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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 the 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 to use in neuroscience. Rather, bioluminescence can be harnessed to activate any photosensory protein, thus enabling the 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 achieved by using 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 experimental execution of bioluminescence activation in cells and organisms and describes the 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, to generate bioluminescence. For applications that require milli- or even femtosecond timescales and/or single-cell spatial resolution, physical light sources (lasers and light-emitting diodes (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 subcellular structures such as mitochondrial tubules[2]. However, many other applications for optical switches have different priorities, including extended spatial control and repeated application non-invasively and without light damage but with 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 the application of the luciferase substrate. The onset is immediate *in vitro* and *in vivo* depending on the route of administration and lasts for ~15–30 min; extended 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 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 the efficiency of activation increasing with higher light emission[6,9]. Ion-moving sensory photoreceptors are a subgroup of the large contingent of sensory photoreceptors found in nature that are non-ion moving[13,14]. The extension of bioluminescence to activating non-ion moving photoreceptors, such as photosensing domains from plants or bacteria, is encouraged by reports[15,16] that non-ion moving photosensors are significantly more light-sensitive than channelrhodopsins, ensuring an 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 a light source for the activation of a variety of photoreceptors, including light-oxygen-voltage-sensing (LOV) domains, blue-light-using-flavin (BLUF) domains, and cryptochromes (CRYs)[3,17–22] (**Table 1**). Applications for bioluminescence-driven activation of optical switches targeted intracellular processes ranging 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 the 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*

1.1. Constructs

1.1.1. Select a luciferase sequence or luciferase-fluorescent protein fusion sequence that will result in the expression of a light emitter producing light of a wavelength matching the photoreceptor to be activated.

NOTE: For example, blue light-emitting luciferases, such as *Gaussia* luciferase variants or NanoLuc, can be paired with blue light-sensing photoreceptors such as CRY/Ca^{2+}- and integrin-binding protein (CIB), LOV, or Vivid (VVD).

1.1.2. If not already available from other investigators or plasmid deposits, use standard molecular biology techniques to clone the DNA into a mammalian expression plasmid.

NOTE: The choice of promoters is dictated by the need to provide strong and constitutive expression of the light-emitting module, such as that provided by the CAG and CMV promoters.

1.1.3. For initial studies, use separate plasmids for co-transfection of the light emitter and the light sensor. Generate fusion proteins of the two moieties as needed and for subsequent studies.

1.1.4. Obtain high-quality plasmid DNAs using mini-, midi-, or maxiprep kits according to manufacturer's protocols.

1.2. Cell culture and transfection

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

1.2.1. Plate cells in formats and numbers according to the desired end use.

NOTE: Specific examples are given in **Table 2**. Cell density at the time of plating will determine how soon cells can be transfected.

1.2.1.1. For assessing bioluminescence-activated transcription by fluorescence microscopy, plate HEK293 cells on poly-D-lysine (PDL)-coated 12 mm coverslips placed in 24-well dishes.

1.2.1.2. For assessing bioluminescence-activated transcription by measuring light emission from an orthogonal reporter luciferase in a luminometer, plate HeLa cells initially in 6- or 12-well dishes for transfection but re-plate them after transfection (see step 4).

1.2.1.3. If repeated bioluminescence stimulation will be carried out in live-cell imaging chambers, select coverslips of the appropriate size and place them into multi-well plates of the appropriate size (24-well plates for 12 mm coverslips; 12-well plates for 15 mm and 18 mm coverslips). Seed the cells on top of the coverslips using the cell numbers specified in **Table 2**. If the cell type selected does not adhere well to the culture surface, plate the cells on PDL-coated dishes.

1.2.2. Perform transfection by lipofection according to the manufacturer's recommendation or use any transfection method appropriate for the cell type selected can be used.

NOTE: **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.

1.2.3. After transfection, place the cells in an incubator that is completely light-sealed (**Figure 1**).

1.2.4. Depending on the desired end use, use the cells for bioluminescence stimulation the next day in their original wells/dishes, or re-plate them 3–4 h after lipofection. For reading transcription of a firefly luciferase reporter gene in a luminometer, re-plate the cells in white 96-well plates.

NOTE: Carry out all manipulations in a light-tight room in a laminar flow hood illuminated by red light (**Figure 2**).

1.2.4.1. Wash the transfected cells once with Dulbecco's modified Eagle medium (DMEM) or phosphate-buffered saline (PBS).

1.2.4.2. Add the minimum volume of a trypsinizing reagent to the wells (24-well: 100 µL; 12-well: 150 µL; 6-well: 300 µL) and incubate the cells for 3 min at 37 °C.

1.2.4.3. Add culture medium to achieve a cell concentration that will yield the appropriate cell density for the next plating step (for example, resuspend cells in a 24-well in a final volume of 1.2 mL for plating in 10 wells of a 96-well plate; resuspend cells in a 12-well in a final volume

of 2.4 mL for plating in 20 wells of a 96-well plate). Pool the transfected cells from several wells depending on the number of wells needed in the end.

1.2.4.4. Plate the transfected cells in their final format and return the plates to the light-protected incubator.

1.3. Bioluminescence activation *in vitro*

1.3.1. Prepare the luciferase substrate (luciferin).

1.3.1.1. Prepare 50 mM stocks by dissolving 5 mg of lyophilized coelenterazine (CTZ) in 250 µL of its specific solvent. Ensure that all the CTZ along the walls of the vial is dissolved by pipetting or vortexing. Protect the vial from direct light.

1.3.1.2. Prepare 50 µL aliquots in 0.5 mL black microcentrifuge tubes and store at -80 °C for future use.

NOTE: 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.

1.3.2. 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**).

1.3.2.1. Prepare a working solution of luciferin in cell culture medium (DMEM or NeuroBasal). Adjust the concentration of the luciferin such that the final concentration is 100 µM. Prepare all dilutions of CTZ in medium shortly before adding to the cells, as CTZ oxidizes over time.

NOTE: 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 of medium containing 300 µM luciferin to 100 µL of medium in the well will result in a 1:3 dilution and thus in a 100 µM final concentration of luciferin).

1.3.2.2. Add luciferin-containing medium to the cells and incubate for the desired duration of light stimulation.

NOTE: This can be as short as 1 min or as long as 15 min 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.

1.3.2.3. Monitor light emission at 100 µM final luciferin concentration by eye after turning off the red light; wait for a few seconds until eyes have adjusted to complete darkness. Document the light emission by taking a photograph (even with a cell phone).

1.3.2.4. Terminate the light stimulation by removing the luciferin-containing medium and replacing it with culture medium. Depending on the sensitivity of the experiments, wash the cells with culture medium once or twice after removing the luciferin-containing medium to eliminate all luciferin. If the cells do not adhere well to the culture surface, plate them on PDL-coated dishes to avoid losing the cells during washes.

1.3.2.5. Return the cells to the light-protected incubator for 16–24 h.

1.3.3. 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.

1.3.3.1. Set up the live-cell imaging chamber. 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 the light sources present inside the light-tight compartment and the room (e.g., LED indicators on the microscope or instruments).

1.3.3.2. Set up the perfusion system with the desired solution for intake and the chamber outport leading to a waste container.

NOTE: For example, the imaging solution can be 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)).

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

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

1.3.3.5. 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.

1.3.3.6. As soon as the luciferin solution has been taken up, place the inlet tube back into the intake beaker. Repeat this process as many times as needed and at intervals of several minutes to hours, depending on the physiological pattern to which the cells are supposed to be exposed.

1.3.3.7. Return the cells to the light-protected incubator for 16–24 h 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*

2.1. Constructs

2.1.1. Select a luciferase sequence or luciferase-fluorescent protein fusion sequence that will result in the expression of a light emitter producing light of a wavelength matching the photoreceptor to be activated.

2.1.2. Use standard molecular biology techniques to clone the DNA into a pAAV plasmid, if not already available from other investigators or plasmid deposits.

2.1.3. Choose strong promoters for the expression of the light-emitting modules, such as CAG or CMV.

2.1.4. Use standard approaches for preparing high-titer viral stocks[6] or have viral vectors commercially prepared.

2.1.5. For initial studies, use separate viral vectors for co-transduction of the light emitter and the light sensor to allow for adjustment of the ratios of the different components if needed.

2.2. AAV transduction

2.2.1. Inject the target organ of the experimental animal with viral vectors of the light emitter, light sensor, and reporter analogous to the concentration ratios used for the *in vitro* transfections (**Table 3**).

2.2.2. Return the animals to their home cages for at least 2 weeks to allow maximal expression of all the components.

NOTE: If the target organ is inside the body and protected from ambient light, the animals can be housed under normal light conditions.

2.3. Bioluminescence activation *in vivo*

2.3.1. Prepare the luciferase substrate (luciferin).

2.3.1.1. Take out a vial of water-soluble CTZ from the -80 °C freezer and let it warm to room temperature. Keep it protected from light.

2.3.1.2. Per 500 µg vial, add 250 µL of 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.

2.3.1.3. Incubate the reconstituted glass vial in a 55 °C water bath for a few minutes to completely dissolve the powder.

2.3.1.4. Transfer the solution into a black microcentrifuge tube. Rinse the walls of the glass vial to retrieve all CTZ.

2.3.1.5. Remove the amount of solution needed for the day. Store the remaining solution at 4 °C for use the next day. Do not freeze!

2.3.1.6. Carry out the same steps (2.3.1.1.–2.3.1.5) for a vial of vehicle.

2.3.2. Bioluminescence light stimulation

2.3.2.1. Remove the volume of luciferin/vehicle needed for the size of the animal and application route chosen (**Table 4**).

2.3.2.2. Inject the animals with luciferin or vehicle. Repeat the bioluminescence light stimulation as per the experimental design. For example, if activation of a recombinase is desired during a specific behavioral paradigm, inject the animals just before the behavioral testing. If phasic transcription of a molecule is the goal, inject the animals repeatedly over days.

2.3.2.3. Collect data from the 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 light-emitting diode (LED) application, the luciferase chosen, or with respect to concentrations and timing of luciferin application.

Fast light- and activity-regulated expression (FLARE) is an optogenetic system that allows the 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 proximity to the protease cleavage site accessible only with light stimulation, resulting in the release of the transcription factor. HEK293 cells were co-transfected 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 the exposure of cells to 2 µM ionomycin and 5 mM calcium chloride ($CaCl_2$), the application of blue LED led to robust expression of the fluorescence reporter compared to cells left in the dark, as well as to the 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 the 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.

Different components were combined to generate a light-induced transcription system based on the 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 the expression of FLuc and dTomato, as shown in **Figure 4A**. 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 the expression of FLuc as measured in an *in vivo* imaging system (IVIS). 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 the expression of FLuc as measured in an *in in vivo* imaging system (IVIS).

In another set of experiments, NanoLuc (luciferin: furimazine or hCTZ) was employed for the optogenetic regulation of transcription through the dimerization of CRY/CIB and the photosensitive transcription factor, EL222[25–27]. **Figure 5A,B** show the schematics of the different components in the dark and light states and 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 min, was more efficient in driving reporter transcription than 20 min 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 min of bioluminescence. In contrast, for EL222, even 60 min of LED were barely half as effective as a brief exposure to bioluminescence. There were no significant differences in the transcription efficacy between the two systems when co-transfected, although the fusion proteins of CRY/CIB were more efficient than those of EL222. For both systems, the fusion proteins led to significantly higher transcription levels than the co-transfected components. CRY/CIB showed consistently higher background levels with vehicle application compared to EL222, which had negligible background transcription. Increasing concentrations of hCTZ alone had no effect on the 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 the 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 the 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 the illuminated control panel (top arrow). Light-impermeable cover over the glass door of the incubator (bottom arrow) to protect the cells from light exposure.

Figure 2: Laminar flow hood illuminated by red light. Setup 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 setups 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 setup (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) from entering 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 the resulting proximity of the protease to the protease cleavage site, either bioluminescence or LED will lead to the 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 min; 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 min; 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 min; control plates were left in the dark. Transcription of the FLuc reporter was measured in an IVIS. 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. Abbreviations: FLARE = Fast light- and activity-regulated expression; LOV = light-oxygen-voltage-sensing; LED = light-emitting diode; CTZ = coelenterazine; FLuc = firefly luciferase; dTom = dTomato; CRY2 = cryptochrome 2; CRY2PHR = CRY2 photolyase homology region; CIB1 = Ca^{2+}- and integrin-binding protein 1; CIBN = N-terminus of CIB1; IVIS = *in vivo* imaging system.

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 min; 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 the 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 – $p < 0.05$. Abbreviations: UAS = upstream activating sequence; LED = light-emitting diode; CTZ = coelenterazine; FLuc = firefly luciferase; CRY = cryptochrome; CIB = Ca^{2+}- and integrin-binding protein; VEH = vehicle.

Figure 6: Bioluminescence for optogenomic manipulation. (**A**) Schematics of bioluminescence-driven optogenomic manipulation using sbGLuc, the split iCreV components, and an 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, the cells were treated for 30 min 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 min; 14.81 mW light power, 20 mW/cm^2 irradiance) as a positive control. Microscopic images of tdTomato fluorescence using conditions as indicated. Scale bar = 100 µm. Abbreviations: LSL = lox-stop-lox; CTZ = coelenterazine; LED = light-emitting diode; VVD = Vivid.

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 g mouse).

DISCUSSION:

There is a range of luciferases and luciferins with light emission wavelengths matching the activation spectra of photosensory proteins from blue to red light[14,29]. Apart from aligning emission and excitation wavelengths, 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 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 setting 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 the 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 the 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 the 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 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. In contrast, 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 the 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 from being applied to different species, from non-human primates to fish or flies. Depending on the permeability of the organism for the luciferin, the 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 must 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 (fluorescence-activated cell sorting, microscopy image analysis). While the basic effects should be reproducible across testing platforms, the quantitative aspects of the effects might vary considerably.

The 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 naturally occurring luciferases and with kinetic features tunable to different applications. These advances are paralleled by the 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.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

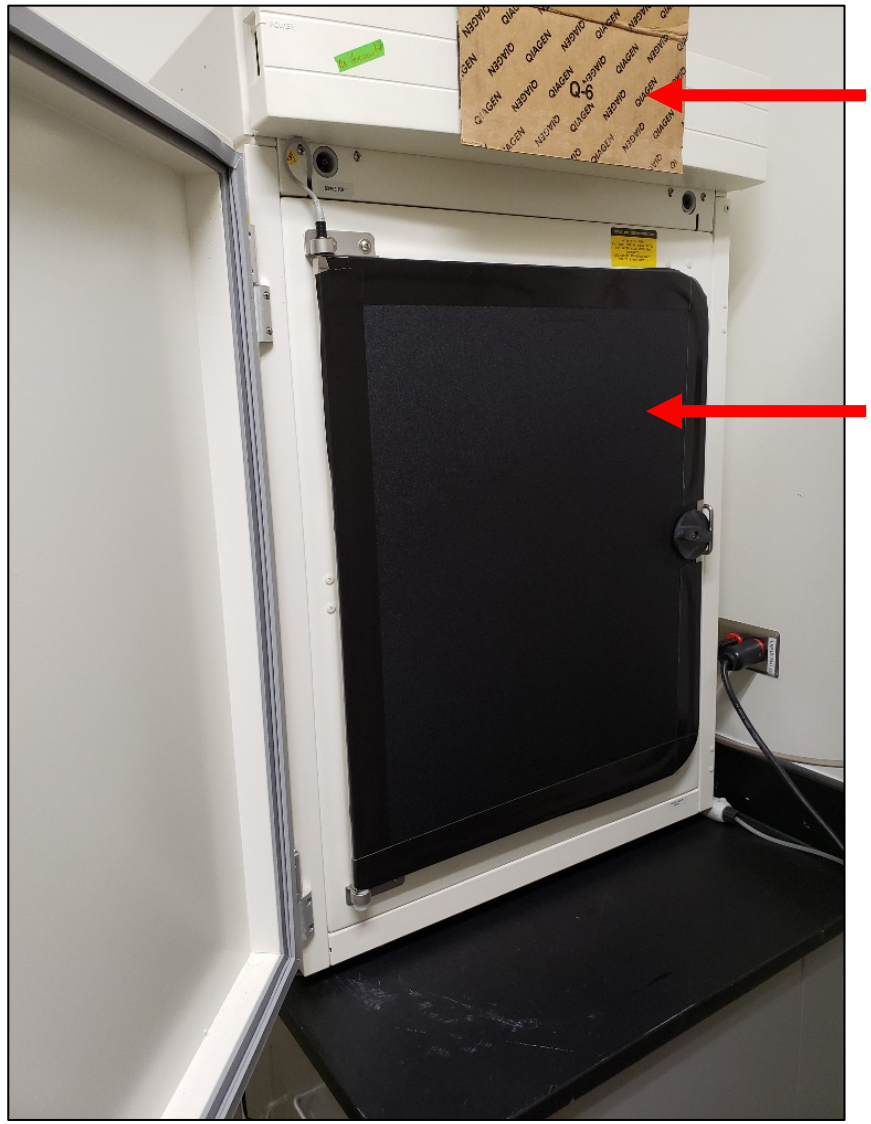

Figure 2

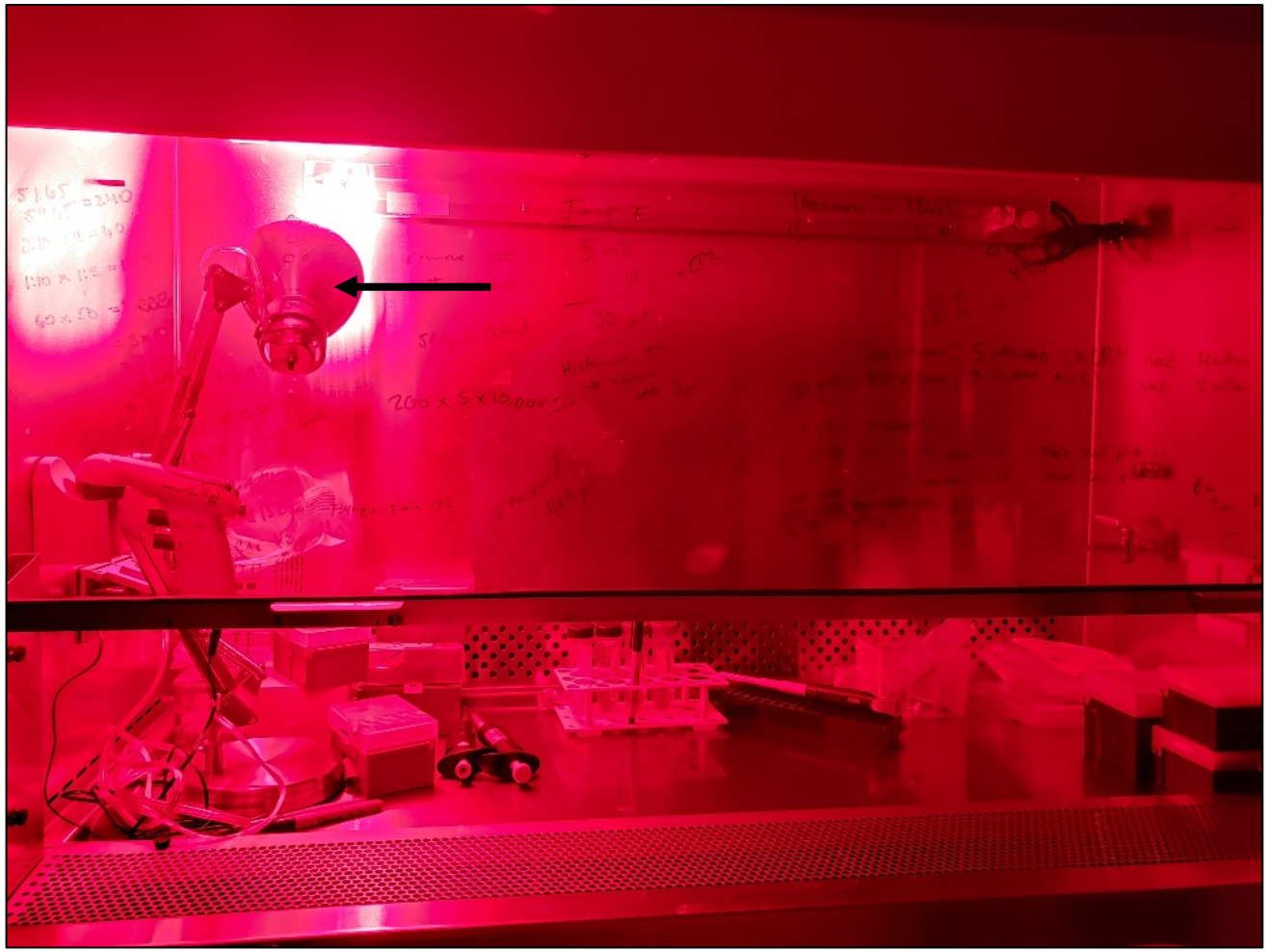

Figure 3

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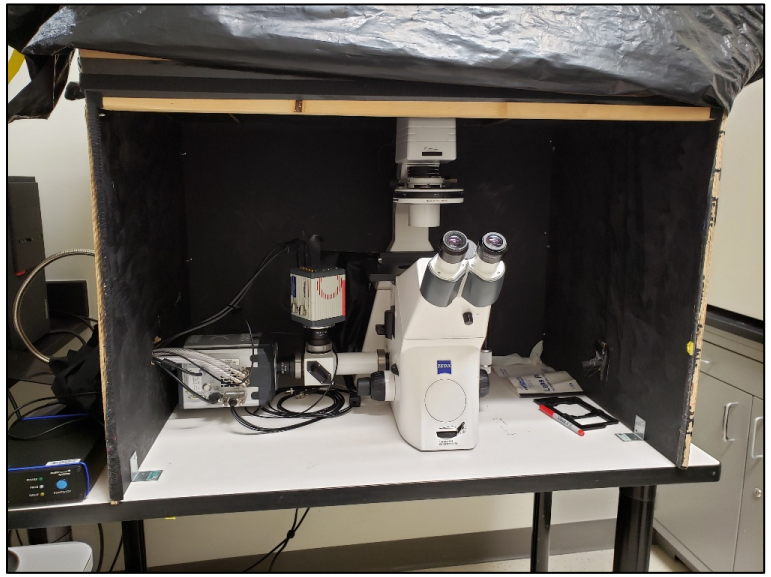

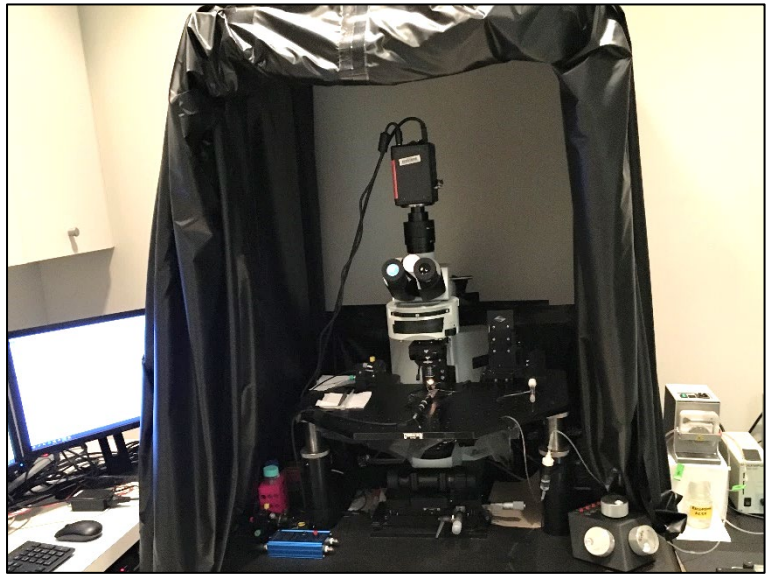

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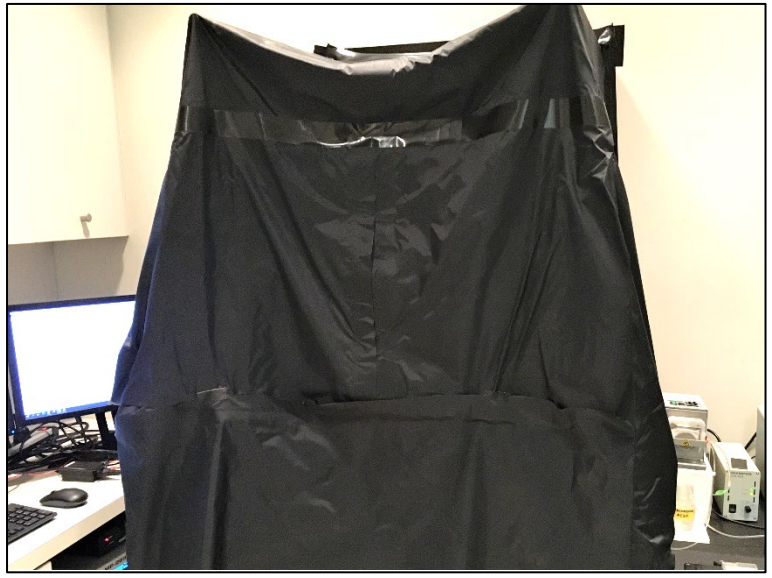

Figure 4

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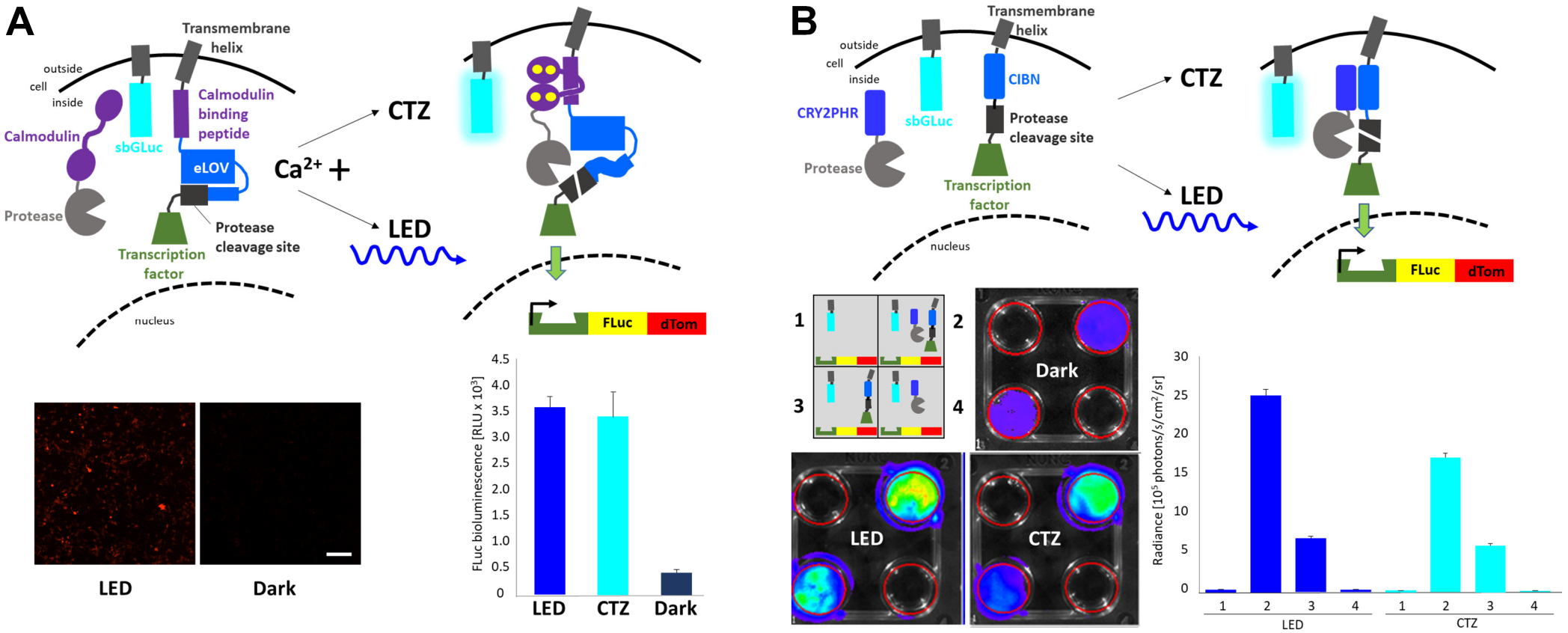


Figure 5

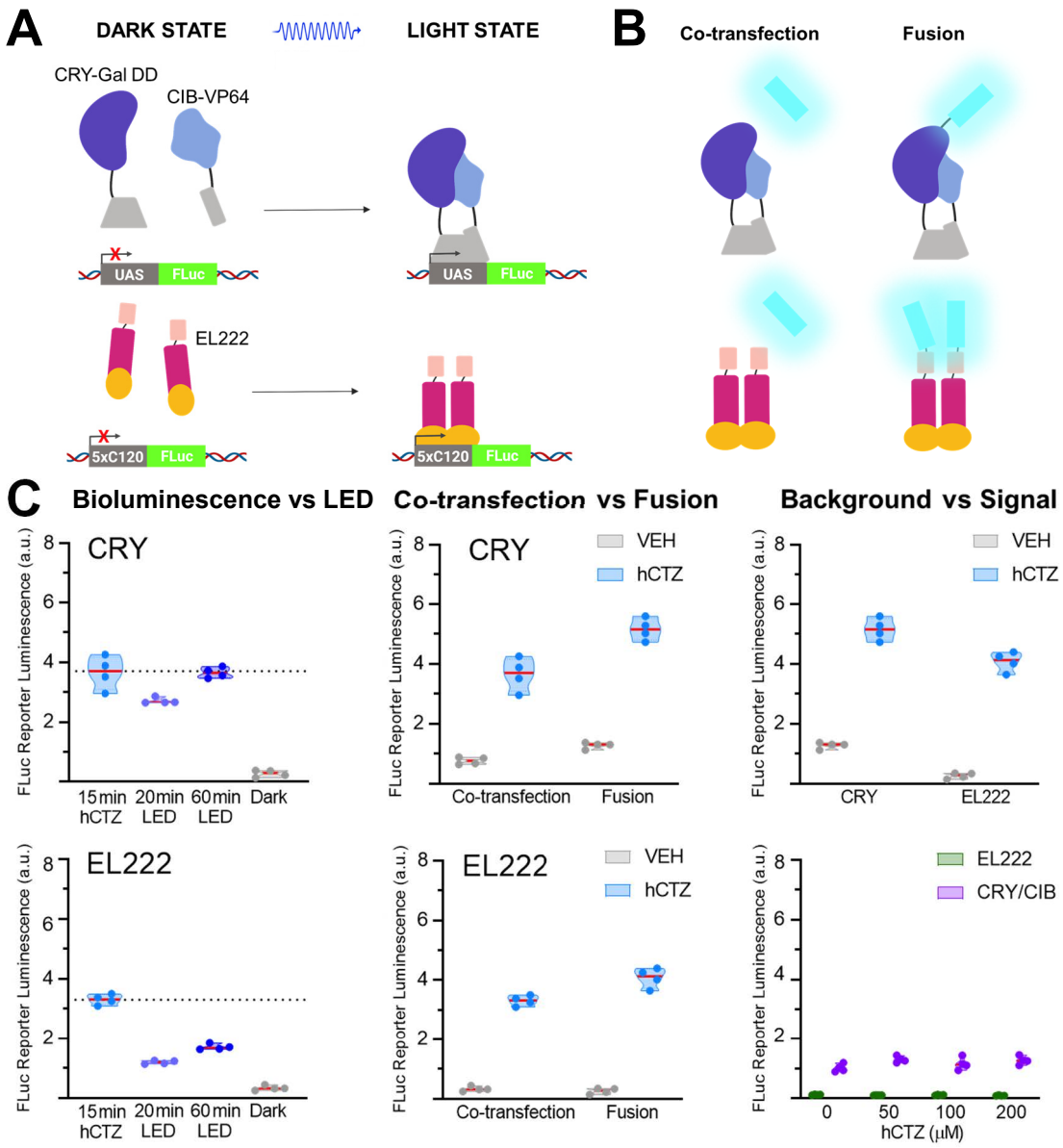
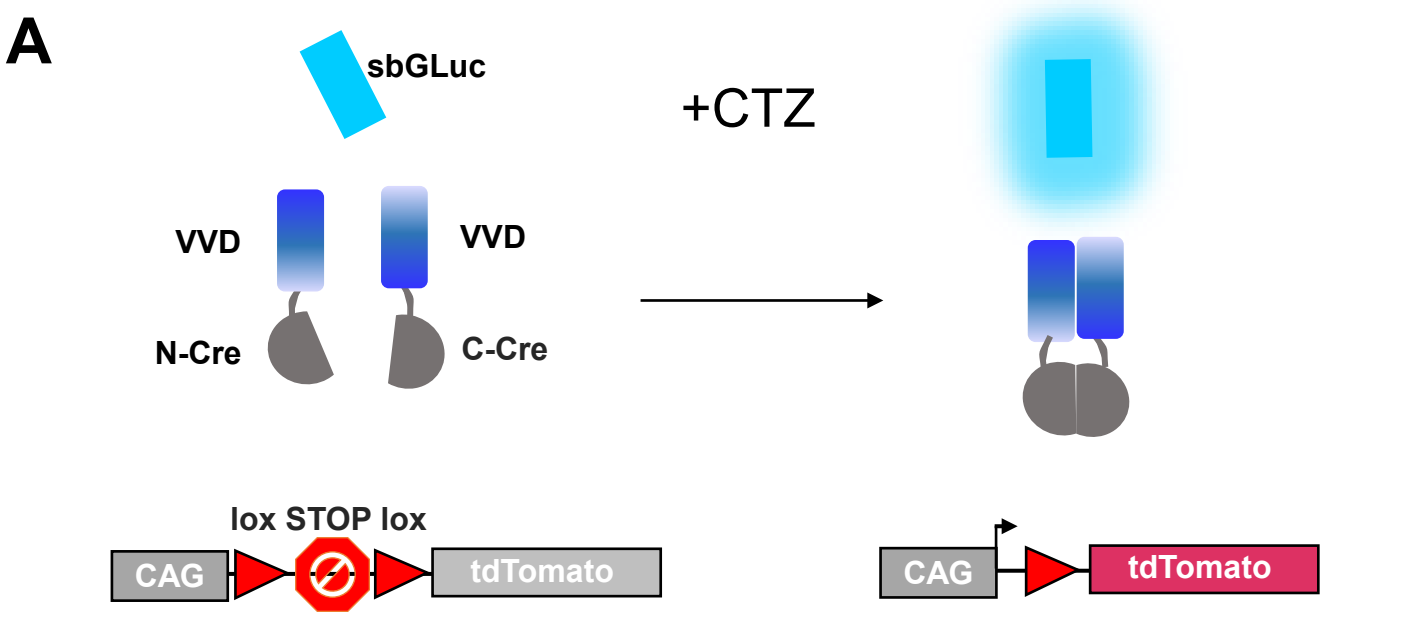


Figure 6



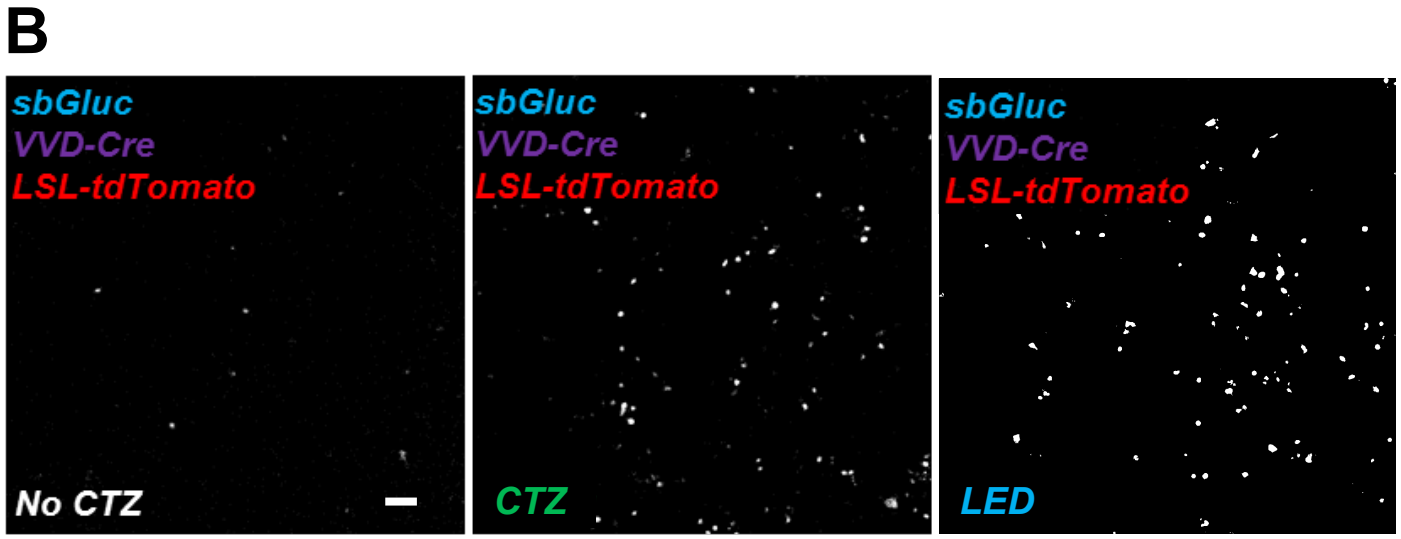


Table 1

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	Intracellular 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/Transfection medium
6-well	1-2 x 10^6	2 mL	5 µg/250 µL
12-well	4-8 x 10^5	1 mL	2 µg/100 uL
24-well	2-4 x 10^5	0.5 mL	1 µg/50 µL

Transfection reagent/Transfection medium
10 µL/250 µL
4 µL/100 µL
2 µL/50 µL

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

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Done.

2. Please provide an email address for each author.

Done.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Lipofectamine 2000; TrypLE Express; NanoFuel Solvent; THOR LABS etc

Removed from text – specified in Materials Table.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns removed from protocol sections.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Checked and done.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough

detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We believe we have detailed the steps so they can be easily followed.

7. After including a ONE-LINE SPACE between each protocol step, highlight UP TO 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Done.

8. As we are a methods journal, please mention any limitations of this technique in the discussion section.

Done.

9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

Done.

10. Please sort the Materials Table alphabetically by the name of the material.

Done.

Response to Reviewers' Comments

We thank the reviewers for their careful reading of the manuscript, their many thoughtful comments, and the great suggestions that all-in-all have made this protocol much better. Detailed responses to the issues raised are below.

Reviewers' comments:

Reviewer #1:

Crespo et al. summarized methods and protocols for bioluminescent optogenetics. They introduced five different optogenetics tools and described how to prepare cells and gene transfection for these experiments. In addition, they also explained how to set up an experimental environment such as a bench and a microscope. This manuscript will be better if the authors improve the following points.

1) The authors use "Biolight" as bioluminescence by luciferase. However, the word is not familiar in the category. They should be change it to "bioluminescence".

Changed throughout the manuscript.

2) In the manuscript, they focused on "non-ion moving photoreceptors" compared to "ion moving receptors" such as channels and pumps in neurons. If so, the title should be more limited. At this point, readers interested in luminopsin may misunderstand.

Bioluminescent optogenetics uses bioluminescence to activate photosensory proteins – opsins (ion moving molecules) are a subset of the much larger class of photosensory proteins. There are ample reports in the literature of applying bioluminescence for activating opsins. This collection of JoVE protocols is focused on non-opsin photoreceptors. However, the principle experimental instructions within this protocol apply to using bioluminescence for activating photosensory proteins including opsins.

3) It is important to match the wavelength of bioluminescence and the active wavelength of photoreceptor (sensor). Each information (the peak wavelength, spectrum, and spectral overlap) can help readers for developing new their own tools.

We added the information on emission wavelength of light emitters and activation wavelength of light sensors to Table 1.

4) Bioluminescent substrate, luciferin is essential for the reaction. In particular, coelenterazine species are easily oxidized in serum. They should add more explanation about the composition of the medium and the timing of substrate addition.

Added information on both aspects to protocol (lines 180-189):

Prepare a working solution of luciferin in cell culture medium. Use the medium the cells are normally grown in (DMEM, NeuroBasal, etc.). The concentration of the luciferin should be adjusted such that the final concentration of the luciferin 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.

5) Toxicity of the substrate should also be considered. Even temporarily, 100 µM coelenterazine is toxic to cells and in vivo. It should be understood that the assay will be carried out under conditions different from the normal environment.

We have addressed this by including a new paragraph in the Discussion (lines 458-467):

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.

6) It would be appreciated if they mention what animal species are tested in vivo assay (mouse, zebrafish etc.) and what operational differences exist.

We have addressed this by including a new paragraph in the Discussion (lines 469-476):

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, intracerebrally, 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.

Reviewer #2:

Manuscript Summary:

The MS describes a useful method allowing novel and potent control of optogenetic systems by utilizing light emerging from in cellulo reactions. Importantly, the results show that light emitted by nanoluc (or

other luciferases), upon addition of the substrate is enough to turn on different optogenetic systems within minutes to levels that are comparable (or actually higher) than what can be obtained by classic blue-light stimulation with LEDs. Relevantly, the use of blue-light (at high intensity and for prolonged times) can have a negative effect of cell viability, something that should not occur with this biolight system.

Major Concerns:
- There are several clarifications that are needed in order to make this article more self-contained and extensively useful to a broad range of scientists. In particular, the authors do not provide a measurement of the amount of biolight that is generated and that is capable of activating the optogenetic systems. This is key in order to compare that to the classic LED stimulation.

We included the specific information reporting such measurements for luciferase-opsin combinations (Lines 65-66):

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].

Such measurements have not been conducted for the activation of other photoreceptors, and these would be experiments beyond the scope of this protocol. The goal of this protocol is to provide detailed information enabling other investigators to carry out bioluminescence-driven activation of photoreceptors. By starting with the luciferases, photoreceptors, and conditions used in the experiments described in this paper together with those described in the literature referred to in this paper (Table 1), other researchers will be able to utilize bioluminescence for activating light sensing domains.

We also make clear that bioluminescence versus LED is not a question of what is better in an absolute sense, but what works best under the priorities of individual experiments (Lines 44-53):

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. // 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.

- Since biolight appears to be so efficient. Could the authors comment on whether shorter times of CTZ addition/removal could also work fine?

We point out in the protocol that timing will have to be established for the individual experiments (Lines 191-195):

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.

In addition, we picked up the aspect of timing again in the Discussion (Lines 458-467):

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.

- For experiments depicted in 4, 5 and 6 it is key to establish a parallel on: a) how much LED light was used to activate the optogenetic system and b) how much bioluminescence was being generated upon CTZ addition.

We added the details on the settings for LED application and the concentrations of luciferase substrate used in each of the figure legends for figures 4-6 (lines 384-413):

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)

Again, it is beyond the scope of this paper to quantitatively compare luciferase and LED light emission. Rather, the examples illustrate the feasibility of using bioluminescence. We added this information to the beginning paragraph of Representative Results (lines 310-317):

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.

- Figure 6: There is no control showing what degree of activation is achieved with LED

We added this positive control to the panel and took out the less informative “no Cre” control (lines 418-421):

Twenty-four hours later cells were treated for 30 minutes with just medium (no CTZ) as a negative control, 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.

- Figure 5C. It is puzzling why vehicle gives higher signals than what was obtained in the dark (left panels)

We clarified the difference between ‘dark’ and ‘vehicle’ (lines 408-410):

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.

- Figure 4. The experiment misses the dark control IVIS image

We added the image.

- Figure 4: Explicitly mention the intensity of LED light (and for how long was applied)

We added this information to the figure legend (lines 388-389):

duty cycle 33%, 2 s on/4 s off for 40 minutes; 3.5 mW light power, 4.72 mW/cm^2 irradiance

- In table 1 please indicate the wavelength emission of the different luciferases utilized as biolight modules. In addition, indicate the wavelength of emission of the reporter system (firefly luciferase)

We added the information on emission wavelength of light emitters and activation wavelength of light sensors to Table 1.

GLuc and FLuc produce light only with their specific luciferins (CTZ and D-luciferin, respectively), they do not cross-react. CTZ is added for light emission from GLuc for activation of photoreceptors. The effect of this light activation is measured by how much FLuc is produced. This is tested by adding D-luciferin at the terminal stage of the culture. The wavelength of FLuc emitted light is 550-570 nm, and it is very weak compared to GLuc and NanoLuc variants. Even if the light were to be strong enough and even if the wavelength were to match perfectly with the light sensor, such effect on FLuc transcription would not be picked up during the 1 second measurement of FLuc light emission.

We added a sentence making the reader aware of the absence of cross-reactivity between GLuc and FLuc (lines 329-331):

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.

Minor Concerns:
- Line 284: "However, the presence of the transcription factor component alone resulted in considerable background signal."
Could the author comment whether this was due to spontaneous proteolysis or other cause?

We added this suggestion and rephrased to point out the increase in transcription with light (lines 337-339):

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 experiments depicted in Fig 5. Please indicate how where the fusion proteins achieved (luc fused at the N or C-terminus?)

We added this information to the figure legend (lines 404-405):

NanoLuc was either co-transfected or fused to the light-sensing moieties as depicted (N-NanoLuc-CRY-GalDD-C; N-NanoLuc-VP16-EL222-C).

- Please indicate Ionomycin and CaCl2 concentrations, as well as light intensity and pulse duration. Likewise, indicate what device (LED system/brand/duty cycle) was utilized as a source of light.

We added this information to the description of the experiment (lines 323-325) and to the figure legend (lines 387-389) and to the Materials Table:

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

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)

LED Array Driver	Amuza	LAD-1
LED Array for Multiwell Plates	Amuza	LEDA-x
Prizmatix USB Pulser TTL Generator for Optogenetics	Goldstone Scientific	

- Since the system is ready to act upon addition of the substrate, can the authors comments on the choice of promoters to provide strong and constitutive expression of the biolight (luciferase) modules?

We added this aspect to the protocol (lines 105-107 and 258-259):

The choice if promoters is dictated by the need to provide strong and constitutive expression of the light emitting module, such as enabled by the CAG and CMV promoters.

Choose strong promoters for expression of the light emitting modules, such as CAG or CMV.

- Regarding the step involving removing the luc substrate and changing media, could the authors comment on whether this step a) alters the status of the cells under analysis (stress), b) leads to loss of cells from the wells/plates.

Regarding the first point (stress for the cells): this depends on the cell type used and the experimental goal. It seems likely that the cells will experience stress. Depending on the goal of the experiment the investigator will have to weigh stress due to media change and stress due to LED exposure.

Regarding the second point (loss of cells): we have added a sentence in the protocol section about Cell Culture (lines 130-132) and the section about Bioluminescence Activation (lines 206-207):

If the cell type selected does not adhere well to the culture surface, plate cells on PDL-coated dishes.

If cells do not stick well to the culture surface, plate cells on PDL-coated dishes to avoid losing cells during washes.

- Red-light: Please indicate vendor and model of red-light in the Table of Materials, and if possible the intensity,

We added this information to the Table of Materials:

ABI 25W Deep Red 660nm LED Light Bulb

- black microcentrifuge tubes. Provide brand/vendor in the Table of materials.

We added this information to the Table of Materials:

Black Microcentrifuge Tubes, 0.5 ml, Argos Technologies	Fisher Scientific	03-391-166
Black Microcentrifuge Tubes, 1.5 ml, Argos Technologies	Fisher Scientific	03-391-161

- Line 179: "Light emission at 100 µM final luciferin concentration can usually be observed by eye when the red light is turned off."
Could the authors indicate the expected amount of light that is generated (dark-adapted eyes, or eyes just adapting to light have different sensitivities). It is key to have a more objective estimate of the amount of light (by luminometer, plate reader etc) that the cells in the wells are yielding, as this is the "amount of light" giving responses that are then compared to the ones obtained with the LED system.

We specified when after turning off the red light bioluminescence becomes visible (lines 197-200):

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.

As to comparing responses obtained with bioluminescence versus the LED system:

Quantitative comparative measurements have not been conducted by us or others, and such experiments would be beyond the scope of this protocol. The goal of this protocol is to provide detailed information enabling other investigators to carry out bioluminescence-driven activation of photoreceptors. By starting with the luciferases, photoreceptors, and conditions used in the experiments described in this paper together with those described in the literature referred to in this paper (Table 1), other researchers will be able to utilize bioluminescence for activating light sensing domains.

We underlined this by adding the following sentence to the first paragraph of the Representative Results section (lines 310-317):

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.

- Figure 6B: It would help switching the order of the panels no CTZ first followed by no VVD-CRE and then at the right the key panel with VVD-CRE.

We improved this figure by re-ordering the panels and swapping the 'no VVD-Cre' panel with the LED induced response as a positive control (negative control = no CTZ; experimental = CTZ; positive control = LED).

Reviewer #3:

Manuscript Summary:

The authors of this manuscript provide a detailed description of step-by-step protocols and equipment for the use of bioluminescence to activate photosensory proteins in vitro and in vivo. They describe how to set up the tissue culture room, incubator, and microscope in order to perform Bioluminescent Optogenetics experiments. Moreover, they provide detailed information regarding the preparation and administration of luciferin for single, or multiple stimulations, for specific in vitro or in vivo applications. They also present several examples of the application of this method. I recommend this manuscript for publication in the journal JoVE after addressing the following comments:

Major Concerns:

-The authors use bioluminescence and biolight as synonyms. They provide a definition of biolight in the summary followed by the exact same definition for bioluminescence in the abstract. The two words are alternatively used in the title and within the body of the text. This is very confusing for the reader. I would recommend the author to use only the term bioluminescence consistently throughout the text, since this is the conventionally used definition for the tool used throughout the manuscript.

Changed throughout the manuscript.

-I would encourage the authors add a resource, preferentially a table, listing the possible combinations of luciferase-luciferin pairs that can be used to activate available photoswitches; specifically, they should list wavelength of light emitted by the pair, intensity of bioluminescence emitted, flash or glow kinetics etc… This table would help the reader when determining the best luciferase-luciferin pair to use for their desired application.

We added the information on emission wavelength of light emitters and activation wavelength of light sensors to Table 1.

While one could list all available light emitters and light sensors, we wanted to focus Table 1 on those combinations used successfully.

-In the introduction the authors state that "biolight emission can be tuned by varying the concentration of luciferin". However, in the majority of the experiments described in this manuscript, the photoreceptors are co-expressed in transient transfection with the luciferase encoding construct. With this experimental paradigm, different cells can express heterogenous level of luciferase enzyme resulting in inhomogeneous levels of activation despite the use of the same amount of luciferase. I recommend the authors discuss this point in the protocol or in the discussion. I suggest expanding upon this concept highlighting the advantage of generating a construct capable of expressing the "light emitter and light sensor" at a 1 to 1 ratio that could overcome this issue.

We alert the reader to the need for optimizing plasmid ratios (lines 137-139):

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.

Ultimately, stable cell lines or transgenic animal lines will always be preferable for better quantitative experiments. In the Discussion section we suggest co-transfection as a starting point (lines 449-456):

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 actuator.

-I would suggest the authors articulate the concept of "luciferase-luciferin combination with flash or glow kinetics" mentioned in lines 176-177 and the effect of experimental consequences. If possible, I would specify in table 1 which combination was used for the experiments mentioned. If the author will add a table with a list of luciferin-luciferase pairs, this detail should be included.

We eliminated the distinction of flash and glow kinetics, as it was more confusing than informative in the context of bioluminescence used for photoreceptor activation. For activating a photosensitive domain the light emitted over the time between adding and removing the luciferin will determine activation, regardless of the types of kinetics (glow or flash). The distinction of flash vs glow is of more practical importance for luminometer plate readings: under flash kinetics the luciferin has to be injected right before the read in each well, while under glow kinetics the luciferin can be added to an entire 96 well plate, as light emission will be stable over the time needed to read all of the 96 wells.

Minor Concerns:
* Check the text for consistency (i.e. Hela line 106 vs HeLa in line 112).

Done

* For reproducibility the authors should specify the origin of the cell lines utilized (i.e ATCC reference number in the table of materials).

ATCC reference number added to table of materials:

HEK293	ATCC	CRL-1573
HeLa	ATCC	CCL-2

* In table 2: DNA/OPTI. Specify what is OPTI (I guess OPTI-MEM-I?). This reagent, source and code is not reported in the reagents list.

Spelled out in Table 2 and added to Table of Materials:

Opti-MEM	Thermo Fisher	11058021

* Line 111: Fluorescence microscopy is more commonly used instead of "fluorescent microscopy "

Changed

* Use of acronyms. The authors should list the full name of a reagent the first time they mention it (PDL line 112, CTZ line 150 etc…).

Done

* The authors say that they use Lipofectamine 2000 for transfection, but the specific protocol used is not reported. Add "accordingly to manufacturer's instruction" if the transfection procedure can be found there (line 121).

Added (line 133-134):

Transfection is done by lipofection according to the manufacturer’s recommendation

* The authors should report the recipe of PBS and Tyrode solution or list the provider in the table of materials. They are not reported in the reagents table.

Added information on Tyrode’s solution to protocol (lines 219-224) and information on PBS to Table of Materials:

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)).

D-PBS, no calcium, no magnesium Thermo Fisher 14190144

* Line 256: Inject experimental animals with luciferin and controls with vehicle.

This sentence was confusing and we removed it (experimental animals are those expressing the light emitter and sensor and receiving luciferin; control animals are those expressing the light emitter and sensor and receiving vehicle or animals not expressing the emitter and/or receiver and receiving luciferin). Instead, we added (line 301):

Inject animals with luciferin and vehicle, respectively.

* Line 272-275: Edit for clarity. The need of coincident Ca2+ increase and blue-light irradiation is not clear.

We changed the sentences to (lines 318-321):

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.

* Line 365. Add reference at the end of this sentence.

Added references (line 434):

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].