Published in final edited form as:

Methods Mol Biol. 2014 ; 1148: 89–107. doi:10.1007/978-1-4939-0470-9_7.

Light-Inducible Gene Regulation with Engineered Zinc Finger Proteins

Lauren R. Polstein¹ and Charles A. Gersbach^{1,2,3,*}

¹Department of Biomedical Engineering, Duke University, Durham, North Carolina, United States of America, 27708

²Institute for Genome Sciences and Policy, Duke University, Durham, North Carolina, United States of America, 27708

³Department of Orthopaedic Surgery, Duke University Medical Center, Durham, North Carolina, United States of America, 27710

Abstract

The coupling of light-inducible protein-protein interactions with gene regulation systems has enabled the control of gene expression with light. In particular, heterodimer protein pairs from plants can be used to engineer a gene regulation system in mammalian cells that is reversible, repeatable, tunable, controllable in a spatiotemporal manner, and targetable to any DNA sequence. This system, Light-Inducible Transcription using Engineered Zinc finger proteins (LITEZ), is based on the blue light-induced interaction of GIGANTEA and the LOV domain of FKF1 that drives the localization of a transcriptional activator to the DNA-binding site of a highly customizable engineered zinc finger protein. This chapter provides methods for modifying LITEZ to target new DNA sequences, engineering a programmable LED array to illuminate cell cultures, and using the modified LITEZ system to achieve spatiotemporal control of transgene expression in mammalian cells.

Keywords

optogenetics; transcription; gene regulation; gene expression; zinc finger protein; protein engineering; genetic engineering; synthetic biology; gene circuits

1. Introduction

The growing complexity of scientific research requires the development of advanced gene regulation systems. The ability to easily manipulate gene expression with spatial and temporal control in a tunable and reversible manner is critical to many potential applications in gene therapy, regenerative medicine, tissue engineering, metabolic engineering, synthetic biology, and basic research. The most common gene regulation systems are controlled by the presence of a small molecule such as an antibiotic or steroid analogue [1–9]; however,

* Address for correspondence: Charles A. Gersbach, Ph.D., Department of Biomedical Engineering, Room 136 Hudson Hall, Box 90281, Duke University, Durham, NC 27708-0281, Phone: 919-613-2147, Fax: 919-668-0795, charles.gersbach@duke.edu.

because small molecules quickly diffuse throughout tissues and cell culture, these systems cannot be used to achieve spatial control over gene regulation. Furthermore, the small molecule must be removed from the system to reverse its effect, making it difficult to dynamically control gene expression for some applications.

Recently, the emerging field of optogenetics has provided tools to engineer gene regulation systems that are controlled by light rather than small molecules, thus enabling precise spatiotemporal genetic control [10,11]. Several of these systems have been reported to modulate transcription of an exogenous transgene by the presence of blue [12–16] or red [17–19] light. This makes gene activation and deactivation easier and faster compared to conventional systems, and it also eliminates the possibility of pleiotropic effects due to a chemical activator molecule. In general, most of these systems take advantage of plant proteins that form a homo- or heterodimer when illuminated by a specific wavelength of light (Figure 1). [FIG 1 NEAR HERE] Fusion of one of the plant dimerizing proteins to a DNA binding protein, such as the GAL4 DNA-Binding Domain, localizes that plant protein to the corresponding DNA target sequence. Fusion of the other plant protein to a transcriptional activation domain, such as the herpes simplex virus protein VP16 [20,21], results in translocation of the activation domain to the DNA binding sequence upon illumination at the activating wavelength. This then activates transcription of a transgene located downstream of the DNA binding sequence.

We engineered a system that can regulate any transgene under the control of any arbitrary promoter sequence using blue light [22]. This system, Light-Inducible Transcription using Engineered Zinc finger proteins (LITEZ), takes advantage of the interaction between GIGANTEA (GI) and the Light-Oxygen-Voltage (LOV) domain of FKF1 [23]. These two proteins exist in the *Arabidopsis thaliana* plant and form a heterodimer in the presence of blue light (450 nm). In the LITEZ system, GI is fused to the N-terminus of a synthetic zinc finger protein (GI-ZFP), which makes this system unique because the ZFP is highly programmable and can theoretically be engineered to bind any desired DNA sequence through a variety of different techniques [24–37]. The other protein domain, LOV, is fused to three tandem copies of the minimal VP16 activation domain (LOV-VP16) [20,21,23]. When expressed in cells, GI-ZFP localizes to the ZFP target DNA sequence upstream of the transgene. In the presence of blue light, LOV binds to GI, which translocates VP16 to the gene of interest and activates transcription. Engineering of the ZFP provides the opportunity to target GI-ZFP to any DNA sequence. This flexibility in design enables the regulation of novel synthetic promoters, the use multiple orthogonal promoters in a single cell, and potentially the ability to control natural endogenous promoters using blue light.

The following protocol describes the steps for engineering and using any GI-ZFP for light-inducible activation of a transgene in a spatiotemporal, reversible, repeatable, and tunable manner. It is assumed that a functional ZFP has already been engineered and determined to be active in the cell type or species of interest as a direct fusion to an activation domain, repression domain, or endonuclease. Many protocols have been published for designing, building, and testing novel ZFPs targeted to new sequences by a variety of methods [24–37], and online webserver have been created to facilitate this process [32,38–40,30]. Novel ZFPs are available commercially through Sigma-Aldrich, and many ZFPs are also available

through the Addgene non-profit plasmid repository (www.addgene.org) [36,37]. A complete volume of *Methods in Molecular Biology* has also been published dedicated to this subject [41].

2. Materials

2.1. Construction of GI-ZFP expression plasmid

1. Polymerase chain reaction (PCR) primers: The 3' end of these primers must be designed to anneal to the cDNA encoding the particular ZFP that will be cloned downstream of GI. For the forward primer (NotI-ZFP fwd), replace the “N” sequence shown below with a sequence that has a melting temperature of 55–60°C (about 14–21 base pairs long) and is homologous to the leading strand of the target ZFP gene, starting with the ATG start codon. It also may be necessary to add one or two additional base pairs between the *NotI* restriction endonuclease site and the homologous sequence to keep the ZFP in frame with the upstream GI gene once it is cloned into the final vector (*see* Note 1). For the reverse primer (XbaI-ZFP rev), replace the “N” sequence with a sequence that has a melting temperature of 55–60°C and is homologous to the antisense strand of the target ZFP gene. Make sure to include the stop codon in this homologous sequence. If desired, an epitope tag, such as HA or FLAG for ensuring expression or localization by western blot or immunofluorescence staining, can also be cloned between the GI and ZFP genes without affecting the activity of the fusion protein. To insert an epitope tag, insert the leading strand sequence of the epitope tag between the “ATG” and the next (fourth) base pair of the sequence that is homologous to the ZFP in the NotI-ZFP fwd primer. Ensure that the downstream ZFP gene remains in frame after adding the epitope tag.
 - a. NotI-ZFP fwd: 5'-taagcgccgcaNNNNNNNNNNNN-3' (*see* Note 2)
 - b. XbaI-ZFP rev: 5'-ttctagannnnnnnnnnnn-3' (*see* Note 3)
2. Expression plasmid pcDNA3-GI-ZFP2: This construct contains a Cytomegalovirus (CMV) immediate early promoter driving expression of a gene encoding GI-ZFP2 [22]. This plasmid can be obtained from Addgene (Plasmid 42215).
3. Restriction endonucleases and appropriate buffers: *NotI* and *XbaI*.
4. Sequencing primers (*see* Note 5):
 - a. CMV fwd: 5'-ATTGACGCAAATGGGCGGTAGGCGTGT-3'

¹One easy way to ensure this is to first perform the cloning procedure *in silico* using software such as SerialCloner®; if the cloning process causes the ZFP to be out of frame, add one or two base pairs to correct the frame.

²The NotI-ZFP fwd primer contains three random base pairs followed by the *NotI* restriction enzyme recognition site. The first three base pairs enable the *NotI* restriction enzyme to bind the DNA and cleave at the restriction site.

³The XbaI-ZFP rev primer contains one random base pair followed by the *XbaI* restriction site. Unlike *NotI*, *XbaI* only requires one extra base pair to achieve acceptable DNA cleavage efficiency. Information on how many base pairs different restriction endonucleases require upstream of their target site for efficient DNA cleavage can be found at: <https://www.neb.com/tools-and-resources/usage-guidelines/cleavage-close-to-the-end-of-dna-fragments>.

⁵While it is highly recommended that the entire GI-ZFP gene is sequenced for clone verification, only the GI fwd 5 primer is necessary to verify the sequence of the inserted ZFP gene.

- b. GI fwd 1: 5'-GCTGTTGCTAATGGAGCTG-3'
- c. GI fwd 2: 5'-CCACTGCAACCTCCTATC-3'
- d. GI fwd 3: 5'-GGACTCCATGGAGTTACAGT-3'
- e. GI fwd 4: 5'-AGCCTTGGATCGCCAAAG-3'
- f. GI fwd 5: 5'-ACTGGGAAGCTCACAGCTT-3'

5. Standard equipment and reagents for agarose gel electrophoresis.
6. Standard equipment and reagents for purification of DNA from agarose gels following electrophoresis, such as the QIAquick Gel Extraction Kit (Qiagen).
7. Standard equipment and reagents for purification of PCR products, such as the QIAquick PCR Purification Kit (Qiagen).
8. Calf intestinal alkaline phosphatase (CIP).
9. T4 DNA Ligase and appropriate buffer.
10. Chemically competent Stbl3 *E. coli* (Invitrogen) (*see* Note 4).
11. LB-Agar plates containing 100 µg/mL ampicillin for bacteria culture.
12. LB media containing 100 µg/mL ampicillin.
13. Standard equipment and reagents for purification of bacterial plasmid DNA, such as the Qiagen Spin Miniprep Kit.

2.2. Construction of reporter plasmid

1. Reporter plasmid pGL3-Basic-9xSeq2-Luc: This plasmid contains 9 copies of the DNA binding site for GI-ZFP2 (5'-AAACTGCAAAAG-3') upstream of a minimal CMV promoter and luciferase gene [22]. This plasmid can be obtained from Addgene (Plasmid 42214).
2. Oligonucleotides: The oligonucleotides below will be annealed and phosphorylated to create an insert containing three GI-ZFP DNA binding sites. Replace (N)_n with the target binding sequence of the GI-ZFP and (N')_n with the sequence that is antiparallel to the target binding sequence of the GI-ZFP. The double-stranded DNA product that will result from annealing these oligonucleotides contains sticky overhangs at the 5' and 3' ends that are compatible with ligation to DNA cleaved by *XhoI* and *BglII* endonucleases (*see* Note 6).
 - a. GI-ZFP binding fwd: 5'-tcgag(N)_ngtcgag(N)_ngtcgag(N)_na-3'
 - b. GI-ZFP binding rev: 5'-gatct(N')_nctcgac(N')_nctcgac(N')_nc-3'
3. Sequencing primer hCMV rev: 5'-GAGCTCTGCTTATATAGACCTCCC-3'.
4. Restriction endonucleases and appropriate buffers: *XhoI* and *BglII*.

⁴Chemically competent Stbl3 *E. coli* have been engineered for reduced recombination in the presence of direct repeat sequences, such as those found in ZFPs or in promoter sequences containing multiple copies of a DNA-binding protein's target site (i.e., pGL3-Basic-9xSeq2-Luc).

5. Reagents 2.1.5–2.1.13

2.3. Construction of a programmable LED light array

1. 12 blue LEDs centered on a wavelength of 450 nm (*see* Note 7).
2. 6×4×2" project enclosure box (Radio Shack).
3. Arduino™ Uno Microcontroller.
4. BuckPuck AC Driver (700 mA).
5. Male and female wire connectors (MOLEX).
6. Soldering iron.
7. Electrical wire.
8. Wire stripper.
9. Wire cutters.
10. Copper-terminal printed circuit board.
11. Lexan™ plexiglass.
12. 12 VDC, 1.0A power supply.
13. USB cable.
14. Silicone glue.

2.4. Measurement of LITEZ activity

1. Mammalian cell line that can be readily transfected, such as HeLa or HEK293 cells.
2. Lipofectamine 2000 Reagent (Invitrogen), or other suitable transfection reagent.

⁶Greater number of ZFP binding sites in the reporter plasmid will increase the level of gene expression when the system is activated by blue light [22]. If it is desired to have more than three GI-ZFP binding sites in the reporter plasmid, perform a PCR reaction using the following primers:

- a. GI-ZFP binding fwd: 5'-gaggagctcgag(N)_ngtcgcgag(N)_ngtcgcgag(N)_ngtcgcgag(N)_na-3'
- b. GI-ZFP binding rev: 5'-ctctctcagatct(N')_nctcgcac(N')_nctcgcac(N')_nctcgcac(N')_nctc-3'
- c. GI-ZFP outside PCR fwd: 5'-gaggagctcgagN₁N₂N₃N₄N₅-3', where N_i is the *i*th base pair of the GI-ZFP forward binding sequence, starting from the 5' end.
- d. GI-ZFP outside PCR rev: 5'-ctctctcagatctN'₁N'₂N'₃N'₄N'₅-3', where N'_i is the *i*th base pair of the sequence antiparallel to the GI-ZFP binding sequence, starting from the 3' end.

Perform PCR using 0.1 µl of each of the four primers listed above. This PCR step will create double-stranded oligonucleotides of different lengths due to misannealing of the GI-ZFP binding fwd and GI-ZFP binding rev primers. Purify the PCR reaction using a PCR purification kit. Digest the purified PCR product with *XhoI* and *BglII*, purify the digestion product using a PCR purification kit, and then ligate this product into the purified pGL3-Basic-9xSeq2-Luc digestion product from Step 2. Screen clones by sequencing until a clone containing the desired number of binding sites is found. This protocol should yield clones containing up to nine GI-ZFP binding sites (we found one clone containing 9 binding sites and another clone containing 7 binding sites by screening about 25 clones by colony PCR [42]).

Alternatively, commercial gene synthesis can be used to generate the desired insert of DNA-binding sites.

⁷We used Blue Rebel LEDs centered on a wavelength of 470 nm that were pre-soldered to a Saber 10 mm square base (Luxeon® Star LEDs). LOV domains typically have a relatively broad absorbance spectrum around 450 nm [43,44], so an LED of the exact 450 nm wavelength is not required. We have achieved similar levels of activation using LEDs centered at 447.5 and 470 nm.

3. OptiMEM® Reduced Serum Media (Life Technologies).
4. DMEM supplemented with 10% Fetal Bovine Serum (FBS).
5. DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (P/S)
6. 0.25% trypsin.
7. 2×24-well tissue culture treated plates.
8. Transfection plasmids:
 - a. pcDNA3-GI-ZFP
 - b. pcDNA3-LOV-VP16 (can be obtained from Addgene (Plasmid 42499, [23]))
 - c. Reporter plasmid
 - d. Empty⁸ expression plasmid (i.e., a plasmid containing a promoter but no expressed gene).
9. Lamp containing red flood lights (*see* Note 8).
10. Luciferase assay reagents, such as the Bright-Glo Luciferase Assay System (Promega).

2.5. Patterning gene expression using LITEZ

1. *Green fluorescent protein* (GFP) cDNA.
2. Reporter construct engineered in method 3.2 or pGL3-Basic-9xSeq2-Luc.
3. Polymerase chain reaction (PCR) primers:
 - a. GFP fwd: 5'-ACCATGGTGAGCAAGGGCGA-3'
 - b. GFP rev: 5'-CCTCTAGATTACTTGTACAGCTCGTCC-3'
4. Sequencing primer hCMV fwd: 5'-TAGGCGTGTACGGTGGG-3'.
5. Restriction endonucleases and appropriate buffers: *NcoI* and *XbaI*.
6. Reagents 2.1.5–2.1.13.
7. Lipofectamine 2000 Reagent (Invitrogen), or other suitable transfection reagent.
8. OptiMEM® Reduced Serum Media (Life Technologies).
9. DMEM supplemented with 10% FBS.
10. DMEM supplemented with 10% FBS and 1% P/S
11. 0.25% trypsin (Life Technologies).
12. 35mm glass-bottom tissue culture dish (MatTek Corporation, Ashland, MA).

⁸Cells expressing the LITEZ constructs must be manipulated under a red “safelight.” Working with cells under white light may activate the system. Red and blue light are at opposite ends of the visible light spectrum, and so red light will not induce GI/LOV binding.

13. Transfection plasmids:
 - a. pcDNA3-GI-ZFP
 - b. pcDNA3-LOV-VP16
 - c. Reporter plasmid,
 - d. An “empty” expression plasmid (i.e., a plasmid containing a promoter but no expressed gene)
 - e. A plasmid that constitutively expresses dsRed or another fluorophore distinguishable from GFP (we used pHUbC-dsRed, which is a plasmid containing the *dsRed* gene expressed under the human Ubiquitin C (hUbC) promoter).
14. Lamp containing red flood lights (*see* Note 8).
15. Photomask, such as one designed using computer software (i.e., AutoCAD®) and fabricated using a 3D printer (*see* Note 9).
16. Black electrical tape.
17. 1× phosphate-buffered saline (PBS).
18. 4% paraformaldehyde in PBS (PFA).
19. Epifluorescence inverted microscope, such as the Zeiss Axio Observer Z1 fluorescence microscope.

3. Methods

3.1. Construction of GI-ZFP expression plasmid

1. Amplify the target ZFP gene via PCR using XbaI-ZFP fwd and NotI-ZFP rev primers.
2. Isolate the PCR product by gel electrophoresis and subsequent column purification using the QIAquick Gel Extraction Kit according to the manufacturer’s directions.
3. Digest the purified PCR product and pcDNA3-GI-ZFP2 with restriction endonucleases *NotI* and *XbaI*. Also add the appropriate amount of CIP to the digestion reaction containing pcDNA3-GI-ZFP2 (we use 1 µl in 50 µl total volume). Allow the digestion to proceed for 1–3 hours.
4. Isolate the 8,858 base-pair digestion product from pcDNA3-GI-ZFP2 via gel electrophoresis and subsequent column purification using the QIAquick Gel Extraction kit. Purify the PCR digest reaction using the QIAquick PCR Purification kit according to the manufacturer’s directions.

⁹The photomask can be made using a 3D printer (Figure 4) or simply by cutting a pattern out of foil or other opaque material. For precision and repeatability between experiments, a 3D printer or other method for automated photomask fabrication is preferred.

5. Ligate the purified PCR digestion product into the purified pcDNA3-GI-ZFP digestion product using T4 DNA Ligase (*see* Note 10). Incubate at room temperature for 1 hour or overnight.
6. Transform 50 µl of chemically competent Stbl3 cells with 4 µl of the ligation reaction. Spread cells on ampicillin-containing LB-agar plates and culture overnight at 37°C.
7. Inoculate 5 mL of LB media containing 100 µg/mL ampicillin with one colony from the ampicillin-containing LB-agar plates, and culture 12–16 hours with shaking at 37°C.
8. Purify plasmid DNA from the bacterial culture using a plasmid purification kit, such as the Qiagen Spin Miniprep Kit.
9. Verify the new construct by sequencing with CMV fwd, GI fwd 1, GI fwd 2, GI fwd 3, GI fwd 4, and GI fwd 5 primers.

3.2. Construction of reporter plasmid

1. Anneal GI-ZFP binding fwd and GI-ZFP binding rev oligonucleotides: Combine 500 ng of each oligonucleotide and 1× T4 DNA Ligase Buffer in water to a final volume of 50 µl. Heat sample in a thermal cycler to 95 °C for 2 min, then cool to 25°C over a period of 45 min. Add 1 µl of T4 PNK and incubate at 37°C for 30 min. Sample can be stored overnight at 4 °C (*see* Note 11).
2. Digest pGL3-Basic-9xSeq2-Luc with restriction endonucleases *XhoI* and *BglII*. Also add the appropriate amount of CIP to this digestion reaction (we use 1 µl in 50 µl total volume). Allow the digestion to proceed for 1–3 hours.
3. Isolate the 4,863 base-pair digestion product via gel electrophoresis and subsequent column purification using the QIAquick Gel Extraction kit. Purify the PCR digest reaction using the QIAquick PCR Purification kit.
4. Ligate the annealed DNA product from step 1 into the purified pGL3-Basic-9xSeq2-Luc digestion product using T4 DNA Ligase (*see* Note 10). Incubate at room temperature for 1 hour or overnight.
5. Repeat steps 3.1.5–3.1.8
6. Verify the new construct by sequencing with the hCMV rev primer.

3.3. Construction of a programmable LED array

1. Cut the printed circuit board into a rectangle that fits inside the project enclosure box.

¹⁰When setting up the ligation reaction, add the PCR insert and pcDNA3-GI-ZFP backbone to the reaction in a 3:1 (insert:backbone) molar ratio. If cloning fails, it is likely that the Ligase Buffer was not fresh; obtain fresh buffer and try the reaction again. If cloning still fails, increase the insert:backbone ratio to 5:1 or more.

¹¹Alternatively, the annealing reaction can be placed in a heat block at 90–95°C for 3–5 minutes. After heating, turn off the heat block and allow the sample to cool to room temperature in the heat block.

2. Connect four blue LEDs in series onto the printed circuit board using the soldering iron and electrical wire. When soldering the LEDs to the circuit board, create a line running down one of the long edges of the board. Solder a second series of four LEDs along the opposite long edge of the printed circuit board. Solder the third and final series of four LEDs down the center of the circuit board. Space the LEDs to the dimensions of a 12-well tissue culture plate such that each LED is positioned approximately where the center of each well would be located.
3. Connect the three series of LEDs in parallel: solder the three wires at the anodic terminal of the circuit together (one from each LED series). Also solder the three wires at the cathodic terminal of the circuit together. Place the circuit board with attached LED array into the project box.
4. Extend the anodic and cathodic terminals by soldering a long piece of wire to each terminal. Cut a hole in the side of the project box and pass the wires through the hole to the outside of the box. Connect the free ends of these wires to a male wire connector, keeping track of which wire is the cathode and which is the anode. The wire connector enables separation of the LED array from the BuckPuck AC Driver and Arduino™ UNO microcontroller, allowing easy installation into an incubator while keeping the electrical control components outside of the humidity of the incubator environment.
5. Cut two new wires and install them into the female wire connector.
6. Fit the female wire connector into the male wire connector to determine which wire in the female connector connects to the anode and which connects to the cathode. Solder the cathodic wire from the female wire connector to the “LED (-)” pin and the anodic wire to the “LED (+)” pin of the BuckPuck AC Driver. An electrical diagram for this LED circuit can be seen in Figure 2. [FIG. 2 NEAR HERE]
7. Strip both ends of a new piece of wire. Solder one end onto the “Ctrl” pin of the BuckPuck AC Driver, and insert the other end into the “Pin 13” port of the Arduino™ UNO microcontroller. Wires can simply be stripped and inserted into the Arduino™ UNO microcontroller ports or soldered into the ports for a sturdier connection.
8. Strip a new piece of wire on both ends, and solder one end onto the “Vin (+)” pin of the BuckPuck AC Driver. Insert the other end into the “Vin” port of the Arduino™ UNO microcontroller.
9. Strip a new piece of wire on both ends, and solder one end onto the “Vin (-)” port of the BuckPuck AC Driver. Insert the other end into the “GND” port on the Arduino™ Uno microcontroller.
10. Cut the Lexan™ plexiglass to the dimensions of the project enclosure box, and glue the Plexiglass to the top of the box using silicone glue.
11. To program the LED array, download the Arduino™ coding software (available free for download at <http://arduino.cc/en/main/software>). Design a computer code that will pulse the LED array as desired (*see* Note 12). Install the LED code onto

the Arduino™ UNO Microcontroller by connecting the Arduino™ board to the computer via a USB cable and following the manufacturer's uploading instructions.

12. To use the LED array, disconnect the Arduino™ UNO microcontroller from the computer. Place the LED array inside the incubator and run the wires connected to the male wire connector out through the incubator's port (usually located in the back wall of the incubator with a plug—ensure the plug is reinserted into the incubator port to maintain environmental conditions). Place the Arduino™ UNO microcontroller and BuckPuck AC Driver outside of the incubator. Insert the male wire connector into the female wire connector, and plug the 12 VDC, 1.0 A power supply into the Arduino™ UNO microcontroller power port. Plug the other end of the power supply into an electrical outlet. The programmed code should now run on the LED array.

3.4. Measurement of LITEZ activity

1. The day before transfection (Day 0), seed cells into wells of 2×24-well plates such that confluency will be ~80% in 24 hours. If using HeLa cells, seed 160,000 cells/well. Make sure to seed and incubate cells in medium that does not contain antibiotic; presence of antibiotic during transfection will decrease transfection efficiency and cell viability.
2. The next day (Day 1), transfect cells according to the transfection reagent manufacturer's instructions. We transfected each well of HeLa cells using 2 µl of Lipofectamine 2000 reagent and 800 ng total plasmid DNA in 100 µl OptiMEM Reduced Serum Medium. Conditions that should be included are:
 - a. Control 1: mock transfected cells (cells that receive no plasmid or an “empty” expression plasmid)
 - b. Control 2: reporter construct only (*see* Note 13)
 - c. Control 3: reporter construct + pcDNA3-LOV-VP16
 - d. Control 4: reporter construct + pcDNA3-GI-ZFP (*see* Note 14)
 - e. Experimental sample: reporter construct + pcDNA3-LOV-VP16 + pcDNA3-GIZFP (*see* Note 15)
3. Wrap both plates in aluminum foil to shield cells from exposure to light.

¹²For assistance writing a computer code, there are free sample codes on the Arduino™ website designed for controlling LEDs. Furthermore, the code used to control the LITEZ system can be obtained from the authors upon request; in our published experiments we used a code that pulses the light for 3 seconds every 3 minutes [22].

¹³This sample is important because it reveals the background expression of luciferase from the reporter construct alone. To facilitate comparison across all samples, make sure to keep the total amount of transfected DNA constant across all samples; thus, if samples that receive only reporter construct will be transfected with 80 ng of the reporter, make sure to also transfect this “reporter only” sample with 720 ng of “empty” expression plasmid DNA.

¹⁴Controls 3 and 4 reveal activation of the reporter by the GI-ZFP or LOV-VP16 alone. There should not be any significant activation of the reporter in these samples when compared to cells that only received the reporter construct.

¹⁵It may be necessary to optimize the ratio of transfected reporter construct, pcDNA3- LOV-VP16, and pcDNA3-GI-ZFP for different cell lines. In HeLa cells, we found that a ratio of 1:1:3 (reporter construct:pcDNA3-LOV-VP16:pcDNA3-GI-ZFP) was optimal.

4. Exchange the media containing transfection reagent with fresh media 4–6 hours posttransfection (cells can now be incubated in media that contains antibiotic). While this media change is not necessary for some cell types, other cell types will die if left overnight in the transfection reagent-containing medium. Make sure to work with cells under a dim red safelight so as to avoid activating the LITEZ system during cell manipulation.
5. Wrap one plate in foil and return to the incubator. Place the other plate on top of the upward-facing LED array (Figure 3). We typically wait 12–24 hours after transfection to achieve stable protein expression levels and then turn on the LED array to initiate blue light-induced activation of the luciferase reporter gene (*see* Note 16). [FIG 3 NEAR HERE]
6. Measure luciferase activity at desired time points using a luciferase assay kit, such as the Promega Bright-Glo Luciferase Assay System, according to the manufacturer's instructions. Compare luciferase activity between cells that received illumination to cells that did not receive illumination, as well as between the different control and sample conditions.

3.5. Patterning gene expression using LITEZ

1. Amplify the cDNA of GFP using the GFP fwd and GFP rev primers (*see* Note 17).
2. Isolate the PCR product via gel electrophoresis and subsequent column purification using the QIAquick Gel Extraction kit.
3. Digest the purified PCR product and the reporter plasmid with restriction endonucleases *NcoI* and *XbaI*. Also add the appropriate amount of CIP to the digestion reaction containing the reporter plasmid (we use 1 μ l in 50 μ l total volume). Allow the digestion to proceed for 1–3 hours.
4. Isolate the 3,366 base-pair digestion product via gel electrophoresis and subsequent column purification using the QIAquick Gel Extraction kit. Purify the PCR digest reaction using the QIAquick PCR Purification kit.
5. Ligate the purified PCR digestion product into the purified reporter construct digestion product using T4 DNA Ligase. Incubate at room temperature for 1 hour or overnight.
6. Repeat steps 3.1.5–3.1.8.
7. Verify the new construct by sequencing with the hCMV fwd primer.
8. The day before transfection (Day 0), seed cells into glass-bottom 35 mm dishes such that confluency will be ~80% in 24 hours. If using HEK293 cells, seed 1.8×10^6 cells/dish. Make sure to seed and incubate cells in media that does not

¹⁶In the original LITEZ manuscript [22], the LED array was set to pulse “on” for 3 seconds every 3 minutes. This illumination regimen may require optimization for different cell types, GI-ZFPs, and applications.

¹⁷Genes other than GFP can be used to pattern gene expression as long as the gene product can be visually imaged by microscopy or immunofluorescence staining. Other example reporter genes include *beta-galactosidase*, *alkaline phosphatase*, and fluorescent proteins other than GFP.

contain antibiotic; presence of antibiotic during transfection will decrease transfection efficiency and cell viability (*see* Note 18).

9. The next day (Day 1), transfect cells according to the transfection reagent manufacturer's instructions. We transfected each dish of HEK293 cells using 10 μ l of Lipofectamine 2000 reagent and 2 μ g total plasmid DNA in 250 μ l OptiMEM Reduced Serum Medium. We transfected cells with 200, 120, 120, 120, and 1440 ng of reporter plasmid, LOV-VP16, GI-ZFP, hUbC-dsRed, and empty plasmid respectively.
10. Wrap dish in aluminum foil to shield cells from exposure to light.
11. Exchange the media containing transfection reagent with fresh media 4–6 hours posttransfection (cells can now be incubated in media that contains antibiotic). Make sure to work with cells under a dim red safelight so as to avoid activating the LITEZ system during cell manipulation.
12. Cover the sides and top of the dish with black electrical tape to prevent light from illuminating cells through the sides and top of the dish (cells should only be able to be illuminated by light passing up through the photomask at the bottom of the dish). Place the dish on top of the photomask. The tape can also be used to secure the plate onto the photomask. Place the plate and photomask on top of the upward-facing LED array, and turn on the LED array 12–24 hours after transfection to initiate blue light-induced activation of the GFP reporter gene.
13. To image patterned GFP expression, remove dish at desired time point. Carefully rinse cells 2 $\times$ with PBS, and then fix cells in 4% PFA for 10 minutes.
14. Rinse cells 2 $\times$ with PBS and image using a fluorescence microscope. Compare dsRed and GFP expression levels; while all transfected cells should express dsRed, only the cells illuminated through the photomask should express GFP (Figure 4). We acquired images using the scan and stitch feature of the Zeiss Axio Observer Z1 fluorescence microscope (5 $\times$ magnification). Images were analyzed using ImageJ software. [FIG 4 NEAR HERE]

Acknowledgments

Ricardo E. Dolmetsch provided the original GI and LOV-VP16 plasmid constructs and Carlos F. Barbas, III provided ZFP constructs used to characterize the original LITEZ system. Graeme O'Connell and Chandra Tucker assisted in the design and programming of the custom LED array. This research was funded by an NSF CAREER Award (CBET-1151035), NIH Director's New Innovator Award (1DP2-OD008586), a Ralph E. Powe Junior Faculty Enhancement Award from Oak Ridge Associated Universities. L.R.P. was supported by an NIH Biotechnology Training Grant to the Duke Center for Biomolecular and Tissue Engineering (T32GM008555) and the Duke Biomedical Engineering McChesney Fellowship.

References

1. Gossen MFS, Bender G, Muller G, Hillen W, Bujard H. Transcriptional Activation by Tetracyclines in Mammalian Cells. *Science*. 1995; 268(5218):1766–1769. [PubMed: 7792603]

¹⁸To increase resolution of activated and non-activated cells at the borders of the pattern, perform the experiment using media that does not contain phenol red or other colorimetric pH indicator; light will diffract less in clear media.

2. No D, Yao TP, Evans RM. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc Natl Acad Sci U S A*. 1996; 93(8):3346–3351. [PubMed: 8622939]
3. Rivera VM, Clackson T, Natesan S, Pollock R, Amara JF, Keenan T, Magari SR, Phillips T, Courage NL, Cerasoli F Jr, Holt DA, Gilman M. A humanized system for pharmacologic control of gene expression. *Nat Med*. 1996; 2(9):1028–1032. [PubMed: 8782462]
4. Beerli RR, Schopfer U, Dreier B, Barbas CF 3rd. Chemically regulated zinc finger transcription factors. *J Biol Chem*. 2000; 275(42):32617–32627. [pii]. [PubMed: 10924515]
5. Fussenegger M, Morris RP, Fux C, Rimann M, von Stockar B, Thompson CJ, Bailey JE. Streptogramin-based gene regulation systems for mammalian cells. *Nat Biotechnol*. 2000; 18(11):1203–1208. [PubMed: 11062442]
6. Weber W, Fux C, Daoud-el Baba M, Keller B, Weber CC, Kramer BP, Heinzen C, Aubel D, Bailey JE, Fussenegger M. Macrolide-based transgene control in mammalian cells and mice. *Nat Biotechnol*. 2002; 20(9):901–907. [pii]. [PubMed: 12205509]
7. Dent CL, Lau G, Drake EA, Yoon A, Case CC, Gregory PD. Regulation of endogenous gene expression using small molecule-controlled engineered zinc-finger protein transcription factors. *Gene Ther*. 2007; 14(18):1362–1369. [PubMed: 17637799]
8. Magnenat L, Schwimmer LJ, Barbas CF 3rd. Drug-inducible and simultaneous regulation of endogenous genes by single-chain nuclear receptor-based zinc-finger transcription factor gene switches. *Gene Ther*. 2008; 15(17):1223–1232. doi:gt200896 [pii] 10.1038/gt.2008.96. [PubMed: 18528430]
9. Schwimmer LJ, Gonzalez B, Barbas CF 3rd. Benzoate X receptor zinc-finger gene switches for drug-inducible regulation of transcription. *Gene Ther*. 2012; 19(4):458–462. [PubMed: 21796215]
10. Pathak GP, Vrana JD, Tucker CL. Optogenetic control of cell function using engineered photoreceptors. *Biol Cell*. 2013; 105(2):59–72. [PubMed: 23157573]
11. Muller K, Weber W. Optogenetic tools for mammalian systems. *Mol Biosyst*. 2013; 9(4):596–608. [PubMed: 23412367]
12. Yazawa M, Sadaghiani AM, Hsueh B, Dolmetsch RE. Induction of protein-protein interactions in live cells using light. *Nat Biotech*. 2009; 27(10):941–945. doi:http://www.nature.com/nbt/journal/v27/n10/supinfo/nbt.1569_S1.html.
13. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker CL. Rapid blue-light-mediated induction of protein interactions in living cells. *Nat Meth*. 2010; 7(12):973–975. doi:<http://www.nature.com/nmeth/journal/v7/n12/abs/nmeth.1524.html-supplementary-information>.
14. Ye H, Baba MD-E, Peng R-W, Fussenegger M. A Synthetic Optogenetic Transcription Device Enhances Blood-Glucose Homeostasis in Mice. *Science*. 2011; 332(6037):1565–1568. [PubMed: 21700876]
15. Ohlendorf R, Vidavski RR, Eldar A, Moffat K, M glich A. From Dusk till Dawn: One-Plasmid Systems for Light-Regulated Gene Expression. *Journal of Molecular Biology*. 2012; 416(4):534–542. [PubMed: 22245580]
16. Wang X, Chen X, Yang Y. Spatiotemporal control of gene expression by a light-switchable transgene system. *Nat Meth*. 2012; 9(3):266–269. doi:<http://www.nature.com/nmeth/journal/v9/n3/abs/nmeth.1892.html-supplementary-information>.
17. Shimizu-Sato S, Huq E, Tepperman JM, Quail PH. A light-switchable gene promoter system. *Nat Biotech*. 2002; 20(10):1041–1044.
18. Levskaya A, Lim WA, Voigt CA, Weiner OD. Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature*. 2009; 461(7266) 997+
19. Muller K, Engesser R, Metzger S, Schulz S, Kampf MM, Busacker M, Steinberg T, Tomakidi P, Ehrbar M, Nagy F, Timmer J, Zubriggen MD, Weber W. A red/far-red light-responsive bi-stable toggle switch to control gene expression in mammalian cells. *Nucleic Acids Res*. 2013; 41(7):e77. [PubMed: 23355611]
20. Beerli RR, Dreier B, Barbas CF 3rd. Positive and negative regulation of endogenous genes by designed transcription factors. *Proc Natl Acad Sci U S A*. 2000; 97(4):1495–1500. [pii]. [PubMed: 10660690]

21. Beerli RR, Segal DJ, Dreier B, Barbas CF 3rd. Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc Natl Acad Sci U S A*. 1998; 95(25):14628–14633. [PubMed: 9843940]
22. Polstein LR, Gersbach CA. Light-inducible spatiotemporal control of gene activation by customizable zinc finger transcription factors. *J Am Chem Soc*. 2012; 134(40):16480–16483. [PubMed: 22963237]
23. Yazawa M, Sadaghiani AM, Hsueh B, Dolmetsch RE. Induction of protein-protein interactions in live cells using light. *Nat Biotechnol*. 2009; 27(10):941–945. doi:nbt.1569 [pii] 10.1038/nbt.1569. [PubMed: 19801976]
24. Pabo CO, Peisach E, Grant RA. Design and selection of novel Cys2His2 zinc finger proteins. *Annu Rev Biochem*. 2001; 70:313–340. doi:70/1/313 [pii] 10.1146/annurev.biochem.70.1.313. [PubMed: 11395410]
25. Beerli RR, Barbas CF 3rd. Engineering polydactyl zinc-finger transcription factors. *Nat Biotechnol*. 2002; 20(2):135–141. [PubMed: 11821858]
26. Maeder ML, Thibodeau-Beganny S, Osiaik A, Wright DA, Anthony RM, Eichtinger M, Jiang T, Foley JE, Winfrey RJ, Townsend JA, Unger-Wallace E, Sander JD, Muller-Lerch F, Fu F, Pearlberg J, Gobel C, Dassie JP, Pruett-Miller SM, Porteus MH, Sgroi DC, Iafrate AJ, Dobbs D, McCray PB Jr, Cathomen T, Voytas DF, Joung JK. Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol Cell*. 2008; 31(2):294–301. [PubMed: 18657511]
27. Sander JD, Dahlborg EJ, Goodwin MJ, Cade L, Zhang F, Cifuentes D, Curtin SJ, Blackburn JS, Thibodeau-Beganny S, Qi Y, Pierick CJ, Hoffman E, Maeder ML, Khayter C, Reyon D, Dobbs D, Langenau DM, Stupar RM, Giraldez AJ, Voytas DF, Peterson RT, Yeh JR, Joung JK. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods*. 2011; 8(1):67–69. [PubMed: 21151135]
28. Gonzalez B, Schwimmer LJ, Fuller RP, Ye Y, Asawapornmongkol L, Barbas CF 3rd. Modular system for the construction of zinc-finger libraries and proteins. *Nat Protoc*. 2010; 5(4):791–810. [PubMed: 20360772]
29. Carroll D, Morton JJ, Beumer KJ, Segal DJ. Design, construction and in vitro testing of zinc finger nucleases. *Nat Protoc*. 2006; 1(3):1329–1341. doi:nprot.2006.231 [pii] 10.1038/nprot.2006.231. [PubMed: 17406419]
30. Gupta A, Christensen RG, Rayla AL, Lakshmanan A, Stormo GD, Wolfe SA. An optimized two-finger archive for ZFN-mediated gene targeting. *Nat Methods*. 2012; 9(6):588–590. [PubMed: 22543349]
31. Bhakta MS, Segal DJ. The generation of zinc finger proteins by modular assembly. *Methods Mol Biol*. 2010; 649:3–30. [PubMed: 20680825]
32. Bhakta MS, Henry IM, Ousterout DG, Das KT, Lockwood SH, Meckler JF, Wallen MC, Zykovich A, Yu Y, Leo H, Xu L, Gersbach CA, Segal DJ. Highly active zinc-finger nucleases by extended modular assembly. *Genome Res*. 2013; 23(3):530–538. [PubMed: 23222846]
33. Thibodeau-Beganny S, Maeder ML, Joung JK. Engineering single Cys2His2 zinc finger domains using a bacterial cell-based two-hybrid selection system. *Methods Mol Biol*. 2010; 649:31–50. [PubMed: 20680826]
34. Zhu C, Gupta A, Hall VL, Rayla AL, Christensen RG, Dake B, Lakshmanan A, Kuperwasser C, Stormo GD, Wolfe SA. Using defined finger-finger interfaces as units of assembly for constructing zinc-finger nucleases. *Nucleic Acids Res*. 2013; 41(4):2455–2465. [PubMed: 23303772]
35. Perez-Pinera P, Ousterout DG, Brown MT, Gersbach CA. Gene targeting to the ROSA26 locus directed by engineered zinc finger nucleases. *Nucleic Acids Res*. 2012; 40(8):3741–3752. doi:gkr1214 [pii] 10.1093/nar/gkr1214. [PubMed: 22169954]
36. Perez-Pinera P, Ousterout DG, Gersbach CA. Advances in targeted genome editing. *Curr Opin Chem Biol*. 2012; 16(3–4):268–277. doi:S1367-5931(12)00076-2 [pii] 10.1016/j.cbpa.2012.06.007. [PubMed: 22819644]

37. Sander JD, Yeh JR, Peterson RT, Joung JK. Engineering zinc finger nucleases for targeted mutagenesis of zebrafish. *Methods in cell biology*. 2011; 104:51–58. [PubMed: 21924156]
38. Mandell JG, Barbas CF 3rd. Zinc Finger Tools: custom DNA-binding domains for transcription factors and nucleases. *Nucleic Acids Res*. 2006; 34(Web Server issue):W516–W523. doi:34/suppl_2/W516 [pii] 10.1093/nar/gkl209. [PubMed: 16845061]
39. Sander JD, Maeder ML, Reyon D, Voytas DF, Joung JK, Dobbs D. ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. *Nucleic Acids Res*. 2010; 38(Web Server issue):W462–W468. [PubMed: 20435679]
40. Fu F, Sander JD, Maeder M, Thibodeau-Beganny S, Joung JK, Dobbs D, Miller L, Voytas DF. Zinc Finger Database (ZiFDB): a repository for information on C2H2 zinc fingers and engineered zinc-finger arrays. *Nucleic Acids Res*. 2009; 37(Database issue):D279–D283. doi:gkn606 [pii] 10.1093/nar/gkn606. [PubMed: 18812396]
41. Engineered Zinc Finger Proteins. *Methods in Molecular Biology*. 2010; 649
42. Packeiser H, Lim C, Balagurunathan B, Wu J, Zhao H. An Extremely Simple and Effective Colony PCR Procedure for Bacteria, Yeasts, and Microalgae. *Appl Biochem Biotechnol*. 2013; 169(2): 695–700. [PubMed: 23271627]
43. Kasahara M, Swartz TE, Olney MA, Onodera A, Mochizuki N, Fukuzawa H, Asamizu E, Tabata S, Kanegae H, Takano M, Christie JM, Nagatani A, Briggs WR. Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and *Chlamydomonas reinhardtii*. *Plant physiology*. 2002; 129(2):762–773. [PubMed: 12068117]
44. Nash AI, McNulty R, Shillito ME, Swartz TE, Bogomolni RA, Luecke H, Gardner KH. Structural basis of photosensitivity in a bacterial light-oxygen-voltage/helix-turn-helix (LOV-HTH) DNA-binding protein. *Proc Natl Acad Sci U S A*. 2011; 108(23):9449–9454. [PubMed: 21606338]

Figure 1.

Schematic of a general light-inducible gene regulation system. Many light-inducible gene regulation systems take advantage of homo- or heterodimerization between two light-inducible plant proteins. The first plant protein ("Light-Inducible Plant Protein 1") is usually fused to a DNA-binding domain, such as the GAL4 DNA-binding domain, which localizes the fusion protein to the DNA-binding domain's target DNA sequence. This target DNA sequence is located upstream a gene of interest. The second plant protein ("Light-Inducible Plant Protein 2"), is usually fused to a transcriptional activation domain, such as VP16. In the presence of an activating wavelength of light, the two light-inducible plant proteins

interact, which translocates the transcriptional activation domain to the gene of interest and activates transcription.

Figure 2.
Circuit diagram for a custom 3×4 LED array controlled by an Arduino UNO® microcontroller.

Figure 3.

Illumination of cells inside an incubator. A 24-well plate containing cells transfected with the LITEZ system was placed atop an upward-facing LED array inside an incubator. The wire extension connected to the male wire connector was connected through the back of the incubator via the incubator port. The male wire connector was then plugged into the female wire connector outside of the incubator.

Figure 4.

Patterned green fluorescent protein (GFP) expression using LITEZ. (a) HEK293 cells were illuminated with blue light through a photomask in the shape of a happy face or dot array pattern. Top two images show the AutoCAD® images used to design and fabricate the photomasks using a 3D printer. Bottom two images show resulting pattern of GFP-expressing cells when illuminated through the corresponding photomask. All cells were transfected with a plasmid that constitutively expressed dsRed. (b) HEK293 cells were illuminated through a photomask containing slits with widths of 2, 1, 0.5, and 0.3 mm. Features as small

as 0.5 mm were readily achievable. Inset (b, lower right) shows the sharp contrast of GFP-positive and -negative cells at the border of the 2 mm slit. (c) Photograph of a glass-bottom 35 mm dish on top of the photomask containing slits of various widths. The photomasks were painted black to decrease reflectance. Reprinted with permission from [22]. Copyright 2012 American Chemical Society.