry-tagged combined PA-Cre2.0 construct, mCherry-CIB1-CreC(106)-IRES-CRY2(L348F)-CreN(104) (p1082), is in the same vector backbone as p688, with CIB1-CreC(106) inserted as a fusion with mCherry upstream of the IRES element. The full sequence of p1082 is provided in Appendix A.

2.2. Cell culture and luciferase quantification

HEK293T cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) (Corning, 10-013-CV) supplemented with 10% FBS (Sigma, F0926) and 1× Penicillin-Streptomycin (Corning, 30-002-CL). Cells were split using Trypsin (Corning, 25-053-CL) and Dulbecco's Phosphate Buffer Saline (DPBS) (Corning, 21-031-CV). Cells were plated on 12-well tissue culture plates. Calcium phosphate or Lipofectamine 2000 (Thermo Fisher, 11668027) transfection was performed. Additional chemicals included 4% paraformaldehyde (Sigma, 1004965000) and Fluoromount-G mounting media (Southern Biotech 0100-01). Luciferase (Flue) activity was quantified using the Luciferase Assay System (Promega) and Modulus microplate reader (Turner Biosystems). Flue activity was normalized using a cotransfected RSV-lacZ control in which β -galactosidase activity was determined using an ONPG assay (Clontech Laboratories, protocol #PT3024-1) using *o*-nitrophenyl- β -d-galactopyranoside (Thermo Scientific) as a substrate, and represented as fold increase over a control containing only the luciferase reporter.

2.3. Imaging

Quantification of Cre DNA recombinase activity was carried out in live cells using a Leica DM IL LED Fluo microscope with L5 ET and TX2 ET filters. Images were taken with an

iPhone7 (Apple) equipped with an iDu microscope adaptor (iDu Optics) and visualized using ImageJ. For imaging of fixed cells, images of slides were acquired on a Dragonfly spinning disc confocal microscope (Andor Dragonfly) equipped with a 1024×1024 EM-CCD camera, 60× oil immersion hi NA objective, on an Olympus IX73 inverted stand. Data acquisition and analysis was performed using Fusion (Andor) and ImageJ software.

2.4. LED light source

Materials used to construct the LED light source included: Raspberry Pi 3 Model B, microSD UHS-I card (SanDisk ultra 16GB), 5 inch HDMI touch screen LCD for Raspberry Pi 1/2/B+ (Elecrow, RPA05010R), HDMI connector cable (AmazonBasics, HL-007306), Power supply for Raspberry Pi 5 V, 2.5 A (Adafruit, 1995), Wireless keyboard (Riitek i8 EPSI8), MiniPuck Driver 3021-D-E-1000 from LUXdrive (3 each), Logic Level Converter Bi-Directional (SparkFun, BOB-12009), array of seven NPN Darlington transistors (Mouser, 595-ULN2003AN), Perma-Proto HAT for Pi MiniKit (Adafruit, 2314), Perma-Proto Full-sized Breadboard PCB (Adafruit, 1606), Transistors (3 each) PNP 2N3906 (Jameco, 38375), Trim potentiometer 5K Ω (3 each) (Digi-Key, 3296W-502-ND), Resistor 330 Ω a 0.25 watt at 5% (3 each) (Jameco, 690742), Resistor 5.1 K Ω a 0.25 watt at 5% (6 each) (Jameco, 691032), power supply (12 V, 1A) (3 each), Royal Blue or Blue XLamp XP-E2 Color High Power LED Star (12 per LED array) (LEDSupply, CREEXPE2-COL-X), heat sink (1 per LED array) (LED Supply, MarkersSLIM 6 in), Solder roll (Jameco, 141786), 22 AWG cable (Jameco, 36792), male-to-male jumper wires (Adafruit, 758).

3. Methods

3.1. Control of Cre DNA recombinase activity using CRY2/CIB1 interaction

Cre recombinase from bacteriophage P1 has been used to control gene expression by inducing site-specific recombination at DNA regions flanked by two 34-base pair loxP motifs [19]. To gain light control over Cre recombinase activity, we previously developed a split protein system where the N-terminus (amino acids 19-104) of Cre DNA recombinase was fused to the photoreceptor CRY2 (CRY2-CreN), and the C-terminus of Cre recombinase (amino acids 106-343) was fused to a N-terminal domain (CIBN, residues 1-170) of CIB1 (CIBN-CreC) [18]. Light-induced heterodimerization of CRY2 and CIBN leads to reconstitution of the split Cre fragments and induction of enzyme activity. To evaluate recombinase efficiency, we used a reporter containing a loxP-flanked stop cassette at the N-terminus of EGFP (loxP-STOP-loxP-EGFP). After 15 min of pulsed light treatment, the light-activated split Cre recombinase yielded a 25-fold increase in the percentage of cells expressing GFP, which was increased to 158-fold after 24 h of pulsed light treatment. While the system was tightly controlled, a limitation was the recombination efficacy, as even after 24 h of light treatment only $16.4\% \pm 1.6\%$ of transfected cells showed recombination.

To improve efficacy of the photoactivatable Cre recombinase (PA-Cre), a second-generation version (PA-Cre2.0) was developed [16]. PA-Cre2.0 incorporates a mutant version of CRY2 (L348F) with an extended photocycle half-life of ~24 min, along with substitution of full length CIB1 in place of the truncated CIBN domain. Use of this light dimerizer pair results in 50-90% recombination rates after 24 h of light treatment. It is also important to note that

high rates of recombination are achievable with this new system even after a single (4s) flash of light ($20.7 \pm 4\%$ quantified in Taslimi et al., 2016 [16]).

The PA-Cre2.0 light-activatable Cre recombinase system has robust versatility for *in vitro* and *in vivo* systems allowing for high spatiotemporal control of gene regulation. In previous studies, we characterized Cre activity using a fluorescent reporter with a floxed-STOP cassette, but the practicality of our system encases an optogenetic toolbox that can manipulate genes via deletion, insertion, exchange, or inversion through insertion and orientation of site-specific flox sites into the genome [19,20]. A particular advantage of the PA-Cre system is its low anticipated toxicity. The Cre-LoxP system can show potential toxicity in mammalian cells due to off-target recombination of pseudo LoxP sequences, or incomplete gene excision of floxed loci [21][22]. This toxicity should be less of a problem when using PA-Cre2.0, as the enzyme is non-functional during periods of dark incubation.

3.1.2. A new combined PA-Cre2.0 vector with improved function—To reduce expression level variation due to use of multiple plasmids, the two fragments of the split PA-Cre2.0 recombinase were cloned into a single vector. Initially, this vector also contained an enhanced green fluorescent protein (EGFP) reporter fused to a P2A cleavable peptide sequence followed by the N terminus of CIB1-CreC (ie, EGFP-P2A-CIB1-CreC-IRES-CRY2(L348F)-CreN), to allow quantification of expressing cells [16]. With more extensive study we observed that due to the initial GFP-P2A tag, this construct functions optimally only at very high expression levels, which can be achieved with calcium phosphate transfection methods in HEK293T cells but not using other (cationic lipid-mediated) methods or cell lines. Thus, we generated a new combined PA-Cre2.0 vector (p1082, mCherry-CIB1-CreC-IRES-CRY2(L348F)-CreN) that maintains high activity with low background over a wide range of expression levels. As another benefit, this version contains a mCherry fluorescent protein tag fused to the CIB1-CreC fragment, which allows direct tracking of expression and localization of the CIB1-CreC fragment (Fig.2A). The combined system containing the mCherry tag shows equivalent (if not improved) recombination activity as compared with the two construct system using p688 (mCh-IRES-CRY2(L348F)-CreN) and p645 (mCh-IRES-CIB1-CreC) (Fig.2B).

3.1.3. Method for inducing PA-Cre2.0 with light—Below we describe a detailed method for using PA-Cre2.0 to induce recombination at loxP sites with light, using p1082 and the loxP-STOP-loxP-EGFP reporter. By replacing the EGFP reporter with a target protein open reading frame, or using another plasmid or cell line responsive to Cre-mediated recombination, one can obtain induced target protein expression with light. Our protocols below describe use of the system via transient transfection of HEK293T cells using calcium phosphate transfection methods, however other cell types and delivery methods, such as viral transduction, can also be used. It is important to note that differences in dynamic range can be achieved by adjusting the ratio of recombinase to floxed reporter, as well as varying the total amount of DNA transfected. These parameters may need to be optimized when using other cell types or different gene delivery approaches.

1. Clone a protein open reading frame of interest into the loxP-STOP-loxP-EGFP, or similar loxP regulated plasmid, in place of EGFP or use an established cell line responsive to Cre

recombinase. Note that a loxP-STOP-loxP-EGFP or similar construct (eg., DIO-EGFP) can be used as control to validate appropriate recombination rates with light treatment. Alternatively, cells containing an integrated floxed allele can also be used.

2. Split HEK293T or other cultured cells from a confluent plate into a 12-well dish. Cells should be split such that they are not overgrown at time of harvest, 48 hours after transfection. If cells will be fixed for imaging, a #1.5 thickness glass coverslip should be added to the culture well at this time. Ensure the coverslip is resting flat at the bottom of the dish by pressing with a pipette tip.

3. The following day, cells can be transfected. Calcium phosphate methods, which we describe here, can achieve close to a 100% transfection efficiency even when transfecting multiple plasmids, but other methods, such as cationic lipid-mediated transfection reagent (eg, Lipofectamine 2000) can also be used. For transfection into one well of a 12-well dish, we typically use 1 µg of each construct (p1082 and floxed reporter). The plasmid DNA is placed into a microcentrifuge tube, also containing 5 µl of 2.5 M CaCl₂, and brought to 50 µl total with sterile water. A second microcentrifuge tube is prepared in parallel that contains 50 µl of 2× HSB solution (50 mM HEPES, 280 mM NaCl, 2.2 mM NaH₂PO₄, 2.2 mM Na₂HPO₄, pH 7.14) (note that the final pH of this solution is critical). Add the ingredients in the first tube containing the DNA solution dropwise to the second tube while mixing (flicking by hand or gently mixing on a vortexer). After incubating the mixture at room temperature for 20 min., add the solution dropwise to the cells while at the same time gently distributing the media to disperse the precipitate (rotate the plate back and forth to allow even distribution of the precipitated DNA). Wrap the dish loosely in aluminum foil and place in a tissue culture incubator. After 4 to 6 hours, using a red LED safelight to avoid photoactivation, wash the cells with PBS, replace the medium, wrap samples in foil, and return them to the incubator until photostimulation.

4. Approximately 18-24 h post-transfection, treat samples with blue light (450-488 nm). CRY2 shows peak response to excitation wavelengths of 450-460 nm, but can be stimulated with light wavelengths within the range of 400-490 nm. Light treatment can range from a single 4s pulse or an extended pulsed light treatment (2s light / 3 min dark) ranging from 1-24 h depending on the desired efficiency of recombination. Using the above protocol with a 4s light pulse (460 nm, 5-15 mW/cm²), we typically induce recombination in 20-50% of cells. For higher rates of recombination, a 4s pulse can be repeated every 3 minutes for 2-4 hours (resulting in recombination in 50-90% of cells). To achieve lower rates of recombination, the light intensity can also be reduced.

For extended light treatment, pulsed light delivery is recommended, as it allows maintenance of near-maximal photoreceptor excitation while minimizing overall blue light exposure, which can induce cell stress and even death. Constant light illumination is also possible, but should be relatively dim due to potential for light-induced cellular toxicity. Pulsed light delivery can be carried out using the custom-built LED light source described in Section 3.4, or with another commercial or custom light source. To illuminate cells, the LED array is placed above the culture plate (plates and LED array can be loosely wrapped in foil to prevent stray light exposure for other users of the incubator). After completing the light

treatment, samples are further incubated in dark to allow time for reporter expression. To keep samples in dark conditions, loosely wrap the culture dish in aluminum foil.

5. After a period of time allowing for protein expression, cells can be harvested or experimentally tested. The efficacy of recombination of the loxP-stop-loxP-EGFP reporter can be assessed by FACS analysis or by imaging-based quantification of EGFP reporter signal (described here). In this latter case, coverslips are gently removed from the culture dish, washed with PBS, and fixed in 4% paraformaldehyde for 15 min. After washing twice more with PBS, coverslips are mounted on glass slides using Fluoromount-G or another mounting medium. Using the combined PA-Cre2.0 construct (p1082), transfected cells expressing mCherry-CIB1-CreC can be quantified by acquiring images in both mCherry (ex 587 nm / em 610 nm) and EGFP channels (ex 488 nm / em 507 nm). The mCherry expression is confined to the nucleus due to the strong NLS contained within CIB1. To quantify the total recombination efficiency, determine the fraction of cells that express EGFP out of the total number of mCherry-expressing cells. *Note: Cells can also be imaged directly in 12-well plates on an upright tissue culture fluorescent microscope, using appropriate mCherry or EGFP filters.*

3.2. Light-dependent disruption of transcription factor function using CRY2 clustering

Fusion of CRY2 to dimerizing or oligomerizing proteins increases its multivalence, leading to light-dependent oligomerization. After extended periods of light treatment, CRY2 oligomers are removed from the nucleus and cytosolic CRY2 is trapped in clusters, excluding the fused protein from the nucleus (Fig. 3A). It was previously shown that coupling CRY2 to a dimeric transcription factor consisting of the DNA-binding fragment of yeast GAL4 and the activation domain of the herpes simplex virus protein VP16 (CRY2-GalVP16), leads to a 28-fold reduction of transcription after 18 h of light due to exclusion of the protein from the nucleus [17].

To allow faster and more precise control of protein expression levels, the above-mentioned CRY2-transcription disruption system was combined with a previously developed AsLOV2-based light induced degron [23], allowing blockage of new protein synthesis as well as loss of existing protein upon onset of light. In this combined system, a target protein is fused to the AsLOV-degron, and placed upstream of a GalUAS-containing promoter. Initial studies indicated fast reduction of protein levels within four hours of light onset [17].

3.2.1. Testing dependence of CRY2-GalVP16 system on concentration—While the light-disrupted transcription approach allows for a large reduction of transcription in the presence of light, one drawback is the high background inherent to the system. Clustering of CRY2-GalVP16 is thought to be due to liquid-like phase separation reactions (condensates) that occur with the multivalent protein upon light exposure. In such reactions, a fraction of the CRY2-GalVP16 is expected to remain soluble even after condensate formation, leading to background activity in light. We tested whether increasing the valency of the protein (by fusing a tetrameric protein at the C-terminus of CRY2) could decrease the amount of soluble protein remaining in light (and thus decrease the background), however the increased valency induced cluster formation even in the dark and blocked transcription factor function

(Fig. 3B). We also tested whether modulating the concentration of CRY2-GalVP16 could result in altered background of the system. To quantify activity, we used a GalUAS-luciferase reporter, and normalized expression using an RSV-lacZ reporter. When we reduced the concentration of CRY2-GalVP16, we observed a decrease in the dynamic range of the system (Fig. 3C). Significantly, the reduction in the dynamic range was due to a decrease in dark activity, however the activity in light remained unaffected. The observed decrease in dark activity but not light-stimulated activity as the total protein concentration decreases is consistent with the properties of phase transitions separating the CRY2-GalVP16 population into two compartments of different concentration (within condensates and outside condensates). In such a case, the proportion of the CRY2-GalVP16 population remaining outside the condensates would be expected to be maintained at the solubility threshold despite the change in total protein concentration [24], resulting in unchanging activity in light.

3.2.2. Method for light disruption of transcription using CRY2 clustering—

Below we describe a detailed method for using CRY2-GalVP16 to disrupt transcription by light induced clustering, using transient transfection. When using this system, it is important to consider its high background, which can be improved by affixing an AsLOV2-degron [23] at the C-terminus of the target protein. As a positive control for the optogenetic regulation provided by CRY2-GalVP16, any GalUAS-luciferase or GalUAS-FP reporter plasmid can be used.

1. Clone target protein open reading frame under control of Gal4UAS promoter element.
2. Split HEK293T or other cultured cells from a confluent plate into a 12-well or other culture dish. Incubate overnight in a tissue culture incubator until cells achieve ~70% confluence.
3. Transfect cells using a calcium phosphate method (HEK293T cells, see protocol in section 3.1.3) or using a cationic lipid-mediated transfection reagent such as Lipofectamine 2000. Wrap plates loosely in foil and place in 37°C tissue culture incubator.
4. Samples can be illuminated with light beginning 4-6 hours after transfection (when media is replaced), delivering 2 s blue light every 3 min for 12-18 h.
5. When light treatment is complete, samples can be harvested for western blot, microscopy, or other types of analysis.

3.3. Light-dependent reconstitution of transcription factor activity using CRY2-CIB1

Transcription can also be regulated with light by induced reconstitution of a split transcription factor. In previous work, light-dependent interaction of CRY2 and CIB1 was used to bring together DNA binding domains (BD) with activation domains (AD) in mammalian cells, including split versions of Gal4 binding domain and VP16 or VP64 activation domains [17]. To avoid light-induced clustering and clearance from the nucleus of the CRY2-fused Gal4BD, a truncated version of GAL4BD (residues 1-65) missing the dimerization domain was fused to CRY2 (CRY2-GAL(1-65)), and the activation domain

VP64 or VP16 was fused to CIB1 (CIB1-VP64 or CIB1-VP16). Co-expression of CRY2-GAL(1-65) and CIB1-VP64 lead to a 101.8-fold induction of transcription after 18 h of pulsed light treatment. Using a slightly different approach, Quejada et al.[25] also regulated transcription using CRY2, CIB1, Gal4BD, and VP16 [25], but were most successful with CRY2 fused to VP16 and CIB1 fused to Gal4BD. We directly compared these two approaches in the same assay and observed similar dynamic range of activity (Fig. 4). Overall lower dark background was observed using CRY2-GAL(1-65) + CIB1-VP16, but higher light-induced activity was achieved using CRY2-VP16 + CIB1-Gal4BD.

We expect that this general split protein approach could be used to regulate a variety of other DNA binding proteins, such as transcriptional repressors. When fusing proteins to CRY2, it is important to avoid any multivalence of the fused construct, to avoid light-mediated clustering and reduced nuclear localization. Alternately, recent studies have explored use of modified versions of CRY2 that reduce clustering [26], which could be incorporated in such studies.

3.3.1. Method for reconstitution of split Gal(1-65)-VP16AD transcription factor using CRY2/CIB1—Below we describe a method for using CRY2-Gal(1-65) (Addgene #92035) and CIB1-VP64 (Addgene #92037) to induce gene expression with light. This system shows very low dark background and is recommended when reversible and tightly controlled gene regulation is required.

1. Clone target protein of interest under control of GalUAS-responsive promoter (for example, in place of luciferase in pGL2-Gal4UAS-luciferase reporter).
2. Split HEK293T or other cultured cells from a confluent plate into a 12-well culture dish. Incubate in a tissue culture chamber until cells achieve ~70% confluence.
2. Transfect cells by calcium phosphate method (HEK293T cells, see protocol in section 3.1.3) or using a cationic lipid-mediated transfection reagent (eg, Lipofectamine 2000). We typically use 0.5 –1 µg of CRY2-Gal(1-65), CIB1-VP64, and the GalUAS-target protein.
3. Initiate light treatment no earlier than 4 h after transfection, illuminating with a 2 s pulse of blue light every 3 minutes. The light intensity can be modulated to tune the expression level of the target gene. For dark samples, wrap the culture dish in aluminum foil. After light treatment is completed, cells can be fixed or harvested for experiments.

3.4. LED array for customizable delivery of pulsed illumination

Optogenetic tools are dose-dependent, thus for many experiments, the user desires to adjust the duration, intensity, and frequency of light treatment. In addition, using a duty-cycle for light delivery that alternates short pulses of light with periods of dark is recommended to avoid light toxicity. The duration and frequency of light pulses should be adjusted according to the photocycle lifetime of the photoreceptor, also taking into account the photoreceptor light sensitivity. For stimulation of wild-type CRY2, which has a dark reversion half-life of ~5.5 minutes, one or two seconds of saturating blue light (we typically use ~5-10 mW/cm²) every 3 minutes is typically sufficient for stimulation of more than 80% of the photoreceptor

population. If the light source is dim, a longer duration light pulse or even constant light can be used. Constant light doses ranging from 0 to 0.72 mW/cm² were previously used to induce different levels of expression of a target gene using the light-dependent reconstitution of CRY2-GAL(1-65) and CIB1-VP16 [17]. The light box can be used not only with CRY2 approaches, discussed here, but with any other optogenetic approaches. For use with optogenetic systems sensitive to other light wavelengths, alternate color LEDs can be substituted for the Blue or Royal Blue LEDs specified in the materials.

Our light box design provides a simple homemade device for independent control over three LED arrays using a Raspberry Pi processor. Using a customized JAVA program (LEDS.jar), a researcher can control the duration, frequency, number of pulses, and starting time of light treatment. Meanwhile, light intensity is controlled via hardware by tuning the current directed into each LED array. As an additional benefit, the light box can be programmed to start automatically at a set time of the day or night, or remotely controlled using remote desktop software (for example, TeamViewer, www.teamviewer.com).

The light box consists of three elements: a software stage where the parameters of the light pulses are introduced into a JAVA program, an electronic circuit where the current flowing into the LED array is controlled, and finally the LED arrays. The program allows control of the General Purpose Input/Output (GPIO) pins of a Raspberry PI 3 B as switches, providing 0 V in its off state and 3.3 V in its on state with a maximum output current of 16 mA per pin. The output current and voltage provided by the GPIO pins are not sufficient to directly power the LED arrays, thus a pre-amplification and an amplification circuit are required (Fig. 5A).

The circuit and software provided allow up to three independent temporal patterns of light. As an additional benefit, the starting time for initiating a light pattern, and the total number of cycles can be specified. If required, up to eight additional GPIO pins could be used to increase the number of independent temporal patterns using the Pi4J libraries (www.pi4j.com). However, such implementation would require circuit expansion and major changes to the provided software.

3.4.1. Method for building a Raspberry Pi programmable LED device—Initially, three different electronic circuits are required. The electronic components should be soldered following the diagrams presented in Fig. 5 using AWG22 cable or male-to-male jumper wires for the connections. Once the circuits have been soldered, the Perma-proto HAT is installed on top of the Raspberry, and the LED.jar program is installed. Next, the 5 inch HDMI screen is connected, and the LED array is connected to the Amplification circuit. After connecting the power supplies for the Raspberry and the LED array, the LED.jar program can be executed and light parameters entered.

3.4.1.1. Pre-Amplification circuit, Amplification circuit and LED array

1. **Pre-Amplification circuit.** The circuit shown in Fig. 5B is a signal conditioning circuit, and its main purpose is to raise the output voltage and current that comes from the Raspberry GPIO pins. This circuit was settled in a Perma-Proto HAT where pins #18, #27, and #22 correspond to the GPIO1, GPIO2 and GPIO3

respectively. In the Perma-Proto HAT, connect pins #18, #27, and #22 to the logic level converter (BOB-12009) to raise the output voltage of the Raspberry GPIO pins from 3.3 volts to 5.0 volts. Then connect the three logic level converter outputs to a Darlington Transistor array (ULN 2003AN) where the current of each GPIO signal will be increased to allow the activation of a BuckPuck LUXDriver at the amplification circuit. Take the amplified signals coming from GPIO 1, GPIO2, GPIO3 and a ground reference to the amplification circuit.

2. Amplification circuit. The circuit shown in Fig. 5C was set up following the design proposed in the driver's data sheet (Fig. 13 in LUXDrive 3021/3023 BuckPuck data sheet). To allow for independent control over three LED arrays, the same circuit was tripled. It should be noticed that the ON/OFF function proposed in the driver's data sheet was not implemented, since this is done by software. The program provides control over three independent LED arrays, with each array requiring an independent power source. The light intensity provided by the LED array can be adjusted by tuning the potentiometer, thus changing the total current that flows into the LED array.
3. LED array. The voltage and current demand of the LED arrays will vary according to the array configuration. To estimate the required power for a specific array configuration, sum 4 V for each High Power LED (Cree XLamp XP-E2 Color High Power LED Star) connected in series, and consider that the current provided by the amplification circuit splits at each branch in a parallel array. Here we used a 12 LED array configuration. We attached 4 series of 3 LEDs connected in parallel, and used a 12V 1A power source for each 12 LED array (Fig. 5D). To avoid LED diode damage due to overheating, each array is attached to a heat sink.

3.4.1.2. Software implementation

1. To set up the Raspberry Pi 3 B with the LEDS.jar software first install the Rufus software on your computer. This will allow you to use a SD Card as DD image. Download from: <https://rufus.akeo.ie/downloads/> Download the operating system, 2018-04-18-raspbian-stretch, from: <http://downloads.raspberrypi.org/raspbian/images/raspbian-2018-04-19/>
2. Insert a SD Card in your PC and open Rufus. In the Device menu choose the SD Card and for the option "Create a bootable disk using" select DD image. Browse for 2018-04-18-raspbian-stretch.zip. Press Start and accept all the following dialog boxes. When the bar is full, press the "Close" button.
3. Install the screen controller. This will depend on the screen you are using. Here we use a 5 inch HDMI touch screen LCD for Raspberry Pi 1/2/B+, and provide specific instructions for this display. This 5 inch HDMI screen model is convenient for darkroom use since it has an external switch to manually turn it on or off without losing communication with the Raspberry:
 - a. Connect the Raspberry to an auxiliary screen using the HDMI port

- b. Plug in the wireless keyboard using the Raspberry USB port
 - c. From your PC, download the screen driver file from the following address www.spotpear.com/download/diver24-5/LCD-show-171219.tar.gz and use a flash drive to transfer the file to the Raspberry Desktop. Alternately, connect the Raspberry to the internet and download the screen driver file directly to the Raspberry Desktop.
 - d. Open the Raspberry terminal and type the following commands:
 - `>cd Desktop`
 - `>tar xvf LCD-show-171219.tar.gz`
 - `>cd LCD-show/`
 - `>sudo ./LCD5-show`
 - e. Turn off the Raspberry, disconnect the HDMI port from the auxiliary screen and connect it to the 5 inch screen. Do not connect the screen pin socket directly to the Raspberry, instead use a male-to-male jumper cable to connect only the 5V, GND, MISO, MOSI, CE1, SCK and P6 pins from the screen pin socket to the Perma-Proto HAT of the Pre-amplification circuit (Fig. 5E).
4. Download and install the Pi4J libraries that allow LEDS.jar program to interact with the GPIO pins. Connect the Raspberry Pi to the internet, then go to the <http://pi4j.com/download.html> site and download the pi4j-1.2-SNAPSHOT.deb.
5. (*Optional*) Install TeamViewer software by typing the following instructions in the Raspberry terminal:
 - `>sudo apt install -y`
 - `>wget https://download.teamviewer.com/download/linux/teamviewer-host_armhf.deb`
 - `>sudodpkg -i team*.deb`
 - `>sudo apt-get -f install`
6. From your PC or Raspberry, download the LEDS.jar program from <https://pharmacology.ucdenver.edu/tucker/reagents.html>. Copy the LEDS.jar program to the Raspberry Desktop. To open the program, type the following commands from the Raspberry terminal:
 - `>cd Desktop`
 - `>sudo java -jar LEDS.jar`

3.4.1.3. Control of the LEDS.jar program: Once the circuit and the software are set up, connect the LED array with its corresponding power source and launch the LEDS.jar program. Select one of the three GPIO output options displayed at the screen and set the values for the light treatment: time on, time off, and number of cycles. Then click the start

button or click the delay button and set a starting time for the light treatment. To reprogram the parameters of the session, click abort or close the window of the specific GPIO.

Note: To set a delay time, enter the hour in a 24 h format. The program takes the time from the Raspberry clock, so it is important to check the time in the right corner of the screen before setting the delay. If the Raspberry is connected to the internet the time will be updated automatically, otherwise it can be updated manually by clicking the “Update time” button displayed in the main screen of the program.

4. Troubleshooting and key parameters for light-induced gene expression systems

For all three approaches presented here, it is important to avoid exposure of dark samples to any blue or white light. General room lighting is sufficient to photoactivate CRY2. When incubating cells, samples should be wrapped loosely with aluminum foil to prevent light exposure. A red LED safelight can be used during experimental manipulations. When using a microscope to visualize cells, white light often used to focus the microscope will stimulate CRY2. A bandpass filter that blocks wavelengths of light below 514 nm can be applied to the microscope to prevent photostimulation. Visualization of mCherry or RFP fluorescence using standard filters is typically safe and will not photoactivate CRY2.

When applying the methods described above, transfection efficiency is an important parameter since dynamic range can vary with construct expression level. Also, high transfection efficiency is important when three or more different DNA plasmids are transfected. If dynamic range is lower than expected, confirm the transfection efficiency by quantifying the expression of a fluorescent reporter protein such as mCherry. As transfection efficiency is dependent on the amount of total DNA transfected, a control or empty vector should be used to equalize the total amount of DNA transfected.

While the methods described here utilize transient transfections in a single cell type (HEK293T cells), we expect these approaches to be broadly useful with many different cell types and gene delivery systems. Because the dynamic range, activation level, and background activity of these optogenetic systems will vary with expression level of the light-regulated proteins in the system, each new gene delivery approach or new cell type use will likely require individual optimization. Such optimization will be especially important for use *in vivo*. To optimize the dynamic range of the systems for use *in vivo* or in other cell types, stronger or weaker promoters can be utilized, or other gene delivery systems (for example viral transduction with AAV or lentivirus) can be used.

Each of the three approaches presented here have different characteristics that should be considered when selecting a suitable system to modulate gene expression. One of the biggest differences is reversibility: the PA-Cre system is not reversible after light stimulation. In turn, the two transcriptional system allow for reversible modulation of transcription by light vs dark. Another distinctive characteristic of PA-Cre2.0 is its high sensitivity, allowing a ctivation with just a single pulse of blue light. The split transcriptional system is also very

sensitive to light, but cannot be activated to very high levels. Finally, with the PA-Cre system, the background signal will increase as the concentration of the split fragments increases, promoting fragment self-assembly. In contrast, because the split domains in the transcriptional system are completely separable domains with no affinity, fragment self-assembly is not a concern.

When compared to other inducible transcriptional systems, the CRY2/CIB1 split transcriptional activator approach described here offers several advantages, including providing tight control with low background expression in dark, use of a commonly used promoter (GalUAS), and minimal sensitivity to protein expression differences. A disadvantage of the system is the long light treatment required and the overall low level of reporter expression achieved, compared to use of an intact Gal4-VP16 transcription factor.

The CRY2-transcriptional inactivation/clustering approach results in high gene expression in dark and decreased expression in response to light. This system is also sensitive to protein expression level, with higher expression resulting in enhanced dynamic range. A major disadvantage of the system is the high background in light, due to the substantial fraction of proteins that remain soluble after condensate formation. To overcome this issue, the light-inactivation system can be combined with a light-activated degron to achieve faster and more complete protein depletion with light.

Finally, the LED light box described here will be useful not only with the CRY2-based methods described here, but can also be used to induce gene expression using other optogenetic approaches. For example, several groups have developed optogenetic approaches to regulate gene expression at endogenous sites using CRISPR/dCas9 or other enzymes [29–31]. The LED light box can be easily configured for use with these systems. As the LED box allows for precise adjustment of illumination intensity and duration, it will be useful in combination with light-activated transcriptional systems to optimize and tune expression of toxic proteins or proteins that need to be maintained at precise levels within cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Masayuki Yazawa for providing the CRY2-VP16 and CIB1-Gal4BD constructs used in Figure 3D. This work was supported by the National Institutes of Health, grant numbers [R01GM100225, R21GM126253].

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Highlights

- Optogenetic tools allow inducible control of gene expression in mammalian cells
- We describe use of CRY2 to regulate Cre recombinase and transcription factors
- A new Raspberry Pi-based LED array to deliver pulsed light to cells is described

Figure 1. Strategies for regulating gene expression using photoreceptor proteins to transduce a light input.

A) Complementation of a split protein, B) Inducing changes in protein localization, C) Induced protein dimerization and DNA-binding, D) Induced steric or allosteric regulation.

Figure 2. Comparison of single, untagged and combined, tagged PA-Cre2.0 versions.

A) Schematic showing the combined construct (p1082) containing mCherry fused at the N-terminus of CIB1-CreC B) Comparison of Cre recombinase activity. HEK293T cells were transfected with a loxP-STOP-EGFP reporter and either p1082 (mCh-CIB1-CreC-IRES-CRY2(L348F)-CreN) or the two-construct system (p688, mCh-IRES-CRY2(L348F)-CreN and p645, mCh-IRES-CIB1-CreC). Cells were exposed to blue light (2s pulse every 15 min, 450 nm) for 4 h, 24 h after transfection, then placed in dark and quantified at 48 h post transfection by imaging. Data represent mean values $\pm$ s.d. ($n = 3$ independent experiments).

Figure 3. Further testing of CRY2-based transcriptional systems.

A) Schematic showing the light dependent exclusion of CRY2-GalVP16 from the nucleus. B) Representative image of HEK293T cells expressing CRY2-mCh-homer1c-GalVP16 in dark conditions. The tetramerizing protein homer1c increases the valency and induces the formation of condensates even in dark in HEK293T cells. Scale bar, 20 μ m. C) Dependence of CRY2-GalVP16 activity on expression level differences. While the level of activity in dark decreases as the amount of transfected CRY2-GalVP16 DNA decreases, the activity in light remained relatively constant, resulting in a net decrease in the dynamic range of the

system. Cells were transfected with indicated amounts of CRY2-GalVP16, along with RSV-lacZ and GalUAS-luciferase reporters. A pcDNA3.1+ empty vector was included as needed to equalize total DNA transfected. Cells were exposed to 18 h of blue light (2s pulse every 3 min, 450 nm), beginning 4 h after transfection. Data represent mean fold increase of normalized luciferase activity (luciferase activity/ β -galactosidase activity) over a control sample. Error bars represent range ($n=2$ independent experiments).

Figure 4.

Comparison of CRY2/CIB1 split transcriptional systems. HEK293T cells were transiently transfected with mCh-IRES-CRY2-Gal(1-65) and CIB1-VP16 (described in Pathak et al. [17]), or CRY2-VP16 and CIB1-Gal4 (described in Quejada et. al. 2017 [25]), as indicated, along with GalUAS-luciferase and RSV-lacZ reporter constructs (1 μ g each). Cells were exposed to 18 h of blue light treatment (2s pulse every 3 min, 450 nm), beginning 4 h after transfection, then harvested at 22 h. Data represent mean fold increase of normalized luciferase activity (luciferase activity/ β -galactosidase activity) over reporter-only control. Error bars represent range ($n=2$ independent experiments).

Figure 5. Diagram circuits used to implement a programmable LED device for optogenetic applications.

A) Light box elements. B) Pre-amplification circuit. We recommend implementing this circuit in a Perma-Proto HAT where pins #18, #27 and #22 correspond to GPIO 1, 2 and 3. C) Amplification circuit. The pre-amplified signal from each GPIO is connected to a LUXdriver (3021-D-E-1000) to trigger the activation of a LED array. Each LED array uses an independent power source. D) Configuration of a 12 LED array that fits a 12-well culture dish, one LED per well. This LED array requires a 12V and 1A power source. E) When using a 5 inch HDMI screen, wire only the socket pins shown in colors to the Perma-Proto

HAT of the pre-amplification circuit. The red box marks the GPIO1, GPIO2 and GPIO3 pins at the Perma-Proto HAT.

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Table 1.

Optogenetic systems for regulating gene expression in mammalian cells

Name	Optogenetic components	λ_{Ex} (nm), peak	Photocycle half-life	Light regulated protein	Reference
SPLIT PROTEIN					
LAD	FKF/GI	450	62 h	Gal4 DNA binding domain (DBD); VP16 activation domain (AD)	Yazawa et al. 2009 [27]
LITEZ	FKF1/GI	450	62 h	Synthetic zinc finger DBD; VP16AD	Polstein and Gersbach 2012 [28]
	FKF1/GI CRY2/CIB1	450	62 h, 5.5 min	Gal4 DBD; VP16 AD or VP64 AD	Quejada et al. 2017 [25]
PA-Cre, PA-Cre2.0 (CRY)	CRY2/CIBN CIB1	450	5.5 min, 25 min	Cre recombinase (19-104; 106-343)	Kennedy et al. 2010 [18] Taslami et al. 2016 [16]
LITE	CRY2/CIB1	450	5.5 min	TALE DBD; VP64 AD	Konermann et al. 2013 [29]
LACE	CRY2/CIB1	450	5.5 min	CRISPR/dCas9; VP64 AD	Polstein and Gersbach 2015 [30]
	CRY/CIB1	450	5.5 min	CRISPR/dCas9; p65 AD	Nihongaki et al. 2015 [31]
	CRY2/CIB1	450	5.5 min	Gal4(1-65) DBD; VP16 or VP64 AD	Pathak et al. 2017 [17]
PA-Tet-ON PA-Tet-OFF	CRY2PHR/CIBN	450	5.5 min	TetR DBD; p65 AD	Yamada et al. 2018 [32]
paCas9-1	pMag/nMagHigh1 (Magnets)	450	1.8 h	Cas9 (2-713; 714-1368)	Nihongaki et al. 2015 [33]
PA-Cre (Mag)	pMag/nMag (Magnets)	450	1.8 h	Cre recombinase (19-59; 60-343)	Kawano et al. 2016 [34]
BphP1- PpsR2	BphP1/ PpsR2	756 or 678	Inducible (636 nm)	TetR DBD; NLS-VP16 AD	Kaberniuk et al. 2016 [35]
	BphP1/ PpsR2(101-251, Q-PAS1)	756 or 678	Inducible (636 nm)	TetR DBD; NLS-VP16 AD	Redchuk et al. 2017 [36]
	PhyB/ PIF6	660 or 740	Inducible (740 nm)	TetR DBD; VP16 AD	Müller et al. 2013 [37]
	TULIPs	450	17 s	Gal4 DBD; VP16 AD	Müller et al. 2014 [38]
COMPARTMENTALIZATION/LOCALIZATION					
iTango	CRY2/CIBN	450	5.5 min	TetR-VP16	Lee et al. 2017 [39]
	CRY2	450	5.5 min	Gal4-VP16	Pathak et al. 2017 [17]
LINuS	AsLOV2	450	50 s	LexA DBD-VP64-NES	Niopek et al. 2014 [40]
LEXY	AsLOV2	450	50 s	NLS-LexA DBD-KRAB; NLS-p53	Niopek et al. 2016 [41]
FLARE	AsLOV2	450	50 s	TetR-VP16	Wang et al. 2017 [42]
	BphP1/PpsR2 (101-251, Q-PAS1)	756 or 678	Inducible (636 nm)	Gal4-VP16	Redchuk et al. 2017 [36]
	PhyB/ PIF6	660 or 740	Inducible (740 nm)	Gal4-VP16; Gal4-VP64	Noda and Ozawa 2018 [43]
	PhyB/ PIF3	660 or 740	Inducible (740 nm)	TetR-VP16	Beyer et al. 2015 [44]
DIMERIZATION AND DNA-BINDING					

Name	Optogenetic components	λ_{Ex} (nm), peak	Photocycle half-life	Light regulated protein	Reference
EL222	EL222	450	30 s	EL222-VP16	Motta-Mena et al. 2014 [45]
Blue-OFF	EL222	450	30 s and 50s	KRAB-EL222	Baaske et al. 2018 [46]
LightON	VVD	450	2 h	Gal4 DD(1-65)-VVD-p65	Wang et al. 2012 [47] Ma et al. 2013 [48]
	BphP1/PpsR2 (101-251, Q-PAS1)	756 or 678	Inducible (636 nm)	Gal4(68)-QPAS1-VP16	Redchuk et al. 2017 [36]
CarH-VP16	CarH	525	NA	CarH-VP16	Chatelle et al. 2018 [49]
STERIC / ALLOSTERIC					
opto-DN- CREB	PYP	450	20 min	Dominant negative inhibitor of CREB	Ali et al. 2015 [50]
CASANOVA	AsLOV2	450	50 s	Cas9 inhibitor AcrIIA4	Bubeck et al. 2018 [51]