TITLE:

Bioluminescent Optogenetics 2.0: harnessing bioluminescence to activate photosensory proteins *in vitro* and *in vivo*

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SUMMARY:

Bioluminescence, light emitted by a luciferase enzyme oxidizing a small molecule substrate, a luciferin, can be harnessed to activate photosensory proteins, thereby adding another dimension to light stimulation and enabling manipulation of a multitude of light-mediated functions in cells across temporal and spatial scales.

ABSTRACT:

Bioluminescence, light emitted by a luciferase enzyme oxidizing a small molecule substrate, a luciferin, has been used *in vitro* and *in vivo* to activate light-gated ion channels and pumps in neurons. While this bioluminescent optogenetics (BL-OG) approach confers a chemogenetic component to optogenetic tools, it is not limited for use in neuroscience. Rather, bioluminescence can be harnessed to activate any photosensory protein, thus enabling manipulation of a multitude of light-mediated functions in cells. A variety of luciferase-luciferin pairs can be matched with photosensory proteins requiring different wavelengths of light and light intensities. Depending on the specific application, efficient light delivery can be obtained by either luciferase-photoreceptor fusion proteins or by simple co-transfection. Photosensory proteins based on light-dependent dimerization or conformational changes can be driven by bioluminescence to effect cellular processes from protein localization, regulation of intracellular signaling pathways, to transcription. The protocol below details the procedures for experimental execution of bioluminescence activation in cells and organisms and describes results using bioluminescence driven recombinases and transcription factors. The protocol provides investigators with the basic procedures for carrying out bioluminescent optogenetics *in vitro* and *in vivo*. The described approaches can be further extended and individualized to a multitude of different experimental paradigms.

INTRODUCTION:

Photosensory proteins can be activated by light from either a physical light source or from a luciferase enzyme in the presence of its substrate, luciferin, generating bioluminescence. For

applications that require milli- or even femtosecond timescales and/or single cell spatial resolution, physical light sources (lasers and LEDs) are the only ones tunable to these scales. Examples are the spatial restriction of light used for stimulating opposite poles in developing Drosophila larvae with millisecond temporal control[1] or the precise stimulation of single sub-cellular structures such as mitochondrial tubules[2]. However, many other applications for optical switches have different priorities, including extended spatial control, repeated application non-invasively and without light damage, yet defined temporal control in minute timescales and tunable intensities. Here, using luciferases as an alternative light source to activate light-sensing domains has several advantages. In contrast to optical fiber light activation, bioluminescence reaches every light sensing domain expressed in the target cell population as the light source is genetically encoded. Using bioluminescence alleviates concerns over tissue and cell damage by fiber optics and extended physical light exposure. The light is turned on with application of the luciferase substrate. The onset is immediate *in vitro* as well as *in vivo* depending on the route of administration and lasts for ~15-30 minutes; longer presence or phasic stimulation of light can be achieved with different luciferins and with additional or repeated applications of substrate[3]. Lastly, bioluminescence emission can be tuned by varying the concentration of the luciferin.

The use of bioluminescence to activate ion-moving photoreceptors, i.e. optogenetic elements such as channelrhodopsins or pumps has been extensively demonstrated[4–8]. This BioLuminescent OptoGenetics (BL-OG) approach has been employed in *in vivo* experiments in mice and rats[5–7,9–12]. BL-OG activation of opsins was found to require an amount of bioluminescence of at least ~33 $\mu W/mm^2$, with efficiency of activation increasing with higher light emission[6,9]. Ion-moving sensory photoreceptors are a sub-group of the large contingent of sensory photoreceptors found in nature that are non-ion moving[13,14]. The extension of the use of bioluminescence to activating non-ion moving photoreceptors, such as photosensing domains from plants or bacteria, is encouraged by the finding by us and others[15,16] that non-ion moving photosensors are significantly more light-sensitive than channelrhodopsins, ensuring even better drive of light sensors with bioluminescence than already obtained with ion-moving optogenetic elements. Recently, several publications reported the use of bioluminescence as light source for activation of a variety of photoreceptors including LOV-domains, BLUF-domains and cryptochromes[3,17–22] (**Table 1**). Applications for bioluminescence driven activation of optical switches targeted intracellular processes from reactive oxygen species induced cell death, cAMP synthesis, protein recruitment and dissociation, to genomic recombination and induction of transcription.

This protocol outlines the general design of bioluminescence-driven optogenetic tools and details the procedures for experimental execution of bioluminescence activation in cells and organisms. It includes descriptions on how to set up a room, a tissue culture hood and incubator, and a microscope for work with bioluminescence as well as the steps from preparing the luciferin to applying it. This protocol provides investigators with the basic procedures for carrying out BioLuminescent OptoGenetics (BL-OG) *in vitro* and *in vivo*. The described approaches can be further extended and individualized to different experimental paradigms. We anticipate this protocol to facilitate the adoption of the use of bioluminescence in optogenetic biological studies.

PROTOCOL:

All procedures in the current study were performed using Institutional Animal Care and Use Committee (IACUC) approved protocols for animal handling at Central Michigan University, MI.

1. Bioluminescence activation of photosensory proteins *in vitro*

1. Constructs

1. Select a luciferase sequence or luciferase-fluorescent protein fusion sequence that will result in expression of a light emitter producing light of a wavelength matching the photoreceptor to be activated. For example, blue light emitting luciferases such as *Gaussia* luciferase variants or NanoLuc can be paired with blue light sensing photoreceptors such as CRY/CIB, LOV, or VVD.
2. Use standard molecular biology techniques to clone the DNA into a mammalian expression plasmid, if not already available from other investigators or from plasmid deposits.
3. The choice if promoters is dictated by the need to provide strong and constitutive expression of the light emitting module, such as provided by the CAG and CMV promoters.
4. For initial studies, use separate plasmids for co-transfection of light emitter and light sensor. Fusion proteins of the two moieties can be generated as needed and for subsequent studies.
5. Obtain high quality plasmid DNAs using mini-, midi-, or maxiprep commercial kits according to manufacturer's protocols.

2. Cell Culture and Transfection

NOTE: HeLa cells and HEK293 cells are used as examples in this protocol.

1. Plate cells in formats and numbers according to the desired end use. Specific examples are given in **Table 2**. Cell density at the time of plating will determine how soon cells can be transfected. For example, HEK293 cells to be used for assessing bioluminescence-activated transcription by fluorescence microscopy are plated on Poly-D-lysine (PDL)-coated 12mm coverslips placed in 24-well dishes. HeLa cell to be used for assessing bioluminescence-activated transcription by measuring light emission from an orthogonal reporter luciferase in a luminometer are initially plated in 6- or 12-well dishes for transfection but are re-plated after transfection (see step 4). If repeated bioluminescence stimulation will be carried out in live cell imaging chambers, select coverslips of the appropriate size and place into multi well plates of the appropriate size (24 well plates for 12mm coverslips; 12 well plates for 15mm and 18mm coverslips). Seed cells on top of coverslips according to cell numbers specified in **Table 2**. If the cell type selected does not adhere well to the culture surface, plate cells on PDL-coated dishes.

2. Transfection is done by lipofection according to the manufacturer's recommendation, but any transfection method appropriate for the cell type selected can be used. **Table 3** details transfection experiments for two different photoreceptors, EL222 and CRY2/CIB, and their respective reporter plasmids, in addition to different light emitting proteins. The ratios of the various plasmids work well for the selected examples but will have to be optimized for each light emitter/light sensor pair.
3. After transfection, place cells in an incubator that is completely light sealed (**Figure 1**).
4. Depending on the desired end use, cells are ready for bioluminescence stimulation the next day in their original wells/dishes, or they are re-plated 3-4 hours after lipofection. For reading transcription of a firefly luciferase reporter gene in a luminometer cells are re-plated in white 96 well dishes.
   Note: All manipulations are carried out in a light-tight room in a laminar flow hood illuminated by red light (**Figure 2**).
   1. Wells with transfected cells are washed once with plain DMEM or PBS.
   2. The minimum volume of trypsinizing reagent is added to wells (24 well: 100 µl; 12 well: 150 µl; 6-well 300 µl) and cells are incubated for 3 minutes at 37°C.
   3. Culture medium is added to achieve a cell concentration of appropriate cell density for the next plating step (for example: cells in a 24-well are resuspended in a final volume of 1.2 ml for plating in 10 wells of a 96-well plate; cells in a 12-well are resuspended in a final volume of 2.4 ml for plating in 20 wells of a 96-well plate; etc.). Depending on the number of wells needed in the end, transfected cells from several wells can be pooled.
   4. Transfected cells are plated in their final format and plates are returned to the light-protected incubator.

3. Bioluminescence activation *in vitro*

1. Prepare luciferase substrate (luciferin).

   1. Prepare 50 mM stocks by dissolving 5 mg lyophilized coelenterazine (CTZ) in 250 µl of its specific solvent. Make sure to dissolve all CTZ along the walls of the vial by pipetting or vortexing. Protect vial from direct light.

   2. Prepare 50 µl aliquots in 0.5 ml black microcentrifuge tubes and store at -80°C for future use. CTZ dissolved in solvent does not freeze at -80°C. Aliquots can be removed from and returned to the freezer several times for making working solutions as long as exposure to light and room temperature is kept to a minimum.

2. Single bioluminescence light stimulation.

Note: All manipulations are carried out in a light-tight room in a laminar flow hood illuminated by red light (**Figure 2**).

1. Prepare a working solution of luciferin in cell culture medium. Use the medium the cells are normally grown in (DMEM, NeuroBasal, etc.). Adjust the concentration of the luciferin such that the final concentration is 100 µM. If the entire volume of medium will be replaced, the working solution will be 100 µM. If luciferin-containing medium is added to the cells, the concentration will be higher by the dilution factor (for example, adding 50 µl medium containing luciferin at a concentration of 300 µM to 100 µl medium in the well will result in a 1:3 dilution, and thus in a 100 µM final concentration of luciferin). Prepare all dilutions of CTZ in medium shortly before adding to the cells, as CTZ oxidizes over time.

2. Add luciferin-containing medium to cells and leave on for desired time of light stimulation. This can be as short as 1 minute or as long as 15 minutes, and might be even shorter or longer. The length of time for leaving the luciferin-containing medium on the cells depends on the half-life and kinetics of the selected luciferase – luciferin combination.

3. Light emission at 100 µM final luciferin concentration can usually be observed by eye when the red light is turned off and eyes have adjusted to complete darkness for a few seconds. It can also be documented by taking a cell phone picture.

4. Light stimulation is terminated by removing the luciferin-containing medium and replacing it by culture medium. Depending on the sensitivity of the experiments, it might be good to wash the cells with culture medium once or twice after removing the luciferin-containing medium to completely eliminate all luciferin. If cells do not stick well to the culture surface, plate cells on PDL-coated dishes to avoid losing cells during washes.

5. Cells are returned to the light-protected incubator for 16 – 24 hours.

3. Repeated bioluminescence light stimulation.

Note: All manipulations are carried out in a room that can be made light-tight and be illuminated by red light. Create a light tight compartment around the live cell imaging microscope using a box and black plastic sheets or black drapes (**Figure 3**). Cover all light sources present inside the light tight compartment and the room (e.g. LED indicators on the microscope or on instruments).

1. Set up the live cell imaging chamber and perfusion system with the desired solution for intake and the chamber outport leading to a waste container.

Imaging solution can be, for example, Tyrode's Solution (Sodium Chloride (124 mM), Potassium Chloride (3 mM), HEPES (10 mM), Calcium Chloride Dihydrate (2 mM), Magnesium Chloride Hexahydrate (1 mM), D-Glucose (20 mM)).

2. Prepare a working solution of luciferin in imaging solution. Aliquot into as many microcentrifuge tubes as repeat stimulations are desired. Adjust the concentration of the luciferin such that the final concentration in the imaging chamber is 100 µM.

3. Place a coverslip with transfected cells in the chamber.

4. While keeping the pump running, remove the inlet tube of the pump from the intake beaker and quickly immerse it in the luciferin solution, keeping the transition time as short as possible to avoid any air void in the tubing.

5. As soon as the luciferin solution has been taken up, place the inlet tube back into the intake beaker.

6. Repeat this process as many times as needed and at intervals of several minutes to hours, depending on the physiological pattern the cells are supposed to be exposed to.

7. Cells are returned to the light-protected incubator for 16 – 24 hours for transcription, or for the length of time the effect of light stimulation is to be assessed.

2. Bioluminescence activation of photosensory proteins *in vivo*

1. Constructs

1. Select a luciferase sequence or luciferase-fluorescent protein fusion sequence that will result in expression of a light emitter producing light of a wavelength matching the photoreceptor to be activated.
2. Use standard molecular biology techniques to clone the DNA into a pAAV plasmid, if not already available from other investigators or from plasmid deposits.
3. Choose strong promoters for expression of the light emitting modules, such as CAG or CMV.
4. Use standard approaches for preparing high titer viral stocks[6] or have viral vectors commercially prepared.
5. For initial studies, use separate viral vectors for co-transduction of light emitter and light sensor. This allows adjusting ratios of the different components if needed.

2. AAV Transduction

1. Inject target organ of experimental animal with viral vectors of the light emitter, light sensor, and reporter analogous to the concentration ratios used for *in vitro* transfections (**Table 3**).
2. Return animals to home cages for at least 2 weeks to allow maximal expression of all components. If the target organ is inside the body and protected from ambient light, the animals can be housed under normal light conditions.

3. Bioluminescence activation *in vivo*

1. Prepare luciferase substrate (luciferin).
    1. Take out a vial of water-soluble CTZ from the -80 °C freezer and let warm to room temperature. Keep protected from light.
    2. Per 500 µg vial add 250 µl sterile water, using either a syringe or by opening the vial and adding water with a pipette, then putting the rubber stopper back on the glass vial.
    3. Incubate reconstituted glass vial in 55 °C waterbath for a few minutes to completely dissolve powder.
    4. Transfer solution into black microcentrifuge tube. Rinse walls of the glass vial to retrieve all CTZ.
    5. Remove amount of solution needed for the day. Store leftover at 4 °C for use the next day. Do not freeze!
    6. Carry out the same steps (1-5) for a vial of vehicle.
2. Bioluminescence light stimulation.
    1. Remove volume of luciferin/vehicle needed for size of the animal and application route chosen (**Table 4**).
    2. Inject animals with luciferin or vehicle, respectively. Repeat bioluminescence light stimulation as per experimental design. For example, if activation of a recombinase is desired during a specific behavioral paradigm, inject animals just before the behavioral testing. If phasic transcription of a molecule is the goal, inject animals repeatedly over days.
    3. Collect data from bioluminescence stimulated animals as designed.

REPRESENTATIVE RESULTS:

There are numerous intracellular events that can be manipulated with actuators responding to light, and that are amenable to bimodal activation with physical and biological light sources. Below are examples employing a photosensing calcium (Ca^{2+}) integrator, light-induced protein translocation, a light sensing transcription factor, and a photosensitive recombinase. The examples illustrate the feasibility of using bioluminescence to activate various kinds of photoreceptors. The experiments presented were not specifically optimized with respect to LED application, the luciferase chosen, or with respect to concentrations and timing of luciferin application.

FLARE is an optogenetic system that allows transcription of a reporter gene with the co-incidence of increased intracellular Ca^{2+} and light[23] (**Figure 4A**). The presence of Ca^{2+} is required to bring the protease in close proximity to the protease cleavage site that is accessible only with light stimulation, resulting in release of the transcription factor. We co-transfected HEK293 cells with the original FLARE components, a dual Firefly (FLuc)-dTomato reporter construct, and a membrane anchored *Gaussia* luciferase variant, sbGLuc[6]. In the presence of increased intracellular Ca^{2+} through exposure of cells to 2 µM ionomycin and 5 mM calcium chloride ($CaCl_2$) application of blue LED led to robust expression of the fluorescence reporter compared to cells left in the dark, as well as to expression of FLuc determined by measuring luminescence upon adding the FLuc substrate D-luciferin. Similar levels of FLuc expression were achieved with bioluminescence emitted by sbGLuc upon application of the sbGLuc substrate (CTZ) together with ionomycin and $CaCl_2$. Note that the luciferases used for light activation (sbGLuc) and for reporting the effect of light activation (transcription of FLuc) only produce light with their respective luciferins (CTZ vs D-luciferin) and do not cross-react.

We combined different components to generate a light-induced transcription system based on heterodimerization of cryptochromes[23,24] (**Figure 4B**). CRY2 was fused to a protease while the membrane bound CIB was fused to the protease cleavage site and transcription factor. Light induced protein translocation released the transcription factor, leading to expression of FLuc and dTomato as in (A). While the presence of the transcription factor component alone resulted in considerable background signal possibly due to spontaneous proteolysis, both physical light (LED) and bioluminescence (CTZ) robustly increased expression of FLuc as measured in an IVIS system.

In another set of experiments we employed NanoLuc (luciferin: furimazine or hCTZ) for optogenetic regulation of transcription through dimerization of CRY/CIB and the photosensitive transcription factor EL222[25–27]. **Figure 5A** and **5B** show schematics of the different components in the dark and light states, and with the luciferase co-transfected or fused to the light sensor. Various comparisons are shown in **Figure 5C**. Bioluminescence, induced by adding hCTZ to HEK293 cells expressing the constructs and removing it after 15 minutes was more efficient in driving reporter transcription than 20 minutes of LED light exposure for both CRY/CIB and EL222. For CRY/CIB an hour of LED exposure was sufficient to reach a level of transcription comparable to 15 minutes of bioluminescence, while for EL222 even 60 minutes of LED were barely half as effective as brief exposure to bioluminescence. There were no significant differences in transcription efficacy between the two systems when co-transfected, but fusion proteins of CRY/CIB were more efficient than those of EL222. For both systems fusion proteins led to

significantly higher levels of transcription compared to co-transfected components. CRY/CIB showed consistently higher background levels with vehicle application compared to EL222 that had negligeable background transcription. Increasing concentrations of hCTZ by itself had no effect on transcription of the reporter gene.

Photoactivatable recombinases provide a versatile tool for optogenomic manipulations. We tested bioluminescence activation of a photosensitive split Cre recombinase based on the Vivid LOV protein, iCreV[28]. **Figure 6A** shows a schematic of the different components, sbGLuc, iCreV, and a lox-stop-lox fluorescence reporter (tdTomato) before and after application of CTZ. The results from CTZ application relative to controls (no CTZ or LED) are shown in **Figure 6B**. There is some background expression even in the dark (no CTZ); however, in the presence of CTZ expression is robustly increased over background and similar to that induced with LED application.

FIGURE AND TABLE LEGENDS:

Figure 1: Light sealed incubator. Cardboard box flap covering the light from illuminated control panel (top arrow). Light-impermeable cover over the glass door of incubator (bottom arrow) to protect cells from light exposure.

Figure 2: Laminar flow hood illuminated by red light. Set-up showing a standard laminar flow tissue culture hood being illuminated by red light. Arrow indicates a standard desktop lamp with a red bulb. All manipulations under red light are carried out in an otherwise dark light tight room.

Figure 3: Light tight compartments around live cell imaging microscopes. Two examples of live cell imaging microscope set-ups showing the use of either a solid box with plastic drapes only on the front side (left panels: top and bottom) or black drapes all around the imaging set-up (right panels: top and bottom). The front sides in both examples remain open and rolled up when not in use (top panels: left and right). The front black drapes are rolled down to prevent any light in the room (e.g. computer screens) to enter the imaging area when performing live cell bioluminescence stimulation and/or imaging (bottom panels: left and right).

Figure 4: Bioluminescence for integrating intracellular signaling events. (**A**) Schematics of the FLARE components co-transfected with sbGLuc. In the presence of Ca^{2+} and resulting close proximity of the protease to the protease cleavage site either bioluminescence or LED will lead to unfolding of LOV, exposure of the cleavage site and release of the transcription factor. Cells were exposed to LED (duty cycle 33%, 2 s on/4 s off for 40 minutes; 3.5 mW light power, 4.72 mW/cm^2 irradiance) or to bioluminescence (100 µM CTZ final concentration for 15 min) or left in the dark. Microscopic images of HEK293 cells expressing the above components after treatment to increase Ca^{2+} levels and exposure to LED (left). FLuc luminescence measured in a luminometer comparing exposure to LED, bioluminescence (CTZ) or left in the dark (right). (**B**) Schematics of a non-Ca^{2+} dependent transcription system co-transfected with sbGLuc. HEK293 cells in 4-well plates were transfected with four different arrangements of components as depicted in the schematic. Plates were exposed to either LED (duty cycle 33%, 2 s on/4 s off for 40 minutes; 3.5 mW light power, 4.72 mW/cm^2 irradiance) or bioluminescence (100 µM CTZ final concentration)

by adding CTZ and leaving it on for 15 minutes; control plates were left in the dark. Transcription of the FLuc reporter was measured in an IVIS system. IVIS images of representative dishes are shown on the left; radiance measurements from several replicates baselined to the dark controls are shown on the right. Scale bar = 100 µm

Figure 5: Bioluminescence for driving transcription. (**A**) Schematics of two photoactivatable transcription systems in their dark and light states. (**B**) NanoLuc was either co-transfected or fused to the light-sensing moieties as depicted (N-NanoLuc-CRY-GalDD-C; N-NanoLuc-VP16-EL222-C). (**C**) Comparisons using both systems regarding light sources, construct design, and signal to noise. Cells were exposed to LED (duty cycle 33%, 2 s on/4 s off for 40 minutes; 3.5 mW light power, 4.72 mW/cm^2 irradiance) or to bioluminescence for 15 min (100 µM hCTZ final concentration; except where different concentrations are noted). Dark, plates were left untouched in the incubator between initial transformation of plasmids and FLuc measurement; VEH, plates were handled the same as those receiving hCTZ, but received vehicle instead. Differences in transcription levels: hCTZ, co-transfected CRY vs EL222 – not significant; hCTZ, luciferase – photoprotein fusion CRY vs EL222 – $p<0.005$; hCTZ, CRY co-transfection vs fusion – $p<0.005$; hCTZ, EL222 co-transfection vs fusion – $p<0.01$; vehicle, CRY vs EL222 - $p<0.05$.

Figure 6: Bioluminescence for optogenomic manipulation. (**A**) Schematics of bioluminescence-driven optogenomic manipulation using sbGLuc, the split iCreV components, and a lox-stop-lox (LSL) reporter cassette, before and after application of light. (**B**) HEK293 cells were lipofected with plasmids, then kept in the dark. Twenty-four hours later cells were treated for 30 minutes with just medium (no CTZ) or with CTZ (100 µM final concentration) or with LED (duty cycle 25%, 5 s on/15 s off for 5 minutes; 14.81 mW light power, 20 mW/cm^2 irradiance) as a positive control. Microscope images of tdTomato fluorescence using conditions as indicated. Scale bar = 100 µm

Table 1: Bioluminescence activation of photoreceptors.

Table 2: Guidelines for plating and transfecting cells in different formats.

Table 3: Ratios of various plasmids for transfection.

Table 4: Injection routes, volumes, and concentrations of luciferin for *in vivo* applications (25 gram mouse).

DISCUSSION:

There is a range of luciferases and luciferins with light emission wavelengths matching activation spectra of photosensory proteins from blue to red light[14,29]. Apart from aligning emission and excitation wavelength there is no reliable way to determine *a priori* which pairing will work best. Thus, the need to experimentally determine how luciferin-luciferase pairs work in cells and in organisms in driving photosensory systems.

The protocols outlined in this presentation describe how to prepare the luciferin and how to apply it *in vitro* and *in vivo*, together with guidelines on how to set up rooms, tissue culture hoods,

incubators and microscopes for experiments utilizing bioluminescence. In the representative experiments different luciferases (NanoLuc, *Gaussia* luciferase) with several photosensory proteins (CRY/CIB, EL222, VVD, LOV) were used, demonstrating effects of bioluminescence versus physical light, co-transfection versus fusion proteins, signal-to-noise comparisons, and different readout assays. More applications of bioluminescence activating photosensory proteins are described in publications from several groups, targeting induction of cell death, cAMP synthesis, and protein movement in addition to transcription (**Table 1**).

Simply co-transfecting light-emitting and light-sensing components is a good start. Variables are the molar ratios of emitter and sensor; unknowns are background levels of sensor activity in the dark, sensor activity in relation to light intensity and duration, and efficiency of sensor activation comparing physical and biological light. While fusion constructs have the advantage of keeping the molar ratio of emitter and sensor at 1:1 and of bringing the light emitter close to the light sensing domain, other considerations come into play, such as where to tether (N- or C-terminus) and how to link (linker length and composition) without impacting the performance of the photosensory actuator.

For experiments both *in vitro* and *in vivo* there are multiple options for tuning bioluminescent light emission, either by varying the concentration of the luciferin, and/or by varying the time the luciferin is made available to the respective sensor. The minimum amount and time are determined by the presence or absence of the effect expected with light activation, while the respective maxima are mainly determined by the tolerance of cells to high concentrations of luciferin over prolonged times. The concentration of CTZ chosen in the above examples, 100 µM, is close to the upper limit for various cell types, from HEK293 cells to neurons. The goal is to use as low a concentration as possible for the shortest time to achieve activation of the targeted photosensing domain. This will be achieved more readily using luciferases with high light emission and photoreceptors with high light sensitivity.

Bioluminescence for driving photoreceptors has been used in rodents (mice, rats) with photosensing proteins expressed in liver, muscle, spinal cord, and brain as well as via photoreceptor expressing cells transplanted subcutaneously or intraperitoneally. In principle, there are no limits preventing the approach to be applied to different species, from non-human primates to fish or flies. Depending on the permeability of the organism for the luciferin, application may be as easy as applying the luciferin to the surrounding water (e.g. in fish larvae[30]). Before using BL-OG in any new organism pilot experiments need to be conducted to ensure that the luciferin reaches its targets via the chosen application route.

Critical aspects of the experimental design are the various controls that are important for the interpretation of results. Cells expressing a reporter driven by a luciferase acting on a photosensory protein should be compared to cells lacking the luciferase or lacking the photosensory protein. Further, comparisons should be made between cells exposed to luciferin, vehicle, or kept in the dark. It is also important to realize the limitations of different assays for assessing the effects of bioluminescence-driven photoreceptor activation. For example, the efficacy of bioluminescence-activated transcription can be tested in different ways, depending

on whether the reporter gene is an orthogonal luciferase (luminometer, IVIS), or a fluorescent protein (FACS, microscopy image analysis). While the basic effects should be reproducible across testing platforms, the quantitative aspects of effects might vary considerably.

The approach of bioluminescence activation of photoreceptors has been demonstrated thus far for a limited number of luciferases and photosensory proteins, respectively, both *in vitro* and *in vivo*. It can be extended to the large class of photoreceptors for activating many more biological processes. Such expansion of the approach is further promoted by the continuous development of novel luciferases and luciferase-fluorescence protein pairs with much higher light emission than that of naturally occurring luciferases and with kinetic features tunable to different applications. These advances are paralleled by generation of novel luciferins, further adding to increased brightness and color palettes[29]. This tool platform offers applications to manipulate and investigate intracellular dynamics and cell interactions inside living cells, tissues, and organisms.

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The authors have nothing to disclose.

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Figure 1

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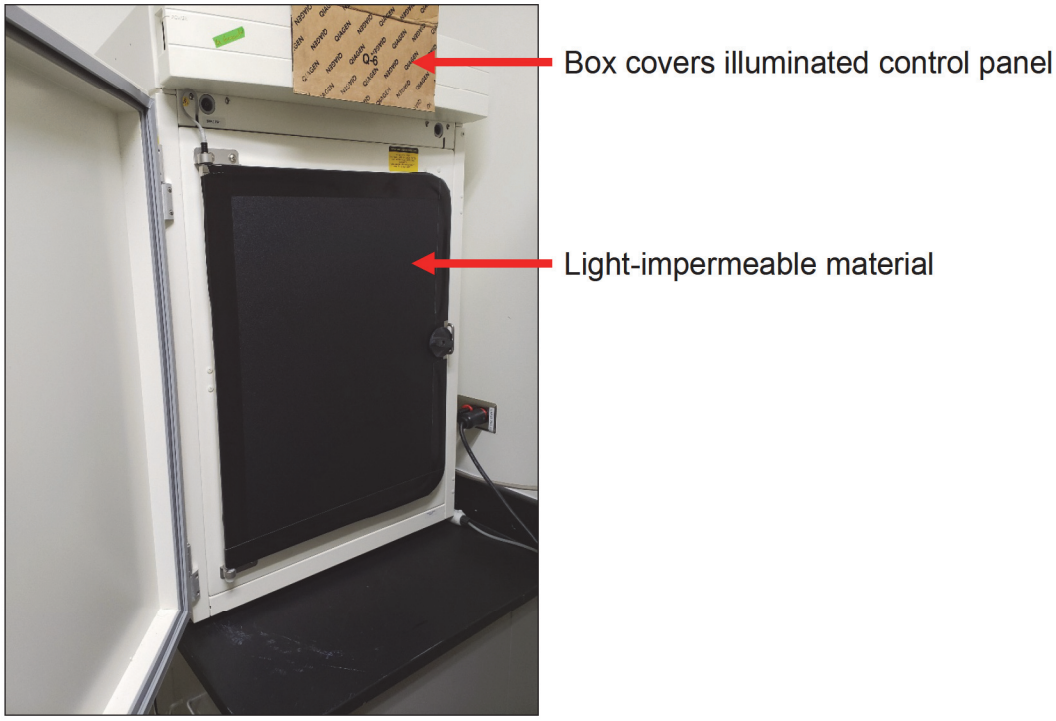

Figure 2

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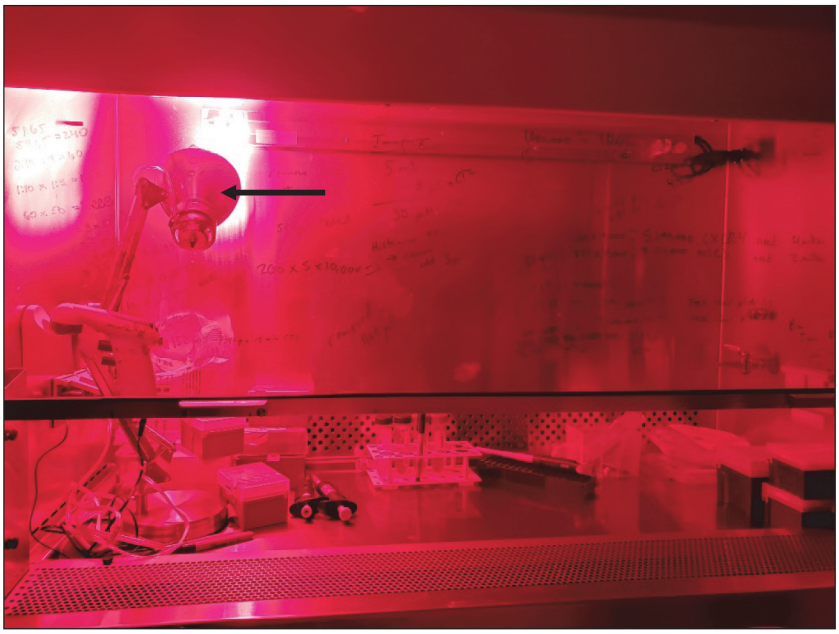

Figure 3

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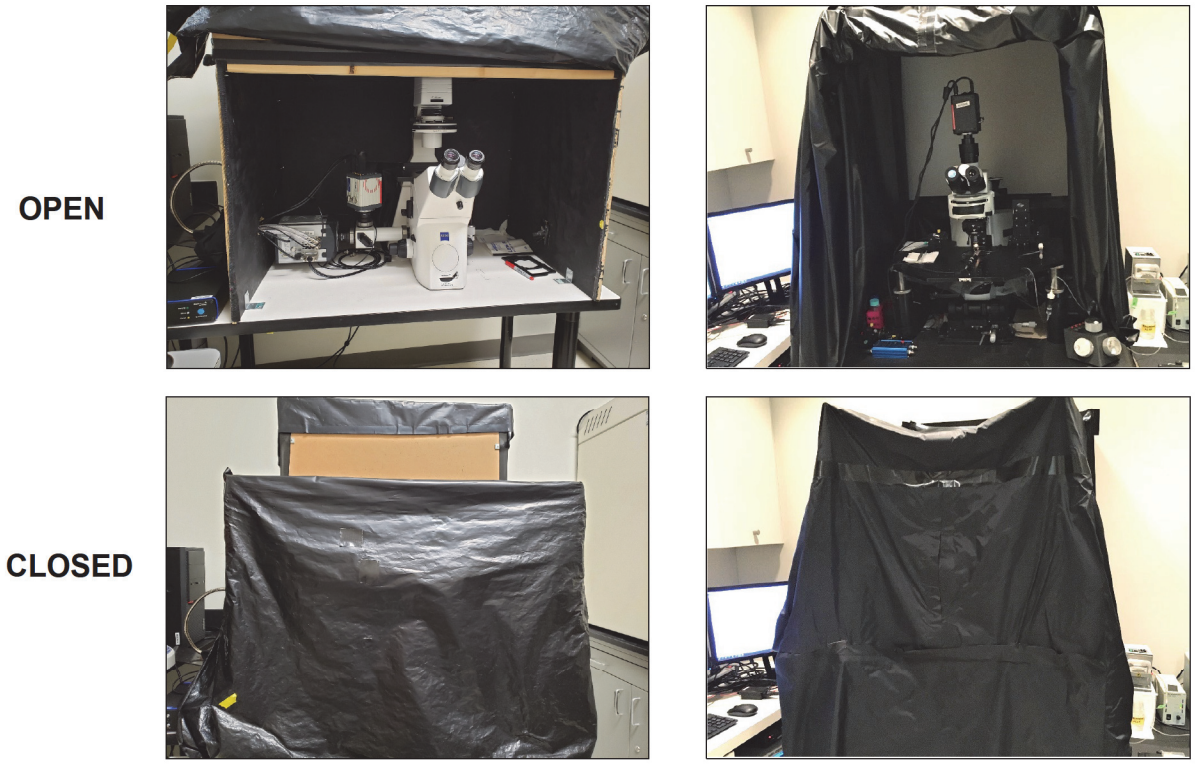

Figure 4

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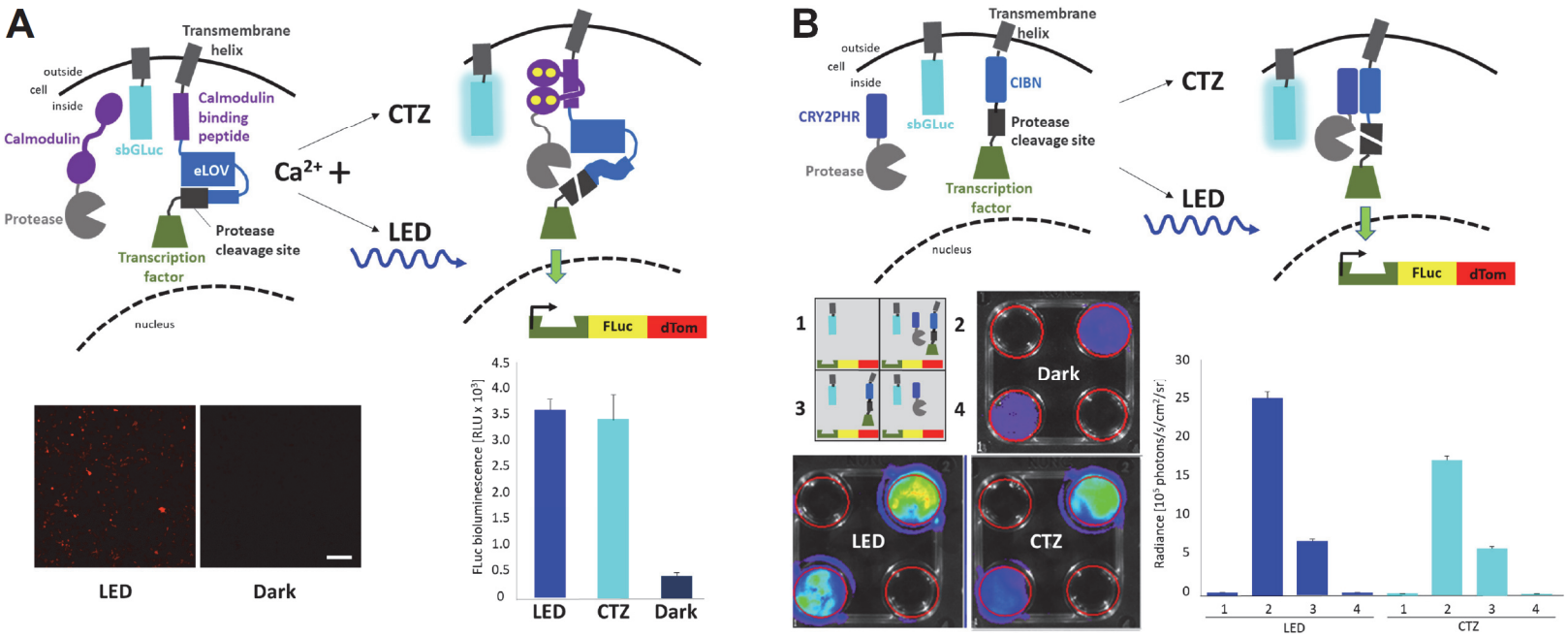

Figure 5

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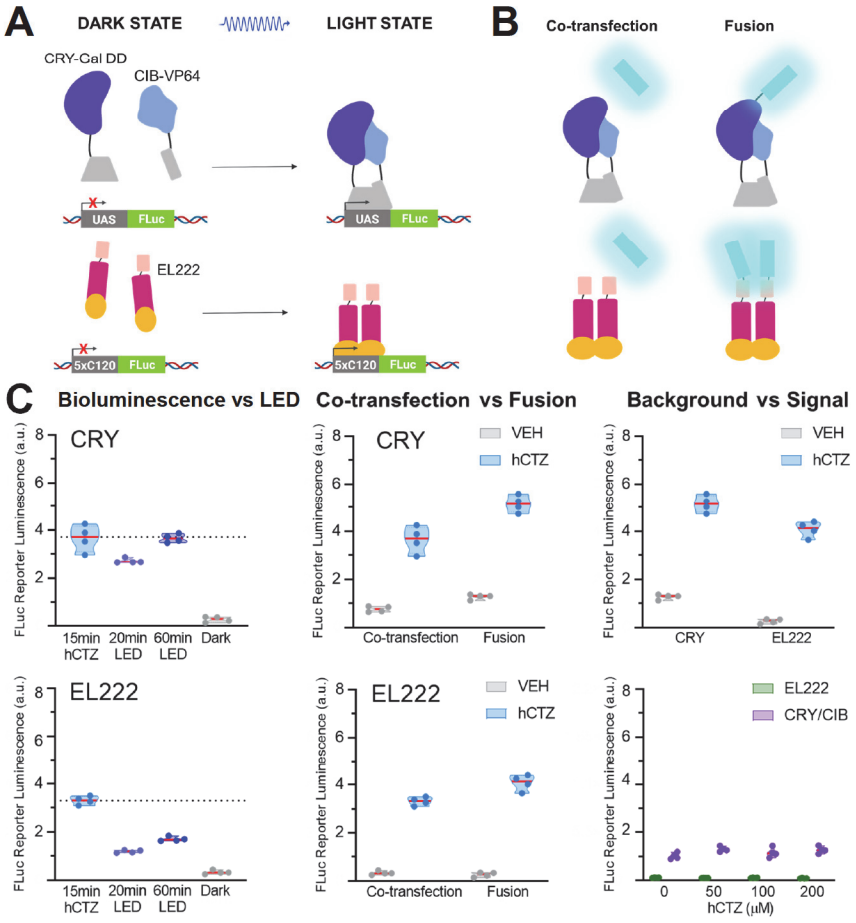

Figure 6

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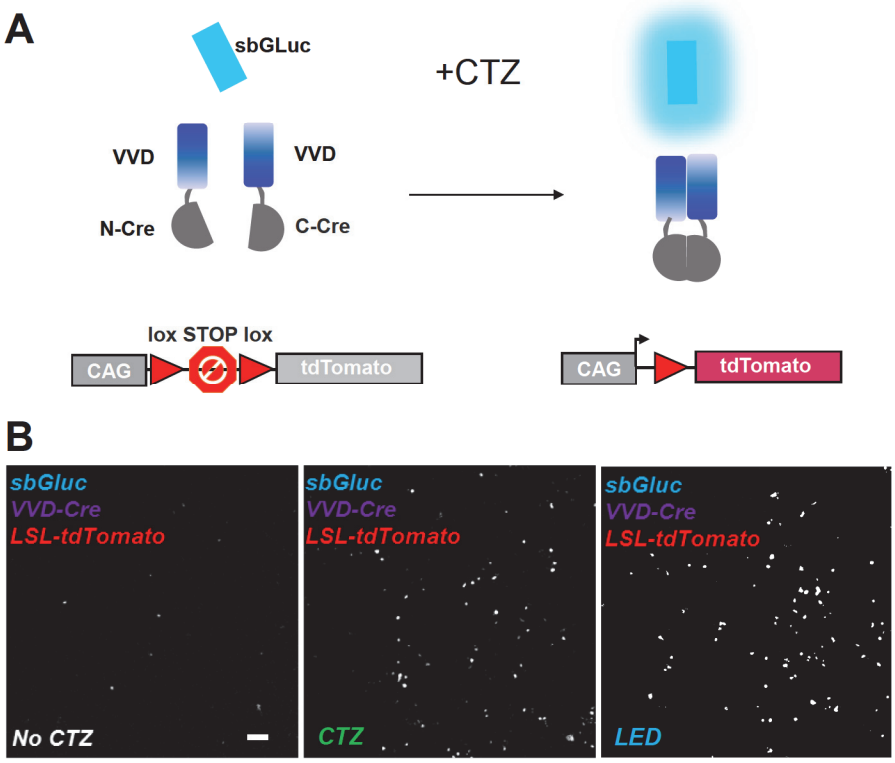

Table 1

Light Emitter	Emission wavelength peak	Light Sensor	Activation wavelength peak	Arrangement of components	Luciferin
NanoLuc	460 nm	LOV	450 nm	Fusion protein	Furimazine
NanoLuc	460 nm	BLUF	450 nm	Fusion protein	hCTZ, Furimazine
NanoLuc	460 nm	LOV	450 nm	Co-transfection	Furimazine
LumiFluor (NanoLuc-FP)	474 nm (CeNLuc) 510 nm (GpNLuc)	LOV, CRY2-CIBN, VVD	450 nm	Co-transfection	Furimazine
RLuc8, RLuc8.6	485 nm (rLuc8) 535 nm (rLuc 8.6)	Fluorescent protein	488nm, miniSOG 585 nm, KillerRed	Fusion protein	hCTZ
NanoLuc	460 nm	LOV	450 nm	Co-transfection	Furimazine
NanoLuc	460 nm	LOV, CRY2-CIBN, VVD	450 nm	Fusion protein	Furimazine
sbGLuc	485 nm	LOV, CRY2-CIBN	450 nm	Co-transfection	CTZ
NanoLuc	460 nm	LOV, CRY2-CIBN	450 nm	Co-transfection Fusion protein	hCTZ
sbGLuc	485 nm	VVD	450 nm	Co-transfection	CTZ

Optogenetic system	Intra-cellular effect
Photoinducible ROS generating protein (miniSOG)	Cell death
Photoactivated adenylate cyclase (from Beggiatoa, bPAC)	cAMP synthesis
Photoinducible protein release (LOVTRAP) Photoinducible protein release (SPARK2)	Protein dissociation Transcription
Photoinducible protein interaction (iLID-mito, FKF1-GI), Cre (pMagnet), dCas9	Recombination, Transcription, Protein recruitment
Photoinducible ROS generating protein (miniSOG, KillerRed)	Cell death
Photoinducible protein release (FLiCRE)	Transcription
Photoinducible protein release (LOVTRAP), protein interaction (CRY2-CIB), transcription (GAVPO)	Protein dissociation and recruitment, Transcription
Photoinducible protein release (FLARE), protein interaction (CRY2-CIB)	Protein dissociation Transcription
Photoinducible transcription and protein interaction (EL222, CRY2-CIB)	Protein recruitment Transcription
Photoinducible protein interaction (iCreV)	Recombination, Protein Recruitment, Transcription

In vitro	In vivo
SK-BR-3	
HC1, PCCL3, HEK293	
HEK293T	
HEK293, HeLa	
Human breast cell lines MCF-7, SK-BR-3, MDA-MB-231, and BT-474, MDA-MB-435, MCF-10A; Primary breast cancer cell from patients	MDA-MB-231 cells subcutaneously implanted in NOD SCID mice
Primary rat neurons (cortical, hippocampal)	
HEK293, HEK293T, HeLa, COS-7, U-87, PC-3, A549 and H1299	Liver, Muscle, IP-transplanted HEK cells in mice
HEK293	
HEK293	
HEK293	

reference

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this paper, Fig 4

this paper, Fig 5

this paper, Fig 6

Table 2

	Cells/well seeded	Plating medium for lipofection	DNA/Opti-MEM
6-well	1-2 x 10^6	2 ml	5 ug/250 ul
12-well	4-8 x 10^5	1 ml	2 ug/100 ul
24-well	2-4 x 10^5	0.5 ml	1 ug/50 ul

Lipofectamine 2000/Opti-MEM

10 ul/250 ul

4 ul/100 ul

2 ul/50 ul

Table 3

	Luciferase	Photoreceptor	Binding partner	Transcription reporter
ratio	1		1	
CRY2/CIB	1	0.33	0.33	0.33
EL222	1	0.7		0.3

Table 4

route	volume	Concentration of injected solution	Final concentration at target area
intravenous	100 µl	1.25 µg/µl (3 mM)	5 mg/kg
intraperitoneal	200 µl	1.25 µg/µl (3 mM)	10 mg/kg
intracerebral ventricle	5 µl	0.68 µg/µl (1.6 mM)	200 µM
intranasal	30 µl	2.5 µg/µl (6 mM)	3 mg/kg