

Near-infrared light-controlled systems for gene transcription regulation, protein targeting and spectral multiplexing

Taras A Redchuk^{1,3}, Andrii A Kaberniuk^{2,3} & Vladislav V Verkhusha^{1,2}

¹Medicum, Faculty of Medicine, University of Helsinki, Helsinki, Finland. ²Department of Anatomy and Structural Biology, Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, New York, USA. ³These authors contributed equally to this work. Correspondence should be addressed to V.V.V. (vladislav.verkhusha@einstein.yu.edu).

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Near-infrared (NIR, 740–780 nm) optogenetic systems are well-suited to spectral multiplexing with blue-light-controlled tools. Here, we present two protocols, one for regulation of gene transcription and another for control of protein localization, that use a NIR-responsive bacterial phytochrome BphP1–QPAS1 optogenetic pair. In the first protocol, cells are transfected with the optogenetic constructs for independently controlling gene transcription by NIR (BphP1–QPAS1) and blue (LightOn) light. The NIR and blue-light-controlled gene transcription systems show minimal spectral crosstalk and induce a 35- to 40-fold increase in reporter gene expression. In the second protocol, the BphP1–QPAS1 pair is combined with a light-oxygen-voltage-sensing (LOV) domain-based construct into a single optogenetic tool, termed iRIS. This dual-light-controllable protein localization tool allows tridirectional protein translocation among the cytoplasm, nucleus and plasma membrane. Both procedures can be performed within 3–5 d. Use of NIR light-controlled optogenetic systems should advance basic and biomedical research.

INTRODUCTION

Non-opsin optogenetic tools allow light-control of a variety of biological processes in cells and in whole organisms^{1,2}. Most of the developed optogenetic tools are operated by a single wavelength, and hence enable control of a single cellular event, such as promoting gene transcription or activation of a signaling pathway^{3,4}. However, the majority of physiological processes are controlled by multifunctional regulatory molecules or their ensembles. Thus, optogenetic tools for spectral multiplexing are required to increase the complexity of light-driven manipulations. Here, we present a protocol for our recently developed approach for gene transcription activation with a NIR (740–780 nm) light-sensing optogenetic system based on a bacterial phytochrome from *Rhodospseudomonas palustris* (*RpBphP1*; hereafter BphP1) and its engineered protein partner QPAS1 (ref. 5). This protocol allows multiplexing of the BphP1–QPAS1 system with blue-light-controllable tools with minimal spectral crosstalk. We also provide a protocol for manipulation of the intracellular protein localization independently controlled by blue and NIR light.

Development of the protocol

Protein–protein interactions (PPIs) have a crucial role in cellular functions and biological processes in all organisms. Control of PPIs by light should promote the progress of basic biology and biomedicine due to noninvasiveness, adjustability, high spatiotemporal resolution and potential for multiplexing. Natural diversity of light-sensing photoreceptors allowed the development of a series of effective tools for regulation of cell functions by light⁶. Among them, the tools based on bacterial phytochromes were shown to be beneficial for use in mammalian cells and whole animals. This is because bacterial phytochromes use biliverdin IX α (BV), which is a ubiquitous heme derivative present in mammalian cells, as a chromophore^{7–9} and are naturally sensitive to far-red and NIR light, which penetrates deep into mammalian tissues through the so-called tissue transparency window between 650 and 900 nm (refs. 10,11).

The first BphP1-based optogenetic system for a light-controllable PPI was designed using its full-length natural binding partner *RpPpsR2* (hereafter referred to as PpsR2)⁴. BphP1 is a light-sensing component of the BphP1–PpsR2 optogenetic system and adopts the Pfr state, absorbing at 760 nm in the dark. Under NIR light of 740–780 nm, it undergoes photoconversion into the Pr state, absorbing at 680 nm and resulting in the binding of PpsR2. This light-controllable reversible PPI was successfully used to regulate cell signaling and gene transcription in cultured cells and in mice⁴. At this stage, it was shown that prolonged expression of BphP1 in mammalian cells allows usage of the system without the requirement for exogenous BV.

Further improvement in the BphP1 system led to the development of an engineered PpsR2 variant, called QPAS1, which is threefold smaller than the natural protein and lacks domains potentially involved in its oligomerization. The resulting BphP1–QPAS1 system was used for both transcription activation and transcription inhibition by NIR light. Moreover, the interaction of BphP1 and QPAS1 was used for light-induced intracellular protein relocalization. Detailed analysis of the spectral behavior of the BphP1-based tools revealed negligible cross-activation with common GFP-like fluorescent proteins. More importantly, this also facilitates spectral multiplexing with blue-light-activatable optogenetic constructs, thereby allowing for fast, reversible, two-color-controlled protein targeting in cells. We have recently combined the BphP1–QPAS1 tool with an optogenetic tool based on a LOV domain from *Avena sativa* phototropin-1 (hereafter AsLOV2), in which the caged nuclear localization signal (cNLS) is uncaged in response to 460-nm light⁵. In this protocol, we describe two multiplexing approaches for light-controlled transcription activation and protein targeting. In the first, gene expression is activated in mammalian cells in response to NIR and blue light with negligible spectral crosstalk. In the second approach, the protein of interest is actively relocalized from the

cytoplasm to either the plasma membrane or the nucleus, being independently controlled by two distinct colors.

Applications of the method

Spatiotemporal optogenetic control of biochemical processes in cells and animals can advance basic biology and biomedicine. An important venue of optogenetic manipulation is the modulation of cellular PPIs to control various cellular processes. NIR-light-controlled tools may be applied to activate almost any physiological process dependent on PPIs.

Genetically encoded optogenetic NIR-light-dependent systems exhibit several advantages over pharmacologically inducible PPI approaches; these include noninvasiveness, high PPI activation rates, fast reversibility and lack of potential side effects of drugs. These systems can be used not only in cell culture but also in animals for controlling intracellular protein localization, induction of enzymatic activity and activation of gene expression⁴. The BphP-based transcription regulation systems are compatible with reporter systems based on a Tet response element (TRE) and a Gal4 upstream activation sequence (UAS). These reporter systems are the most popular for conditional transcription activation in animals and, consequently, various Tet- and Gal4-responder animal lines (including fruit flies, zebrafish and mice) are available^{12–14}, which potentially allows wide application of the BphP1-based transcription activation in animals.

Beyond proof-of-principle applications, non-opsin optogenetic systems have been proven useful for controlling the physiology of various invertebrate^{15,16} and vertebrate organisms^{4,17,18}. Among biological processes, cell differentiation and reprogramming were light-regulated to achieve tissue modeling¹⁹. These types of applications can benefit greatly from multiplexing with visible-light tools, potentially allowing more-complex differentiation scenarios. Indeed, cellular differentiation processes during development are usually guided by transcription factor ensembles rather than single triggering molecules. Although complex gene networks were modeled in bacteria²⁰, substantially fewer multiplexing approaches are known for mammalian cells^{21,22}, making BphP1–QPAS1-based systems beneficial for multicolor control of cell differentiation. Unlike the original PpsR2, the smaller QPAS1 in the BphP1–QPAS1 pair is expected to be preferable in certain size-limited gene-delivery systems, such as adeno-associated viruses. Such virus-mediated delivery is hardly possible for larger light-sensitive proteins such as GIGANTEA²³ or PhyB (ref. 24), or those which require delivery of additional enzymes to provide chromophore synthesis²⁵. Its viral gene delivery will allow use of the BphP1–QPAS1 pair in various difficult-to-transfect cell lines and in tissues of living animals, expanding its applicability.

Moreover, the smaller QPAS1 size enables combination of the BphP–QPAS1-based NIR-sensitive system with the AsLOV2-based blue-light-sensitive optogenetic system. A near-IR-blue-light-inducible shuttle (termed iRIS), based on QPAS1 (NIR) and LOV (blue), allows researchers to target proteins to the nucleus with blue light (460 nm), to the plasma membrane with NIR light and to the cytoplasm in the dark. Among other possible applications, the iRIS could also be used for more stringent regulation of protein localization. For example, a transcription factor could be caged at the plasma membrane using the iRIS. This caging could act synergistically with a nuclear exclusion signal (NES) to additionally reduce the background activity of a transcription

factor in the nucleus. In this case, activation of the transcription factor would require two triggers, dark relaxation of BphP1 for uncaging from the membrane and blue light for active nuclear transport, providing more stringent control. In applications in which protein activity or protein signaling depends on its localization, the iRIS creates new approaches to study functions of signaling molecules in specific subcellular environments. Instead of the membrane-localization peptide sequence in the current iRIS variant, various protein-targeting signals could be used to target a protein of interest to different organelles or allow its secretion. As bacterial phytochrome-based and LOV-based optogenetic tools have been used separately in non-mammalian model animals^{15,26–28}, we anticipate that the iRIS-based approach can be applied in various model animals. Activation of the BphP1 part of the system can be done with NIR light, whereas activation of the LOV part of the system can be done with 460-nm blue light or 900-nm light in two-photon mode.

Similar to other light-sensitive proteins used for optogenetic tool development, the BphP1–QPAS1 pair can be further modified using protein-engineering techniques^{1,29,30}. Potential properties to modify include the BV binding rate (to avoid uncontrolled interaction with QPAS1), background QPAS1 interaction in the Pfr state, the kinetics of BphP1 photoconversion and the stability of the Pr and Pfr states. For the last, experimental data from the area of bacterial phytochrome-based NIR fluorescent proteins can be used for structure-guided rational design^{31,32}.

Comparison with other methods

Many systems using small molecules to control PPIs share common drawbacks. Among these drawbacks are the lack of temporal and spatial precision, potential side effects of the activating drugs³³, slow pharmacokinetics and large consumption of expensive compounds. Some systems are also reported to be leaky or to demand more-specific reagents^{34,35}. Optogenetic systems lack these drawbacks, and inexpensive, widely available LED emitters allow easy custom assembly of low-voltage light sources for illumination in any spectral range, from UV to IR.

Optogenetic tools developed from bacterial phytochromes possess several advantages over other light-controlled systems. First, the BV availability in mammalian cells and tissues makes the application of the bacterial phytochrome-based systems independent of exogenously supplied chromophores, whereas plant phytochrome-derived constructs, such as those based on PhyB, although exploited in a wide range of light-controlled applications^{24,36}, require the phycocyanobilin compound as a chromophore. This compound is not available in eukaryotic cells and should be delivered exogenously or synthesized intracellularly by overexpressing several bacterial enzymes^{25,37}, potentially incurring cytotoxicity and other side effects.

Second, bacterial phytochrome-based tools are sensitive to NIR light, which has a higher penetration depth by virtue of low light absorption and scattering of 650- to 800-nm light by mammalian tissues (**Supplementary Fig. 1**). By contrast, blue-light-controlled tools require much higher light intensities, when applied to animal models, to achieve light-activation in deep tissues³. It is also known that the plant phytochrome-based tools are extremely sensitive to far-red light (660 nm)³⁷. Moderate sensitivity to light and reversibility of BphP1 activation make the BphP1-based tools tolerate low-level ambient light, which makes them easier to work

with in an experimental setting. BphP1-based tools are also non-sensitive to illumination from conventional light sources with an emission peak in the blue–yellow spectral range. However, some precautions with broadband and blue-light sources are required because a partial BphP1 activation through its Soret absorption band, which peaks at 390–420 nm, may occur.

These properties of optogenetic tools based on bacterial phytochromes make them more valuable when combined with other light-sensitive constructs. In this regard, AsLOV2-based tools for protein targeting^{28,38–40} using the peptide caging–uncaging principle are perfect for this combination. Until now, all protein-targeting optogenetic systems could either direct a protein of interest from the cytosol to a single specific compartment (e.g., plasma membrane, mitochondria, nucleus and peroxisomes) or translocate it between two different locations (e.g., nucleus and mitochondria). In this protocol, the two-color-controllable tool iRIS, which uses the AsLOV2 domain, provides tridirectional protein targeting, which is not possible with optogenetic tools of monochromatic light.

Limitations

Despite successful use of the BphP1–QPAS1 pair for light-control of cellular processes, there are several factors that currently narrow its applications. Most of the limitations are shared with similar systems, which use light or drugs to control PPIs.

One potentially limiting feature of the bacterial phytochrome-based tools is dependence on cellular BV, which was shown to vary depending on the particular tissue or cell type⁹. As the

BphP1 apoprotein likely can also interact with PpsR2 in a light-independent way⁴¹, high BphP1 concentrations and a shortage of BV may lead to undesired PPIs in the dark, reducing the light-to-dark signal contrast. This may become especially critical for applications in which absolute signal values are sometimes hard to compare or estimate correctly, and it can be a limiting step, in particular for applications that are sensitive to the background level in the dark.

However, in cell culture, this can be overcome by the generation of a stable cell line with low expression of the BphP1 component (see **Box 1** for detailed procedures). Applicability of the BphP1–QPAS1 systems *in vivo* in tissues with low BV levels, such as neural tissues, must be tested. However, successful visualization of NIR fluorescent proteins incorporating BV as a chromophore in neurons and in the mouse brain⁴² provides reasons for optimism.

Another difficulty with the BphP1–QPAS1 pair is its relatively slow kinetics of thermal relaxation (deactivation) as compared with similar systems. Moreover, the level and kinetics of the cellular response to NIR illumination may depend not only on the well-characterized light-controlled PPI between BphP1 and QPAS1 (refs. 4,5), but also on the characteristics of DNA-binding domains (Gal4 and TetR), transcription regulation modules (VP16 and SID) and the protein-targeting peptide signals (NLS, NES and CAAX) used. Similarly, the output signal values in transcription activation, as well as background signals in protein targeting, are strongly influenced by the expression levels of all components of the system. Thus, pilot studies to determine BV dependence and appropriate expression schemes are unavoidable when

Box 1 | Generation of a cell line stably expressing the BphP1 component of the optogenetic system ● TIMING 6 weeks

BV is an essential factor for the correct functioning of bacterial phytochrome-based optogenetic systems. In mammalian cells, BV exists in equilibrium with bilirubin, and these together act as a part of the protective system against cellular stress⁴⁹. Endogenous BV levels are usually sufficient in experiments with bacterial phytochrome expression over prolonged times^{4,50}. However, if delivered using transient transfection, the high level of BphP1 expression may require additional BV, which can be supplemented exogenously. Alternatively, a cell line stably expressing BphP1 can be generated to bypass the need for exogenous BV supplementation.

The efficiency of stable cell-line generation can be substantially increased by using a transposase system such as Sleeping Beauty⁴⁵. Transposases facilitate the integration of the intact nucleotide sequence of interest into the genome by using a cut-and-paste mechanism. To do so, the BphP1–mCherry–TetR encoding cassette is placed between terminal repeats, which are recognized by the Sleeping Beauty transposase (pT2/SVNeo-103, see **Table 1**). Cells are cotransfected with plasmid pT2/SVNeo-103 and the pCMV(CAT)T7-SB100 transposase-producing plasmid (**Table 1**).

Procedure

1. Seed 4×10^5 HeLa cells in 3 ml of DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin in a 60-mm culture dish. Let the cells adhere overnight.
2. The next day, cotransfect the cells with pT2/SVNeo-103 and pCMV(CAT)T7-SB100 plasmids using Effectene transfection reagent as follows: mix 900 ng of pT2/SVNeo-103 and 100 ng of pCMV(CAT)T7-SB100 plasmids with the DNA-condensation buffer EC, to a total volume of 150 μ l. Add 8 μ l of enhancer and mix by vortexing for 1 s. Incubate at room temperature for 2–5 min.
- ▲ **CRITICAL STEP** To avoid toxic effects after transfection, use endotoxin-free quality plasmid DNA purification kits.
3. Add 25 μ l of Effectene transfection reagent to the DNA-enhancer mixture. Mix the samples by vortexing for 10 s. Incubate the samples for 5 min at room temperature for transfection-complex formation.
4. While complex formation takes place, aspirate the medium from the 60-mm culture dish with cells, wash the cells once with 3 ml of PBS and add 4 ml of DMEM medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin to the cells.
5. Add 1 ml of DMEM media supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin to the tube containing the transfection complexes, and mix by pipetting. Add the transfection complexes dropwise to the cells in the culture dish, and gently shake the dish to ensure uniform distribution of the transfection complexes.
6. 14–16 h after incubation with the transfection complex, replace the transfection complex with DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin.

(continued)

Box 1 | Generation of a cell line stably expressing the BphP1 component of the optogenetic system ● TIMING 6 weeks (continued)

7. After 8 h of cultivation in a tissue culture incubator, trypsinize the cells and transfer them to a 150-mm culture dish containing 20 ml of DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin. Let the cells adhere overnight and replace the culture medium with 20 ml of selective medium: DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin and G418 active component (700 µg ml⁻¹).
8. Change the selective medium every day until colonies appear.
 - ▲ **CRITICAL STEP** Carefully wash the cells with PBS when changing the selective medium, as many dead cells are expected after several days of cultivation with the selective medium.
9. After single colonies appear, decrease the concentration of G418 active component to 470 µg ml⁻¹; this will result in faster cell growth.
10. Once the colonies have increased in size (to ~30–50 cells per colony), trypsinize the cells and transfer them to a 100-mm culture dish containing 10 ml of selective medium. Maintain the cells in selective medium with the concentration of G418 active component at 470 µg ml⁻¹ until confluency.
11. Trypsinize the cells and transfer them to a 150-mm culture dish containing 20 ml of selective medium. Maintain the cells in selective medium with the concentration of G418 active component at 470 µg ml⁻¹ until confluency.
12. The day before scheduled FACS-sorting, seed 2×10^6 nontransfected HeLa cells onto a 25-cm² culture flask. These cells will be used as a negative control to discriminate mCherry-negative cells.
13. Start preparing the cells 2 h before scheduled sorting. Trypsinize the cells from the 150-mm culture dish, transfer them to a 15-ml sterile tube, pellet the cells (180g, 7 min, 4 °C) and resuspend them in 15 ml of PBS without Ca²⁺ and Mg²⁺. Count the cells using a hemocytometer, pellet the cells by centrifugation at 180g for 7 min at 4 °C and resuspend the cells in PBS without Ca²⁺ and Mg²⁺ supplemented with 4% (vol/vol) FBS, 2 mM EDTA and 6 U µl⁻¹ benzonase. Filter the cells through a sterile 30-µm filter and transfer them to a sterile FACS tube. Place the tube with cells on ice.
14. Trypsinize the nontransfected HeLa cells from the 25-cm² culture flask and transfer them to a 15-ml tube. Pellet the cells by centrifugation at 180g for 7 min at 4 °C, resuspend them in 4 ml of PBS without Ca²⁺ and Mg²⁺, filter the cells through a 30-µm filter and transfer them to a sterile FACS tube.
15. Prepare several collection tubes with 500 µl of DMEM supplemented with 60% (vol/vol) FBS.
16. Perform FACS sorting. For this, run nontransfected cells and create a gate to select the mCherry-positive cells. Run the transfected cells and sort the mCherry-positive cells (**Supplementary Fig. 6**).
17. After sorting, seed the cells into an appropriate culture dish depending on the number of sorted cells. Let the cells adhere overnight and replace the culture medium with selective medium.
18. Expand the cells in selective medium in a 150-mm dish. This step may take up to 2 weeks and depends on the amount of sorted cells.
19. To substantially enrich the population of mCherry-positive cells, repeat FACS sorting as described in steps 9–17.
20. Expand the cells in selection medium in a 150-mm dish.
21. Trypsinize the cells from the 150-mm dish, pellet the cells (180g, 7 min, 4 °C) and resuspend them in 10 ml of DMEM supplemented with 50% (vol/vol) FBS and 10% (vol/vol) DMSO. Put 1-ml aliquots in cryotubes and place the tubes into a freezing container. Incubate the container at –80 °C for 24 h, and then store the tubes in liquid nitrogen.
 - **PAUSE POINT** The cells can be stored in liquid nitrogen for up to several years.

developing new applications or applying existing tools to specific biological problems.

Experimental design

This protocol is designed to control gene expression or the cellular localization of a protein of interest with NIR and blue light in cultured mammalian cells. For controlling gene expression (**Fig. 1a**), the cells are transfected with constructs carrying the BphP1-based light-controlled transcription activation system (containing transactivation domain VP16 and DNA-binding domain Gal4, split in a way similar to yeast two-hybrid systems) and the gene of interest under the conditional promoter (Gal4-UAS) (**Table 1**). At the same time, the cells are also transfected with the blue-light-sensing LightOn transcription activation system³, which is based on a homodimerization of the Vivid (VVD) domain of the LOV protein family (**Fig. 1b**).

Then the transfected cells are illuminated with NIR (740 nm) and blue (460 nm) light. Under NIR illumination, the VP16 transactivator and DNA-binding domain associate with each other and relocalize to the cell nucleus (driven by the NLS), where the complex binds the UAS and launches the transcription of a gene of interest. Independently, under blue light, Vivid forms a dimer, allowing Gal4-driven expression in cells expressing the LightOn system. Finally, the cells are analyzed for the expression level of the protein of interest. To achieve the best results, the ratio of plasmids and the transfection procedure should be optimized for each particular cell type. Control cells co-expressing (i) reporter constructs without the activator component and (ii) nonconditional activator with the reporter are needed for estimation of reporter leakage and light-activation dynamic range.

Generation of cells stably expressing the light-sensing component may precede the transient transfection and illumination

step in order to achieve light regulation without the necessity of exogenous BV (Box 1, Supplementary Fig. 2). This may be beneficial for the reproducibility, for coexpression with other constructs and when low expression levels of BphP1 are desirable. We recommend establishing stable cell lines if cells do not tolerate transient transfection or BV addition. Otherwise, transient transfection with additional BV in cell culture medium is, usually, a less laborious way to obtain meaningful results. Kinetics of the protein production can be studied using a secreted reporter, such as secreted embryonic alkaline phosphatase (SEAP) (Box 2). Alternatively, the cell line stably expressing BphP1 may be used for transcription activation in mice (Box 3).

The optogenetic system for protein targeting consists of the membrane-tethered BphP1 and the QPAS1–AsLOV2 fusion, termed iRIS (Fig. 2). Being localized mainly to the cytoplasm in the dark, the iRIS accumulates in the nucleus under blue light, due to the uncaging of the NLS in the AsLOV2 part²⁸. NIR light targets the iRIS to the plasma membrane as a result of the BphP1–QPAS1 interaction. The tridirectional protein targeting can be observed in a single cell with fluorescence time-lapse imaging and analyzed using fluorescent signals in the cytoplasm and the nucleus.

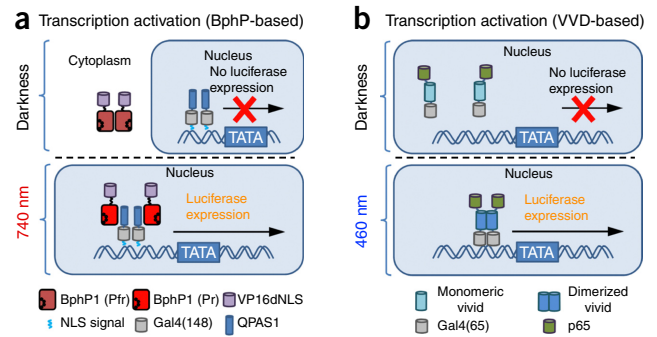


Figure 1 | Mode of function of the NIR- and blue-light-inducible transcription activation systems. (a) In the dark, the BphP1 bacterial phytochrome is in the Pfr state and localized in the cytoplasm. NIR light (740 nm) converts BphP1 to the active Pr state, which is able to bind QPAS1. The formed BphP1–QPAS1 complex translocates to the nucleus where it interacts with a reporter gene construct via its Gal4 DNA-binding domain. Gene transcription occurs from the minimal promoter, activated by the BphP1 fused to the VP16 transactivation domain with NLS deleted (VP16dNLS). (b) After the blue-light activation, the Vivid domains homodimerize and interact with a reporter gene construct via its Gal4 domain (1–65). Transcription from the minimal promoter is activated by the p65 transactivation domain. a adapted from ref. 5, Nature Publishing Group.

TABLE 1 | Plasmids used in the protocol.

Application	Plasmid	Description	Addgene no.	Reference
Transcription activation (two-color), Steps 3, 7A	pQP-T2A	BphP1–VP16–T2A–NLS–GAL4–QPAS1	102583	5
	pGAVP0	GAL4–VVD–p65	Not commercially available ^a	3
	pFR-Luc	UAS-controlled reporter (luciferase)	Not available from Addgene ^b	5
Tridirectional protein targeting, Steps 3, 7B	pQP-iRIS	BphP1–mVenus–CAAX–IRES2–NES–mCherry–QPAS1–AsLOV2NLS	102584	5
Stable cell line generation, Box 1	pT2/SVNeo-103	RpBphP1–mCherry–TetR	79841	4
	pCMV(CAT)T7-SB100	Sleeping Beauty 100× transposase	34879	45
Kinetics of light-induced transcription activation, Box 2	pCMV-104	NLS–PpsR2–VP16	79842	4
	pTRE-Tight-SEAP	TRE-controlled reporter (alkaline phosphatase)	Not commercially available ^a	4
Light activation of gene expression in mice, Box 3	pCMV-104	NLS–PpsR2–VP16	79842	4
	pTRE-Tight-Rluc8	TRE-controlled reporter (luciferase)	Not commercially available ^a	4

^aPlasmids and plasmid sequences should be requested from the corresponding authors of the respective original publications. ^bAvailable from Agilent Technologies, cat. no. 219050.

Box 2 | Determination of the kinetics of light-induced transcription activation using a TetR-responsive system ● TIMING 12 h, over 6 d

To optimize the time of light-induced transcription activation, it is recommended to study the kinetics of protein expression in response to 740-nm light illumination. In this box, we describe how the expression of a reporter protein (SEAP) can be induced by 740-nm light, and how the kinetics of expression can be monitored by detecting secreted SEAP in culture medium samples collected every 6–12 h after the beginning of illumination. For this, HeLa cells stably expressing BphP1–mCherry–TetR (Box 1) are cotransfected with pTRE-Tight-SEAP (7× tetO) reporter plasmid and the pCMV-104 plasmid, encoding the transcription activator VP16 fused with PpsR2 and nuclear localizing signal (Table 1).

Procedure

Culture of HeLa cells ● TIMING 1 h, day 1

1. Start with one 80% confluent 100-mm dish containing HeLa cells stably expressing BphP1–mCherry–TetR (Box 1) grown in DMEM medium supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin–streptomycin and G418 active component (470 µg ml⁻¹).
2. Trypsinize the cells and seed them in six wells of each of two 12-well plates at a density of 1 × 10⁵ per well in 1 ml of DMEM medium supplemented with 10% (vol/vol) tetracycline-free FBS, 1% (vol/vol) penicillin–streptomycin and G418 active component (470 µg ml⁻¹).

▲ **CRITICAL STEP** It is important to use tetracycline-free FBS, as trace amounts of tetracycline may affect the efficiency of light-induced SEAP expression.

Transfection procedure ● TIMING 2 h, day 2

3. The next day, cotransfect cells in three wells in each plate with pCMV-104 and pTRE-Tight-SEAP, and in the other three wells with a mock plasmid (instead of pCMV-104) and the pTRE-Tight-SEAP plasmid with a plasmid ratio of 5:1. Cells cotransfected with the mock and pTRE-Tight-SEAP plasmids may be used as a control to detect the background level of SEAP expression from the pTRE-Tight-SEAP reporter plasmid. Prepare 300 ng of plasmid DNA mix (all the reagent amounts at this step are indicated per well) with the DNA-condensation buffer (EC buffer), to a total volume of 75 µl. Add 2.4 µl of enhancer and mix by vortexing for 1 s. Incubate at room temperature for 2–5 min.
4. Add 6 µl of Effectene transfection reagent to the DNA–enhancer mixture. Mix the samples by vortexing for 10 s. Incubate the samples for 5 min at room temperature for transfection-complex formation.
5. While complex formation takes place, aspirate the medium from the cells, wash the cells once with 1 ml of PBS buffer and add 800 µl of DMEM medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin.
6. Add to the tubes containing the transfection complexes 400 µl of DMEM medium supplemented with 10% (vol/vol) tetracycline-free FBS and 1% (vol/vol) penicillin–streptomycin, and mix by pipetting. Add the transfection complexes dropwise to the cells in the corresponding wells, and gently shake the plate to ensure uniform distribution of the transfection complexes. Transfer the dishes to a tissue culture incubator and protect them from light.

Transcription activation ● TIMING 1 h, days 3–5

7. After 8–12 h, replace the culture medium with pre-warmed DMEM medium supplemented with 10% (vol/vol) tetracycline-free FBS, 1% (vol/vol) penicillin–streptomycin and G418 active component (470 µg ml⁻¹).
8. Place one culture plate into the cell culture incubator equipped with an illumination source as described in the Equipment Setup section and illuminate the plate with a 740-nm LED with 0.2 mW cm⁻² for up to 72 h. Keep the other plate in a separate cell culture incubator or securely covered with black material or aluminum foil.

Sample collection ● TIMING 1 h, days 3–5

9. Collect 35 µl of supernatant from each well every 6 or 12 h into separate microtubes, starting right after changing the culture medium after transfection (considering the first samples as at 0 h). Spin the samples down at 200g for 5 min at room temperature to pellet cell debris, and transfer 30 µl of the supernatant to clean microtubes and store them at –20 °C.

▲ **CRITICAL STEP** Before collecting the samples, gently shake the plate to provide uniform distribution of SEAP in the culture medium.

■ **PAUSE POINT** The supernatants can be stored at –20 °C for several months.

SEAP assay ● TIMING 4 h, day 6

▲ **CRITICAL** To detect the level of SEAP in the collected samples, we suggest using a Great EscAPe SEAP Fluorescence Detection Kit. All buffers described in the procedure below are from this kit.

10. Thaw the collected supernatants, and transfer 25 µl to individual wells of a 96-well plate.
11. Use a multichannel pipette to transfer 25 µl of 1× dilution buffer to each well, and then seal the plate with adhesive tape.
12. Incubate the sealed plate at 65 °C on a heat block or in a water bath for 30 min to inactivate endogenous phosphatases.
13. Cool the plate by placing it on ice for 2–3 min, and then centrifuge the plate at 1,200g for 3 min at room temperature.
14. Use a multichannel pipette to add 75 µl of assay buffer to each well, and incubate the buffer at room temperature for 5 min.
15. Prepare enough 120-µM 4-methylumbelliferyl phosphate (MUP) in 1× dilution buffer and use a multichannel pipette to add 25 µl of 120-µM MUP solution to each well.
16. Seal the plate with transparent adhesive tape.
17. The excitation and emission peaks of MUP fluorescence are 360 nm and 449 nm, respectively; measure the MUP fluorescence for 2 h in 5-min intervals using a plate reader.

Box 3 | Light activation of gene expression in mice ● TIMING 10 h, over 5 d

HeLa cells stably expressing BphP1–mCherry–TetR (Box 1) can be used for light-induced transcription activation after s.c. injection in mouse. For this, cells are cotransfected with the pCMV-104 plasmid, encoding the transcription activator VP16 fused with NLS–PpsR2, and the pTRE-Tight–Rluc8 (7× tetO) reporter plasmid, encoding red-shifted *Renilla* luciferase (Table 1). 24 h after transfection, 3×10^6 cells are s.c. injected into the interscapulum area of an FVB mouse. The efficiency of light-induced transcription activation is detected with an IVIS Spectrum instrument 48 h after injection.

Procedure

Culture of HeLa cells ● TIMING 1 h, day 1

1. Start with one 80% confluent 100-mm dish containing HeLa cells stably expressing BphP1–mCherry–TetR (Box 1) in DMEM media supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin–streptomycin and G418 active component ($470 \mu\text{g ml}^{-1}$). For an experiment on three illuminated animals and three dark-treated control animals, a total of 1.8×10^7 cells are required (3×10^6 cells per animal). To harvest the necessary amount of transfected HeLa cells, cotransfection with pCMV-104 and pTRE-Tight–Rluc8 plasmids (in steps 3–5) should be done on at least four 100-mm dishes (4×10^6 HeLa cells are expected to be harvested from a 100-mm dish after transfection).
2. The day before transfection, seed 2×10^6 cells on each 100-mm dish in 10 ml of DMEM medium supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin–streptomycin and G418 active component ($470 \mu\text{g ml}^{-1}$).

Cotransfection procedure ● TIMING 2 h, day 2

3. The next day, ensure that the cells are at high density (confluency $\geq 80\%$). To achieve higher transfection efficiency, we recommend using TransIT-LT1 transfection reagent as follows: warm the transfection reagent to room temperature and vortex gently before using. Place 6 ml of Opti-MEM medium in a sterile 15-ml tube. Add $15 \mu\text{g}$ of the plasmid DNA mix (pCMV-104 and pTRE-Tight–Rluc8 in a 5:1 ratio), and pipette gently to mix completely. Add $45 \mu\text{l}$ of the transfection reagent to the diluted DNA mixture. Pipette gently to mix completely, and incubate at room temperature for 15–30 min.
4. While complex formation takes place, aspirate the medium from each dish with cells, wash the cells once with 10 ml of PBS and add 15.5 ml of DMEM medium supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin–streptomycin and G418 active component ($470 \mu\text{g ml}^{-1}$).
5. Add the DNA complexes dropwise to different areas of the dish. Gently rock the dish back and forth and from side to side to evenly distribute the DNA complexes. Transfer the dish to a tissue culture incubator and protect it from light.

Preparation of animals for the experiment ● TIMING 2 h, day 2

6. Before injection of the cells, the fur on the interscapular area of each mouse should be removed with a depilatory cream. To facilitate fur removal before applying the cream, fur may be cut away by using scissors or a shaving machine.
- ! CAUTION** Any experiments involving live mice must conform to relevant institutional and national regulations. All animal experiments in this protocol were performed in an AAALAC-approved facility according to permission no. 20160313 from the Albert Einstein College of Medicine Animal Use Committee.

Harvesting and injection of cells ● TIMING 3 h, day 3

7. 24 h after transfection in step 5, trypsinize the cells from the culture dishes, transfer them to a 15-ml sterile tube, pellet the cells ($180g$, $4 \text{ }^\circ\text{C}$, 7 min) and resuspend them in 10 ml of PBS without Ca^{2+} and Mg^{2+} . Count the cells using a hemocytometer, pellet the cells by centrifugation at $180g$ for 7 min at $4 \text{ }^\circ\text{C}$ and resuspend them in 0.6 ml of RPMI-1640 media without phenol red and with 2 mM L-glutamine to a final concentration of $3 \times 10^7 \text{ ml}^{-1}$. Transfer the cells to 0.5-ml syringes with a 28-gauge needle (3×10^6 cells in each syringe). Keep the syringes filled with cells on ice in the dark.
- ▲ CRITICAL STEP** During harvesting of transfected cells, do not expose them to bright sources of light.
8. Before injection of cells, anesthetize the mice with an isoflurane vaporizer. After s.c. injection, keep the animals under 740-nm light at 1 mW cm^{-2} or in the dark for 48 h.
- ! CAUTION** Be extremely cautious when handling isoflurane. The liquid is flammable and combustible, and the vapors are toxic.

Imaging of animals ● TIMING 2 h, day 5

9. Before imaging, prepare the Rluc8 substrate. Dissolve Inject-A-Lume Coelenterazine native with sterile diluent to a final concentration of 2 mg ml^{-1} and prefill 0.5-ml syringes with a 28-gauge needle with $40 \mu\text{l}$ of the diluted substrate.
10. After 48 h, anesthetize the mice with an isoflurane vaporizer, and keep them under anesthesia throughout the imaging. Immediately before imaging, inject the mice i.v. through the retro-orbital vein with the Rluc8 substrate. Determine the efficiency of light-induced transcription activation by imaging the animals with an IVIS Spectrum *in vivo* imaging system.

MATERIALS

REAGENTS

- HeLa cells (ATCC, cat. no. CCL-2) **▲ CRITICAL** The cell lines should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- DMSO (Fisher BioReagents, cat. no. BP231-100)
- Trypsin EDTA (Corning, cat. no. 25-053-CI)
- Penicillin–streptomycin (Corning, cat. no. 30-002-CI)
- DMEM (GE Healthcare Life Sciences, cat. no. SH30022.02)
- PBS (GE Healthcare Life Sciences, cat. no. SH30264.01)
- PBS without Ca²⁺ and Mg²⁺ (GE Healthcare Life Sciences, cat. no. SH30256.01)
- Biliverdin (BV; Frontier Scientific, cat. no. B655-9)
- FBS (Gemini Bio-Products, cat. no. 100-106)
- Effectene Transfection Reagent (Qiagen, cat. no. 301425) **! CAUTION** The individual kit components may be harmful; check the safety data provided by the manufacturer. **▲ CRITICAL** Transfection efficiency may greatly influence the results of the described protocols. Therefore, the transfection reagent should be carefully chosen.
- Plasmid Maxi Prep Kit (Qiagen, cat. no. 12162) **! CAUTION** The individual kit components may be harmful; check the safety data provided by the manufacturer.
- Plasmid Midi Prep Kit NucleoBond Xtra Midi EF (Macherey-Nagel, cat. no. 740420.50) **! CAUTION** The individual kit components may be harmful; check the safety data provided by the manufacturer.
- Live cell imaging solution (Life Technologies, cat. no. A14291DJ)
- Bioluminescence assay reagent (NanoLight Technology, cat. no. 318) **! CAUTION** The individual kit components may be harmful; check the safety data provided by the manufacturer.
- Poly-L-lysine solution (0.01% (wt/vol); Merck, cat. no. A-005-C)
- Tris-HCl (Thermo Fisher Scientific, cat. no. 15506017)
- Glycerol (Thermo Fisher Scientific, cat. no. 17904)
- β-mercaptoethanol (Thermo Fisher Scientific, cat. no. 21985023)
- Triton X-100 (Thermo Fisher Scientific, cat. no. 85111)
- PMSF (Thermo Fisher Scientific, cat. no. 36978) **! CAUTION** This compound is toxic if swallowed. It causes severe skin burns and eye damage. Protect eyes and skin; do not inhale or swallow.
- Plasmids (Table 1)

(Optional) Generation of a cell line stably expressing the BphP1 component of the optogenetic system

- G418 (Corning, cat. no. 61-234-RF; see Box 1)
- Benzonase (Millipore Sigma, cat. no. 70746-4)

(Optional) Determination of the kinetics of light-induced transcription activation of the TetR-responsive system

- Great EscAPE SEAP Fluorescence Detection Kit (Takara Bio, cat. no. 631704; see Box 2) **! CAUTION** The individual kit components may be harmful; check the safety data provided by the manufacturer.

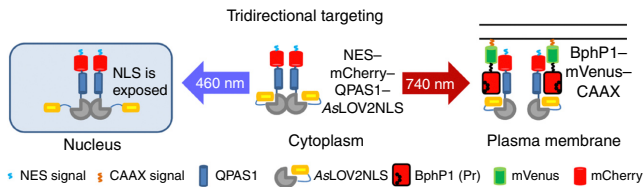


Figure 2 | Mode of function of the light-controllable tridirectional subcellular targeting system. In the dark, the NES–mCherry–QPAS1–AsLOV2NLS construct (iRIS) is localized in the cytoplasm and can be targeted to the nucleus or the plasma membrane with blue (460 nm) or NIR (740 nm) light, respectively. Blue light uncages the nuclear localization signal (NLS), which results in the iRIS translocation to the nucleus. NIR light converts the membrane-anchored BphP1 to the active Pr state and triggers its heterodimerization with QPAS1, which results in the iRIS translocation to the plasma membrane. Adapted from ref. 5, Nature Publishing Group.

- FBS, tetracycline free (Takara Bio, cat. no. 631106)
- (Optional) Light activation of gene expression in mice
- 2- to 3-month-old female FVB mice (20–25 g of body weight; see Box 3) (National Cancer Institute (NIH), strain no. 207) **! CAUTION** Any experiments involving live mice must conform to relevant institutional and national regulations. All animal experiments in this protocol were performed in an AAALAC-approved facility according to permission no. 20160313 from the Albert Einstein College of Medicine Animal Use Committee.
- TransIT-LT1 (Mirus Bio, cat. no. MIR 2304)
- Opti-MEM (Gibco, cat. no. 31985062)
- RPMI-1640 medium supplemented with L-glutamine (Gibco, cat. no. 11835030)
- Inject-A-Lume Coelenterazine (NanoLight Technologies, cat. no. 303-INJ)
- L-glutamine (Corning, cat. no. 25005CI)

EQUIPMENT

- 25-cm² Culture flask (Corning, cat. no. 430639)
- 24-Well culture plate (Corning, cat. no. 3526)
- Six-well culture plate (Corning, cat. no. 3516)
- Inverted microscope (Olympus, model no. IX83 or a comparable epifluorescence microscope (see the Equipment Setup section))
- NIR LED emitter (LED Engin, cat. no. LZ4-00R308) **! CAUTION** If not adjusted properly, LED light sources may produce extremely bright light. Protect your eyes, if necessary.
- Blue LED emitter (LED Engin, cat. no. LZ4-40B200) **! CAUTION** If not adjusted properly, LED light sources may produce extremely bright light. Protect your eyes, if necessary.
- Power source (Tekpower, cat. no. TP6005E)
- 24-gauge stranded wire (Remington Industries, cat. no. 24UL1007STRBLA)
- Thermal adhesive tape (Luxeon Star LEDs, cat. no. LXT-S-12)
- 1-s–60-h pulse-pause timer (Velleman, cat. no. VM188)
- NanoDrop ND-2000 spectrophotometer (Thermo Scientific, cat. no. ND2000USCAN)
- Power meter (Thorlabs, cat. no. PM130D)
- Power sensor (Thorlabs, cat. no. S130C)
- Tissue culture hood (Thermo Fisher Scientific, 1300 Series A2 Biological Safety Cabinet)
- Tissue culture incubators, set to 37 °C, 5% CO₂ (Forma Series 2; Thermo Fisher Scientific, cat. no. 3110)
- Victor ×3 multilabel plate reader (PerkinElmer)
- Hemocytometer (Hausser Scientific, cat. no. 3120)

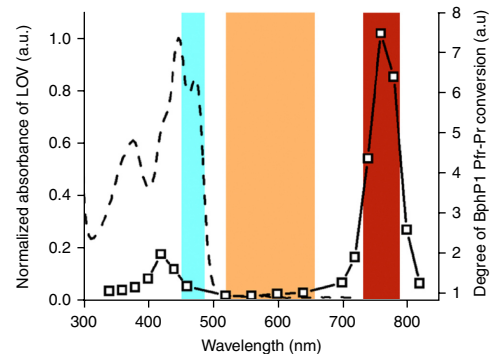


Figure 3 | Light-sensitivity of the iRIS optogenetic tool. Light-sensitivity of the LOV domain is determined by a combination of the absorption spectrum of the LOV domain in a ground state (dashed line) and by the previously measured light-action spectrum of BphP1 (solid line). The LOV part of iRIS can be activated with 460-nm light (light-blue box) without notable activation of the BphP1 part. The BphP1 part can be activated with NIR light of 730–790 nm (dark-red box). The dark-yellow box highlights the spectral region that is considered to be ‘safe’ in terms of a near-absence of iRIS light-activation. a.u., arbitrary units. Adapted from ref. 4, Nature Publishing Group.

- 35-mm cell culture dish (CELLview; Greiner Bio-One, cat. no. 627 860)
- Cell chamber for microscopy (Thermo Fisher Scientific, cat. no. A7816)
- Circle coverslips, 25 mm (Thermo Fisher Scientific, cat. no. 1014355125NR1)
- 96-Well half-area, white, flat-bottom plates (Corning, cat. no. 3693)
- Centrifuge with a holder for 96-well plates
- 200-W metal-halide arc lamp (Lumen, model no. 220PRO)
- 40× or 60× objective lens (e.g., Olympus UPlanSApo 40×, 0.95-NA (numerical aperture) air or 60×, 1.35-NA oil lens)
- Scientific complementary metal oxide semiconductor (sCMOS) camera (e.g., QImaging optiMOS camera)
- OriginPro v8.6 graphing and data-analysis software (<https://www.originlab.com/>)
- ImageJ v1.51J8 image analysis software (<https://imagej.nih.gov/ij/>)
- Fiji v1.50b image analysis software (<https://fiji.sc/>)

(Optional) Generation of a cell line stably expressing the BphP1 component of the optogenetic system

- 150-mm Culture dish (Corning, cat. no. 430599; see **Box 1**)
- 100-mm Culture dish (Corning, cat. no. 430167)
- 60-mm Culture dish (Corning, cat. no. 430166)
- FACS tube (Corning, cat. no. 352063)
- 30- μ m Filter (Sysmex, cat. no. 04-004-2336)
- 15-ml Sterile centrifuge tube (Corning, cat. no. 430790)

(Optional) Determination of the kinetics of light-induced transcription activation of the TetR-responsive system

- 12-Well culture plate (Corning, cat. no. 3513; see **Box 2**)
- 96-Well black plate (Greiner Bio-One, cat. no. 655076)
- SpectraMax M2 fluorescence plate reader (Molecular Devices)

(Optional) Light-activation of gene expression in mice

- 0.5-ml Syringe (BD, cat. no. 329461; see **Box 3**)
- IVIS Spectrum *in vivo* imaging system (Perkin Elmer, cat. no. 124262)

REAGENT SETUP

MidiPrep plasmid DNA purification The purity of plasmid DNA and the absence of endotoxins are critical for transfection efficiency. Use the NucleoBond Xtra Midi EF plasmid purification kit or similar to ensure a pure, endotoxin-free plasmid DNA sample. Purified plasmid can be stored at $-20\text{ }^{\circ}\text{C}$ for several months.

Lysis buffer (for bioluminescence assay) Lysis buffer is 20 mM Tris-HCl, pH 8.0, 10% (vol/vol) glycerol, 0.1% (vol/vol) β -mercaptoethanol, 0.1% (vol/vol) Triton X-100 and 1 mM PMSF. Make fresh lysis buffer before each experiment.

BV stock solution Prepare a 100 mM BV stock solution in DMSO (this can be stored at $4\text{ }^{\circ}\text{C}$ for up to 6 months). Add the BV stock to the cell culture medium >6 h before the medium change to ensure it completely dissolves. Do not add the transfection reagent in culture medium containing BV. BV is rather stable in the culture medium, so BV-containing medium may be stored for several days at $+4\text{ }^{\circ}\text{C}$.

Poly-L-lysine-coated tissue culture dishes or coverslips Prepare 0.1 mg ml^{-1} poly-L-lysine solution in sterile water. Fully cover the surface of the cell culture dish or coverslip. Incubate the solution for 1–12 h at room temperature ($20\text{--}25\text{ }^{\circ}\text{C}$). Aspirate the poly-L-lysine solution and wash the vessel with sterile water. Allow the surface to dry aseptically. Coated vessels can be stored at room temperature for at least several weeks.

EQUIPMENT SETUP

Safelight Preferably, to prevent activation of optogenetic systems by ambient light, a tissue culture hood should be located in a darkroom. If no darkroom is available, cover sources of light, such as windows or doors, with black adhesive vinyl foil or aluminum foil. The tissue culture room can be equipped with a source of orange safelight (**Fig. 3**). For this,

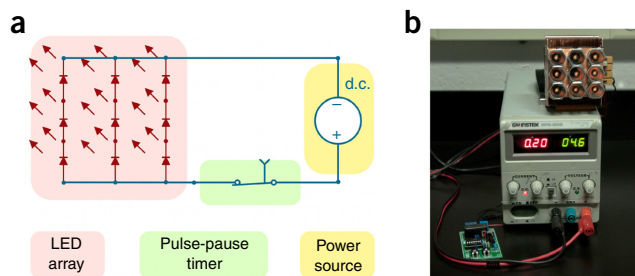


Figure 4 | Setup for the LED illumination. (a) The connection scheme of the major components is shown. Nine LEDs mounted on a CPU heat sink and connected by a parallel circuit of three rows with three LEDs each make up the LED array. To provide a pulsed regimen of illumination, the pulse-pause timer is serially connected between the LED array and the power source. **(b)** Photograph of the assembled setup.

an LED strip or LED bulb emitting light at 550–600 nm may be mounted outside the hood.

LED arrays For illumination of the cells, we designed and used maximally simplified equipment that does not require special skills and that can be assembled in a fast and a cost-effective way (**Fig. 4**). LEDs of one wavelength for the illumination of tissue culture plates may be mounted on the CPU heat sink. For example, we used a CPU heat sink with a mounting surface area of 60 mm \times 70 mm. This heat sink accommodates an array consisting of nine LEDs, each 20 mm in diameter. LEDs are mounted on the heat sink with a thermal adhesive tape. All LEDs are connected by a parallel circuit assembly of three rows, each containing three LEDs. LEDs may be connected to a standard power supply. However, to provide regulation of light flux, the power supply should have adjustable voltage and current. The light power should be measured by the optical power meter. The pulse regimen of the illumination can be provided by a pulse-pause timer serially connected between the LED and the power supply. With the pulse-pause timer, ON and OFF steps can be easily adjusted from 1 s to several hours. A support stand with a rod and a wire support with a clamp can be used to hold the LED array above the culture plate to provide even illumination of wells. Light flux power levels used for activation of optogenetic systems are low enough to avoid the need for special measures directed to LED cooling. The passive dissipation of heat provided by the CPU heat sink is sufficient for the vast majority of experiments. If overheating occurs, a fan may be added to the heat sink to increase heat dissipation.

Microscopy setup Epifluorescence microscopy can be performed with an inverted microscope. A 200-W metal-halide arc lamp can be used for both imaging and activation through the objective, using appropriate filter sets. If activation through the objective is used, check the cutoff wavelength of the IR filter, if installed in a light source to prevent sample overheating, and consider replacing or removing it if the filter does not transmit light within the 700- to 800-nm range. Measure the activation light power density at a back focal plane of the objective lens. Alternatively, LED arrays may be used as a source of activation light. For this, install the arrays instead of the transmitted light source to illuminate the sample from the top, and measure the light intensity at the sample plane. Perform image acquisition using a 40× or 60× objective lens and a sCMOS camera. Do not use the automatic autofocus systems, which exploit NIR light for measurements of the distance to the sample; check the technical specification of the microscope for details.

PROTOCOL

PROCEDURE

Culture of HeLa cells ● TIMING 1 h, afternoon of day 1

1| Culture a sufficient amount of healthy HeLa cells in DMEM medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin to ensure the appropriate transfection efficiency.

▲ **CRITICAL STEP** Deteriorated culture is often the cause of excessive toxic effects of the transfection reagent or other undesired effects. Consistency of the subculture schedule is a key to ensuring reproducible results.

2| Dissociate the cells using 1× trypsin–EDTA solution when subconfluent and seed the cells in a 24-well plate to obtain ~80% confluency after overnight cultivation (~ 1.5×10^5 cells per well). Prepare wells in triplicate for each experimental condition (NIR light, blue light and dark) and for controls. Typically, for transcription activation, at least nontransfected cells and cells transfected with reporter vector and mock plasmid (instead of the light-controlled construct) are used as controls. For estimation of the absolute expression level, cells may be transfected with the transcription activator without a light-sensing component such as Gal4-VP16. Choose additional controls specific to your study, if needed.

Transfection procedure ● TIMING 2 h, day 2

3| To prepare the DNA mix for transcriptional activation and bioluminescence assay, follow option A, and for tridirectional subcellular targeting, follow option B (Table 1).

(A) DNA mix for transcriptional activation and bioluminescence assay

- (i) Prepare 200 ng of the following plasmid DNA mix (all the reagent amounts at this step are indicated per well of a 24-well plate):

Tube	Plasmid	Amount
T2A	pQP-T2A	150 ng
	pFR-Luc	50 ng
GAVPO	pGAVPO	100 ng
	pFR-Luc	100 ng ^a
Control T2A	'Blank' plasmid	150 ng
	pFR-Luc	50 ng
Control GAVPO	'Blank' plasmid	100 ng
	pFR-Luc	100 ng ^a

^aPlasmid ratio according to ref. 3.

▲ **CRITICAL STEP** All mixes should be prepared in a volume sufficient for transfection of cells for three illumination conditions: 740 nm, 460 nm and in the dark, all wells in triplicate.

▲ **CRITICAL STEP** To avoid toxic effects after transfection, use endotoxin-free quality plasmid DNA purification kits (Reagent Setup).

- (ii) Increase the volume to 60 µl using DNA-condensation buffer. Add 1.6 µl of enhancer and mix by vortexing for 1 s. Incubate at room temperature for 2–5 min.

(B) DNA for tridirectional subcellular targeting

- (i) Prepare 200 ng of the pQP-iRIS plasmid DNA (all the reagent amounts at this step are indicated per well of a 24-well plate).

▲ **CRITICAL STEP** To avoid toxic effects after transfection, use endotoxin-free quality plasmid DNA purification kits (Reagent Setup).

- (ii) Increase the volume to 60 µl using DNA-condensation buffer. Add 1.6 µl of enhancer and mix by vortexing for 1 s. Incubate at room temperature for 2–5 min.

4| Add 5 µl of Effectene transfection reagent to the DNA-enhancer mixture. Mix the samples by vortexing for 10 s. Incubate the samples for 5 min at room temperature for transfection-complex formation.

5| Add DMEM medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin to the tube containing the transfection complexes to a final volume of 400 µl and mix by pipetting. Aspirate the medium from the 24-well plates, wash once with warm PBS buffer and add the medium with transfection mixes to the cells. Gently shake the plate to ensure uniform distribution of the transfection complexes. Incubate the plate for 6 h under normal growth conditions.

6| Aspirate the medium containing the transfection complexes. Wash the cells once with 1–2 ml of prewarmed PBS buffer. Add 0.5 ml of fresh DMEM medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin, and also supplemented with 25 μ M BV (for protocol setup without exogenous BV addition, see **Box 1**). Place the cells back into the incubator for another 42 h when performing the transcription activation and bioluminescence assay (Step 7A), or for 18 h when performing the tridirectional subcellular localization assay (Step 7B).

▲ **CRITICAL STEP** Although the Effectene transfection reagent may be kept on cells longer than 6 h for most applications, it is not recommended to add BV-containing media to the cells simultaneously with the transfection complexes, as this may lead to inappropriate BV uptake or even cause cytotoxicity.

Illumination and analysis of results

7| Depending on the experimental setup and plasmids used in Step 3, use option A for bioluminescence assay or option B for tridirectional subcellular targeting.

(A) Transcription activation and bioluminescence assay ● **TIMING 3.5 h, days 3–5**

- (i) Turn on the 740-nm (alternating cycles of 30 s of light and 180 s of dark) and 460-nm (continuous) illumination (recommended intensity: 1 mW cm⁻²) in separate cell culture CO₂ incubators or well isolated from one another. Samples transfected with 'T2A' and 'GAVPO' mixes (see Step 3A) should be illuminated with both 740-nm and 460-nm light separately, so that each illumination condition contains both optogenetic systems for comparison. Keep the dark samples (again both 'T2A' and 'GAVPO') in a separate CO₂ incubator or securely covered with black material or aluminum foil. Control plates can be placed into either an illuminated or dark incubator, as no phototoxicity is expected at the indicated light intensities. Illuminate the cells under normal growth conditions 42 h after the removal of Effectene transfection reagent (Step 6).

▲ **CRITICAL STEP** When arranging the illumination, check for overheating of LED array heat sinks. Monitor the temperature in the CO₂ incubator and water condensation on the plate covers due to excessive warming. If overheating occurs, improve the ventilation (add more space between the arrays and CO₂ incubator surfaces) or reduce light intensity (740-nm light intensity, as measured on a plate surface, may be reduced to 0.2 mW cm⁻² without a noticeable change in transcription activation levels). In addition, separate incubators may be used for NIR and blue light. Pulse illumination regimens help to avoid the overheating.
- (ii) Wash the cells with 1 ml of PBS. Add 100 μ l of freshly prepared ice-cold lysis buffer (see the Reagent Setup section) to each well.

! **CAUTION** Work under a hood while using appropriate protection to avoid PMSF inhalation or contact with skin.
- (iii) Incubate the cells on a gently agitating platform at 4 °C for 30 min to lyse the cells.
- (iv) Transfer 10 μ l of cell lysate from each well to a 96-well half-area nontransparent plate.
- (v) Add 20 μ l of Bioluminescence assay reagent to each well, using a multichannel pipette or a luminometer injector. Measure bioluminescence using a luminometer.

? TROUBLESHOOTING

(B) Tridirectional subcellular targeting in single cells ● **TIMING 6 h, days 3–5**

- (i) Incubate the cells in the dark for 18 h after the medium change after the transfection (Step 6). Wash the cells with 1 ml of PBS and dissociate the cells using 1 \times trypsin–EDTA solution.
- (ii) Seed the cells transfected with pQP-iRIS from Step 6 (4×10^5 to 8×10^5 cells per vessel) in glass-bottom tissue culture dishes (CELLview or similar, see the EQUIPMENT section) or coverslips precoated with poly-l-lysine (Reagent Setup) for better attachment. Incubate the cells under normal growth conditions for 3–24 h to allow them to attach.

▲ **CRITICAL STEP** The incubation step is to ensure a low-density culture of healthy attached cells appropriate for imaging. The duration of incubation depends on the particular cell type and cell culture vessel used, and can also be affected by the expression level of the proteins to be imaged. During the incubation, avoid intensive illumination by avoiding routinely examining the cells under the microscope.
- (iii) Gently aspirate the medium and wash the cells with 3 ml of warm PBS. Be careful if you use nonadherent cells instead of HeLa cells. When using glass coverslips in Step 7B(ii), mount the coverslips into a cell chamber for microscopy according to the chamber manufacturer's recommendations. Ensure the absence of leakage. Add 37 °C live cell imaging solution, 1–2 ml to the coverglass chamber or 2–3 ml to the glass-bottom tissue culture dish.

▲ **CRITICAL STEP** The maximum incubation time for incubating cells in live cell imaging solution without altering the cell physiology is limited to 2–4 h, depending on cell culture and the illumination scheme. Incubation for too long in live cell imaging solution followed by an imaging session may influence cell viability. Therefore, it is recommended to prepare a single dish before each imaging session, avoiding the simultaneous preparation of dish series.

PROTOCOL

▲ **CRITICAL STEP** Cells may be imaged at a different confluence or in a different medium, if this is required by the specific protein of interest or experimental design.

- (iv) Place the glass-bottom dish or cover glass chamber into the stage holder. Ensure an even temperature in the live cell imaging chamber, to eliminate thermal drift during the time lapse.
- (v) Determine the appropriate imaging parameters, allowing sufficient signal-to-noise ratio for the 'red' mCherry channel (excitation: 560/25 nm; emission: 607/36 nm). Check for proper target protein localization at the initial stage in the dark and acquire an image.

▲ **CRITICAL STEP** At this point, exposure to blue excitation light in the 'green' channel (excitation: 485/20 nm; emission: 525/30 nm) should be avoided, to prevent unwanted activation of the LOV-based component of the iRIS. Focusing and target protein observation should be performed using mCherry fluorescence.

▲ **CRITICAL STEP** During the time lapse, the focal plane may be lost due to thermal drift (Step 7B(iv)) or the cells' natural motility. Therefore, z-stack acquisition is recommended to ensure appropriate focusing during time-lapse imaging.

▲ **CRITICAL STEP** Protocols for epifluorescence imaging are microscope-dependent, and here we provide general guidelines for image acquisition. All of the acquisition steps are described for an Olympus IX83 microscope; however, similar functions should be readily available on most inverted epifluorescence microscopes. Please consider the manufacturer's instructions for your particular instrument and read the Equipment Setup section.

- (vi) Determine an appropriate frame rate for the analysis of the target protein relocalization kinetics. We recommend starting from 2 frames per min for the activation of the iRIS (both blue and NIR) and 1 frame per 4 min for the recovery.

▲ **CRITICAL STEP** Practically, the target protein relocalization time depends on the fusion partner's physical properties, the protein diffusion rate in the cytoplasm and the nuclear transport rates (if active nuclear transport is involved). For best performance, pilot trials should be performed to find an optimal frame rate.

- (vii) Turn on the 460-nm illumination (recommended intensity: 1 mW cm⁻²) (see the Equipment Setup section for the description of the illumination setup through the objective lens or using a LED array; also check the recommendations for correct light intensity measurements for both illumination options) and perform time-lapse imaging for relocalization from the cytoplasm to the nucleus.
- (viii) Keep the cells in the dark for 30 min in order to allow LOV-domain thermal relaxation. Check the reporter protein for cytoplasmic localization using the mCherry channel. Perform imaging in the mCherry channel during the incubation time in the dark, if needed.
- (ix) Adjust the frame rate for the analysis of the kinetics of target protein relocalization from the cytoplasm to the plasma membrane, turn on the 740-nm illumination (recommended intensity: 1 mW cm⁻²) and perform time-lapse imaging. Avoid exposure to blue light (excitation: 485/20 nm; emission: 525/30 nm) to prevent activation of the LOV-based component of iRIS.

▲ **CRITICAL STEP** Because the kinetics for the relocalization to the membrane may differ from the kinetics of relocalization to the nucleus, adjust the frame rate appropriately. The frame rate for the relocalization to the membrane may be between 2 and 12 frames per min.

- (x) Keep the cells in the dark for 30 min in order to allow BphP1 thermal relaxation and QPAS1 dissociation. Perform imaging in the mCherry channel during the incubation time in the dark, if needed. The relaxation process may be facilitated by exposure to 620-nm light. 1 mW cm⁻² of 620-nm illumination results in doubling of the relaxation rate.
- (xi) Export the images in an uncompressed format suitable for quantitative analysis. Ensure that the images were not automatically corrected in any way, leading to intensity distribution changes.

▲ **CRITICAL STEP** The procedure details are software dependent but may be performed by using almost any software for operating fluorescence microscopes or by using open-source software for image analysis, such as ImageJ^{43,44}.

- (xii) Subtract the background fluorescence from the captured images.
- (xiii) Make a stack from time points and align the images within the stack using rigid body registration or a similar algorithm to reduce misalignment due to cell motility. Choose an appropriate number of regions of interest (ROIs) in the cytoplasm and/or nucleus.
- (xiv) Perform analysis of the captured images. Analyze the kinetics of fluorescence intensity in the respective compartments. For each time point, normalize the intensity level to the average fluorescence for the whole cell, to account for photobleaching due to exposure during time-lapse imaging.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution	
7A(v)	High background level of transcription in the dark	QPAS1 or PpsR2 binding by BphP1 apoprotein due to insufficient endogenous BV concentration	Consider one of the following options: <ul style="list-style-type: none"> • Add BV to the cell culture medium or increase its concentration. Most cell lines are tolerant to BV concentrations up to 25 μM • Use a cell line stably expressing BphP1 (Box 1) 	
		Expression level of transcription activation system components is too high	The easiest solution is to reduce the amount of plasmids used in the transfection procedure (Step 3), either by using mock plasmid as a substitute, or by adjusting the activator/reporter plasmid ratio. Alternatively, the following modifications may be performed: <ul style="list-style-type: none"> • Use weaker promoters (may depend on the particular cell line), e.g., CMVd1 (Promega) • Use an internal ribosome entry site (IRES) of different efficiency, or use a self-cleaving peptide sequence to adjust the expression levels of system components^{46,47}. • Try different transfection reagents or alternative gene-delivery options that are suitable for the particular experiment 	
7B(xiv)	High background level of plasma membrane-bound iRIS in the dark or under 460-nm light (Supplementary Fig. 5a)	QPAS1 binding by BphP1 apoprotein due to low endogenous BV concentration, or iRIS expression is too high	See troubleshooting advice for Step 7A(v). <ul style="list-style-type: none"> • Reduction of the expression level of the iRIS may help. Try to reduce the intensity of 460-nm light, and reduce exposure to ambient illumination 	
		Shifted cytoplasm–nucleus equilibrium of iRIS localization (e.g., high background in the nucleus under 740-nm light) (Supplementary Fig. 5b)	Nuclear transport machinery performance may differ in certain model organisms or cell lines ⁴⁸	The system may be modified using nuclear localization or nuclear exclusion signals of variable strength ^{28,38–40} . Avoid exposure to blue light or ambient illumination
		Low mCherry fluorescence signal from the iRIS after time-course imaging	Photobleaching	Reduce the exposure to excitation light (excitation: 485/20 nm or excitation: 560/25 nm) by using neutral-density filters. Alternatively, choose a lower frame rate for image acquisition, or capture fewer z-sections for each time point

● TIMING

Steps 1 and 2, culture of HeLa cells: 1 h (day 1)

Steps 3–6, transfection procedure: 2 h (day 2)

Step 7A(i), transcription activation: 1.5 h (days 3–5)

Step 7A(ii–v), bioluminescence assay: 2 h (days 3–5)

Step 7B(i,ii), preparation of cells for tridirectional subcellular targeting: 2 h (days 3–5)

Step 7B(iii–x) tridirectional subcellular targeting in single cells: 3 h (days 3–5)

Step 7B(xi–xiv) export of results and data analysis: 1 h (days 3–5)

Box 1, generation of a cell line stably expressing the BphP1 component of the optogenetic system: 6 weeks

Box 2, determination of the kinetics of light-induced transcription activation using a TetR-responsive system: 12 h, over 6 days

Box 3, light-activation of gene expression in mice: 10 h, over 5 days

ANTICIPATED RESULTS

Gene transcription activation (Step 7A)

This PROCEDURE results in the expression of a target gene in the 740-nm illuminated cells, demonstrating a rather low background activation in the dark. Typically, the signal level in the illuminated samples differs from that of those kept in the dark by a factor of 35–40 for both the bioluminescence and the SEAP reporter assays (Fig. 5, Supplementary Fig. 2). The absolute signal level is comparable to that obtained by activation with the nonconditional transcription activators based on the VP16 transactivation domain. Importantly, the increase in target protein expression level in cells expressing the BphP1-based system following exposure to 460-nm light is negligible (Fig. 5), which makes the BphP1–QPAS1 pair an optogenetic system of choice for spectral multiplexing with numerous blue-light-activatable tools.

A 35-fold light-to-dark contrast of SEAP production can be achieved using mammalian cells stably expressing BphP1, without any supply of exogenous BV (Boxes 1–3).

The SEAP accumulation curve typically has a sigmoid shape and reaches its plateau after 36 h (Supplementary Fig. 2b). A 32-fold increase in bioluminescence, caused by light-controlled transcription activation, may be achieved in the s.c. injected cells stably expressing BphP1 when illuminated in living mice (Supplementary Fig. 3)

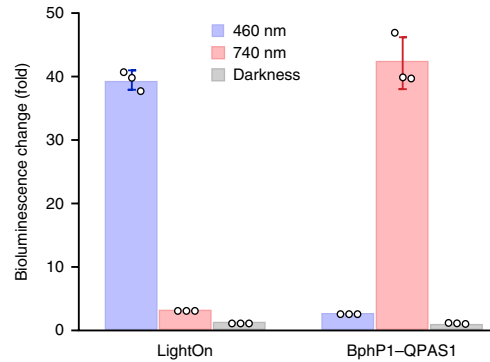


Figure 5 | Spectral compatibility of the BphP1–QPAS1 and blue-light-activatable transcription systems. Light-to-dark ratios of luciferase reporter were detected in HeLa cells transfected with either LightOn or BphP1–QPAS1 transcription activation systems. Samples were illuminated either with continuous 460-nm light of 1 mW cm⁻² or 740-nm pulsing light (30 s ON, 180 s OFF) of 1 mW cm⁻². The results of individual experiments are shown as dots; bars represent the respective mean values; error bars are s.e.m.; *n* = 3. Adapted from ref. 5, Nature Publishing Group.

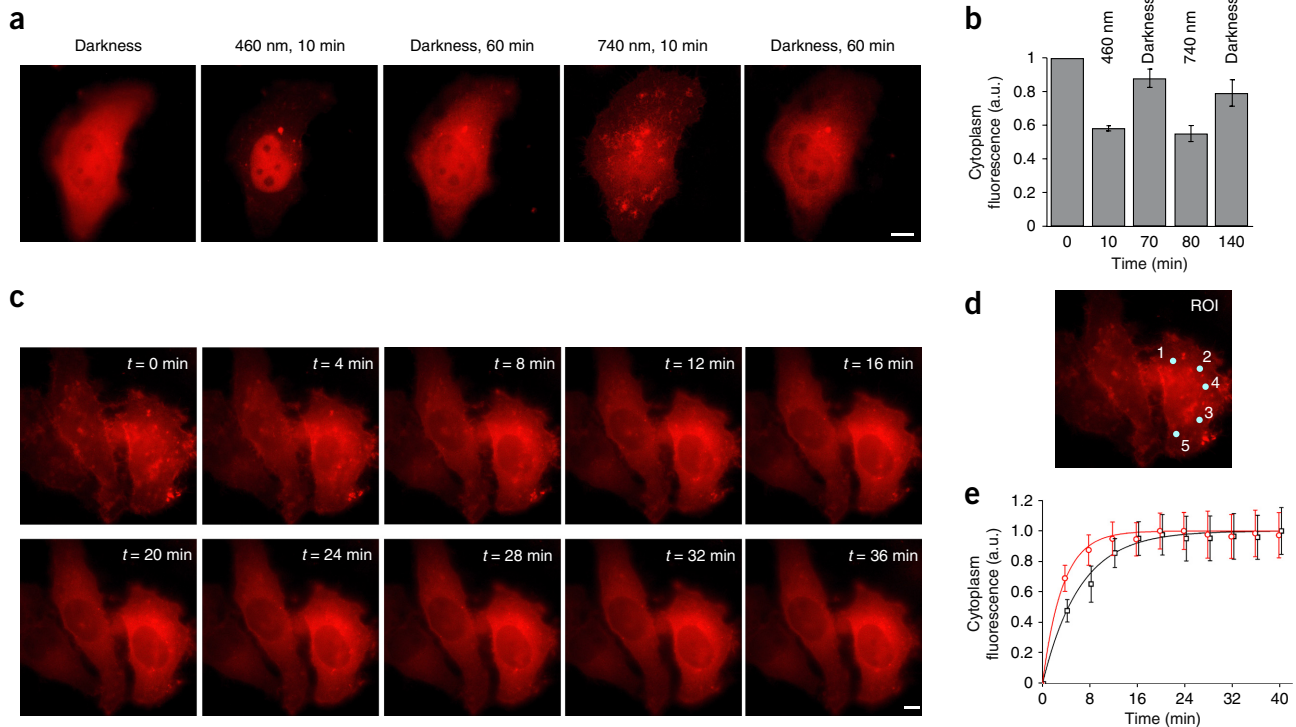


Figure 6 | Two-color light-controlled tridirectional intracellular protein targeting. (a) Sequential targeting of iRIS from the cytoplasm to the nucleus and to the plasma membrane in a single cell after the indicated illumination periods: after 10 min of 460 nm of 1 mW cm⁻² illumination, after 60 min in darkness, after 10 min of 740 nm of 1 mW cm⁻² illumination and after 60 min in darkness again. All images were acquired at 37 °C using an epifluorescence microscope. (b) mCherry fluorescence of iRIS in cells illuminated as shown in a. Error bars represent s.e.m., *n* = 10 cells for each bar. (c) mCherry-tagged protein dissociation from the plasma membrane in the dark due to BphP1 thermal relaxation (time-lapse imaging). (d) Example of region of interest (ROI) placement for quantification of cytoplasmic fluorescence. (e) mCherry fluorescence of iRIS measured in the cytoplasm of HeLa cells pre-illuminated with 740-nm light and subsequently kept in the dark (black line) or under 620-nm illumination (red line). The 1-min pulses of 620-nm light (1 mW cm⁻²) were alternated with 1 min of darkness. Epifluorescence images of cells were acquired every 4 min. The observed dissociation half-time was ~4.4 min in the dark and ~2.3 min under 620-nm illumination. Fitting of the exponential curve was performed using OriginPro 8.6. Error bars represent s.e.m., *n* = 10 cells similar to those shown in c for each time point. Scale bars, 10 μm (apply to d as well). a,b,e adapted from ref. 5, Nature Publishing Group.

Tridirectional targeting in single cells (Step 7B)

The iRIS tool enables the light-controllable translocation of a target protein between the cytoplasm, the nucleus and the plasma membrane in single cells (**Fig. 6a**, **Supplementary Figs. 4 and 5**). The relocalization of the target protein fused with a fluorescent tag to the nucleus is observed in response to 460-nm light. The fluorescence level in the nucleus reaches its maximum after ~20 min of blue-light illumination. In the dark, the target protein returns to the cytoplasm, and it can subsequently be recruited to the plasma membrane with 740-nm light. After translocation to the plasma membrane, fluorescence of the target protein's mCherry tag can be visualized by creating an intensity profile (**Supplementary Fig. 4**), in which the cell perimeter and the filopodia appear as sharp peaks. Quantification of fluorescence at the plasma membrane can be problematic; therefore, the fluorescence decrease in the cytoplasm is recommended as a quantity to measure for characterization of the kinetics and degree of protein relocalization. When using an epifluorescence microscope without optical sectioning, the typical decrease of cytoplasmic fluorescence due to the targeting to the nucleus or to the plasma membrane is 40% (**Fig. 6b**). The light-induced relocalization of the target protein from the cytoplasm is rather quick (1–5 min) and fully reversible (**Fig. 6b–e**). A half-life for the thermal relaxation of the BphP1–QPAS1 system, resulting in returning the protein to the cytoplasm, is ~4.4 min in the dark; this can be reduced to ~2.3 min with 620-nm illumination (**Fig. 6e**).

Further information on experimental design is available in the **Life Sciences Reporting Summary**.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS T.A.R. and A.A.K. performed the experiments. V.V.V. directed and planned the project, and, together with T.A.R. and A.A.K., designed the experiments, analyzed the data and wrote the manuscript.

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► Experimental design

1. Sample size

Describe how sample size was determined.

For bioluminescence and SEAP analyses all cells from a well of the 24-well or 12-well plate were analyzed. All experiments were repeated 3 times. (n = 3).
For quantification of mCherry fluorescence signal upon near-infrared and blue light illumination in microscopy 10 cells were analyzed (n = 10)
Both experimental and control mice groups contained 3 animals (n = 3)

2. Data exclusions

Describe any data exclusions.

No sample was excluded from data analysis

3. Replication

Describe whether the experimental findings were reliably reproduced.

The experiments were reliably reproduced. All experiments were performed at least 3 times.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Randomization method was not applied

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

No blinding was employed.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- | n/a | Confirmed |
|-------------------------------------|---|
| <input type="checkbox"/> | <input checked="" type="checkbox"/> The <u>exact sample size</u> (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> A statement indicating how many times each experiment was replicated |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> The test results (e.g. <i>P</i> values) given as exact values whenever possible and with confidence intervals noted |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> A clear description of statistics including <u>central tendency</u> (e.g. median, mean) and <u>variation</u> (e.g. standard deviation, interquartile range) |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> Clearly defined error bars |

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

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7. Software

Describe the software used to analyze the data in this study.

Data were analyzed using OriginPro 8, and images were analyzed using ImageJ 1.50i software. No custom algorithms were used

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

► Materials and reagents

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8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

The nucleotide and amino acid sequences of the proteins used in the manuscript are already available in public repositories; All major plasmids, their maps and their sequences, generated in this manuscript, will be available via Addgene nonprofit public depository.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

HeLa cell line were purchased from ATCC (CCL2)

b. Describe the method of cell line authentication used.

Cell lines were purchased from ATCC and were not additionally authenticated

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell line was not tested for mycoplasma contamination

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

No commonly misidentified cell lines were used

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

The FVB 2-3 month old female mice (NCI/NIH, USA) of 20-25 g body weight were used for in vivo light-induced transcription activation

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12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants