



(51) International Patent Classification:

C07K 14/555 (2006.01) C07K 14/415 (2006.01)
 C07K 16/00 (2006.01) C07K 14/435 (2006.01)
 C07K 16/28 (2006.01) C07K 14/47 (2006.01)
 C07K 7/08 (2006.01) C07K 14/475 (2006.01)
 C07K 14/00 (2006.01) C12N 9/00 (2006.01)

(21) International Application Number:

PCT/US2018/063447

(22) International Filing Date:

30 November 2018 (30.11.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/593,750 01 December 2017 (01.12.2017) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: LIGHT-RESPONSIVE FUSION PROTEINS FOR CONTROLLING BINDING TO TARGETS

(57) Abstract: Provided herein is an isolated fusion protein comprising a light-responsive domain and a heterologous peptide component. Exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity of the fusion protein and the activity is a binding activity selected from an *in vitro* binding activity, an *in vivo* extracellular binding activity and an *in vivo* intracellular binding activity. Also provided are methods of using and identifying the fusion proteins.

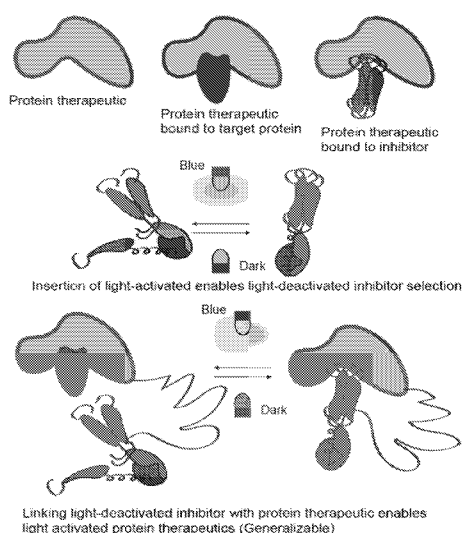


FIG. 3

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *of inventorship (Rule 4.17(iv))*

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

LIGHT-RESPONSIVE FUSION PROTEINS FOR CONTROLLING BINDING TO TARGETS

RELATED APPLICATION(S)

[0001] This application claims the benefit of U.S. Provisional Application No. 62/593,750, filed on December 1, 2017.

[0002] The entire teachings of the above application are incorporated herein by reference.

GOVERNMENT SUPPORT

[0003] This invention was made with government support under Grant No. EB024247 and F32GM128304-01 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0004] Developing stringent control over the activity of biological therapies could potentially unlock a new level of therapeutic potential for already-potent treatments. Unlike traditional therapeutic agents, such controlled therapies could integrate user-defined information to determine activity. For example, a user could define where and when therapeutic behaviors are executed, allowing biological therapies to overcome dosing limitations of their inert pharmaceutical counterparts while at the same time limiting off-target side effects. In theory, all drugs could be maximally dosed (and thereby maximally effective) if they could be selectively delivered only at the right location and at the right time.

[0005] Manufactured proteins are a ubiquitous part of the 21st century economy. These engineered molecules play crucial roles in medicine, manufacturing, biotechnology, energy production, agriculture, and even cosmetics and beauty products. Indeed, beauty companies now produce protein ingredients by the ton and protein-based biopharmaceuticals make up nine of the 10 top-earning drugs. However, while proteins allow for manipulation of complex chemical and biological systems, an ability to control protein activity is lacking. This means that, for instance, once a given skin care product has been applied or a biopharmaceutical has been administered, it will be active everywhere rather than only where it is needed. In almost every context where manufactured proteins are used, this leads to a reduction in efficacy and,

especially in health and cosmetic applications, a reduction in safety, thereby limiting their potential market.

SUMMARY

[0006] There is a need for a platform technology that enables caregivers and/or patients to control therapeutic activity of biological therapies. Developing technology that allows user-defined control over the activity of engineered proteins unlocks a new level of both therapeutic and commercial potential for already-potent products. Unlike traditional engineered proteins, such controlled molecules could integrate user-defined information to determine activity. For example, a user could define where and when therapeutic behaviors are executed, allowing light-switchable biopharmaceuticals to overcome dosing limitations of their inert pharmaceutical counterparts while at the same time limiting off-target side effects. In theory, all drugs could be maximally dosed (and thereby maximally effective) if they could be selectively delivered to the right location at the right time.

[0007] Beyond therapeutic applications, control over protein binding has further potential applications in biomanufacturing. For example, light-switchable binding scaffolds can improve industrial protein purification via single-step purification columns that reduce the time and money required to obtain a pure product.

[0008] Accordingly, provided herein are light-responsive fusion proteins and methods of identifying and using light-responsive fusion proteins, for example, to treat a subject in need thereof.

[0009] A first embodiment is an isolated fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity of the fusion protein and the activity is a binding activity selected from an *in vitro* binding activity and an *in vivo* extracellular binding activity.

[0010] A second embodiment is a method of altering an activity of a fusion protein comprising a light-responsive domain and a heterologous peptide component. The method comprises exposing the fusion protein to light that induces a conformational change in the fusion protein, thereby altering an activity of the fusion protein. The conformational change alters an activity of the fusion protein and the activity is a binding activity selected from an *in vitro* binding activity and an *in vivo* extracellular binding activity.

[0011] A third embodiment is a method for treating a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a therapeutic fusion protein comprising a light-responsive domain and a therapeutic protein or peptide, and delivering a sufficient amount of light to at least a portion of the subject to induce the conformational change, thereby altering the therapeutic efficacy of the fusion protein exposed to the light and treating the subject. Exposure of the fusion protein to light induces a conformational change in the fusion protein that alters its therapeutic efficacy.

[0012] A fourth embodiment is a method of identifying a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change that alters its ability to bind a target protein. The method comprises providing one or more phages displaying a fusion protein intended to bind a target protein. The fusion protein comprises a light-responsive domain and a heterologous peptide component. The immobilized target protein is exposed to the one or more phages in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein, and one or more phages is eluted in the absence of light, thereby eluting one or more phages displaying a fusion protein that binds the target protein upon exposure to light and dissociates from the target protein in the absence of light.

Alternatively, immobilized target protein is exposed to the one or more phages in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein, and one or more phages is eluted in the presence of light, thereby eluting one or more phages displaying a fusion protein that binds the target protein in the absence of light and dissociates from the target protein upon exposure to light. A fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the fusion protein undergoes a light-dependent conformational change that alters its ability to bind the target protein, is thereby identified.

[0013] A fifth embodiment is a method of identifying a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change that alters its ability to bind a target protein. The method comprises providing one or more cells expressing a fusion protein intended to bind a target protein. The fusion protein comprises a light-responsive domain and a heterologous peptide component. One or more cells is sorted using fluorescence-activated cell sorting (FACS). Immobilized target protein is exposed to the one or more cells in the presence of light under conditions sufficient to induce binding of the target protein and the

fusion protein, and one or more cells is eluted in the absence of light, thereby eluting one or more cells expressing a fusion protein that binds the target protein upon exposure to light and dissociates from the target protein in the absence of light. Alternatively, immobilized target protein is exposed to the one or more cells in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein, and one or more cells is eluted in the presence of light, thereby eluting one or more cells expressing a fusion protein that binds the target protein in the absence of light and dissociates from the target protein upon exposure to light. A fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the fusion protein undergoes a photodependent conformational change that alters its ability to bind the target protein, is thereby identified.

[0014] A sixth embodiment is a method of purifying a target protein from a cell lysate. The method comprises providing a substrate comprising an immobilized fusion protein intended to bind a target protein, the fusion protein comprising a light-responsive domain and a heterologous peptide component. Exposure of the fusion protein to light induces a conformational change that alters its ability to bind the target protein. The method comprises exposing the substrate to the cell lysate in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and eluting the target protein in the absence of light, wherein the fusion protein binds the target protein upon exposure to light and dissociates from the target protein in the absence of light, thereby purifying the target protein. Alternatively, the method comprises exposing the substrate to the cell lysate in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and eluting the target protein in the presence of light, wherein the fusion protein binds the target protein in the absence of light and dissociates from the target protein upon exposure to light, thereby purifying the target protein.

[0015] A seventh embodiment is an isolated fusion protein comprising a light-responsive domain, a heterologous peptide component, and a cell-penetrating peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity (e.g., a binding activity) of the fusion protein.

[0016] An eighth embodiment is a method of delivering a fusion protein to a cell, comprising contacting the cell extracellularly with an isolated fusion protein comprising a light-responsive domain, a heterologous peptide component, and a cell-penetrating peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity (e.g., a binding activity) of the fusion protein,

wherein the fusion protein is delivered intracellularly. In some embodiments, the heterologous peptide component also acts as a cell-penetrating peptide. For example, an antimicrobial peptide can serve as both the heterologous peptide component and cell penetrating peptide (e.g., an antimicrobial peptide can insert into a cell membrane and act as an antimicrobial agent, wherein delivery of the antimicrobial peptide is controlled with light).

[0017] An ninth embodiment is a chimeric antigen receptor (CAR), comprising an extracellular antigen-binding domain, a transmembrane domain and an intracellular signaling domain, wherein the extracellular antigen-binding domain comprises a light-responsive domain and a heterologous peptide component, wherein exposure of the CAR to light induces a conformational change in the extra-cellular antigen-binding domain that alters an activity (e.g., an *in vivo* extracellular binding activity) of the extra-cellular antigen-binding domain.

[0018] A tenth embodiment is a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the heterologous peptide component is selected from the group consisting of a nanobody, a monobody, an antibody, a growth factor, and a designed ankyrin repeat protein (DARPin), wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity (e.g., a binding activity) of the fusion protein.

[0019] An eleventh embodiment is a method of delivering a fusion protein to a cell, comprising contacting the cell with a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the heterologous peptide component is selected from the group consisting of a nanobody, a monobody, an antibody, a growth factor, and a designed ankyrin repeat protein (DARPin), wherein the fusion protein is delivered intracellularly.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0021] The foregoing will be apparent from the following more particular description of example embodiments.

[0022] FIG. 1 is a depiction of a yeast 2 hybrid screening of light-switchable protein binding.

[0023] FIG. 2 is a depiction of directed evolution of light-switchable proteins for better kinetics/activity.

[0024] FIG. 3 is a depiction of a generalizable platform for light-activated therapeutics.

[0025] FIG. 4A is a model of photoswitchable mCherry (red/grey) fluorescence through AsLov2 (blue) structural change.

[0026] FIG. 4B is a depiction of the workflow for preliminary studies used to validate library generation using transposon assisted gene insertion technology (TAGIT).

[0027] FIG. 5A is a graph showing that an AsLOV2 domain inserted into the ERK2-specific DARPin E40 retains its ability to undergo blue light-induced conformational shifts.

[0028] FIG. 5B is a graph showing that insertion of the AsLOV2 domain from FIG. 5A into DARPin E40 does not dramatically impact the dark recovery time of the AsLOV2 domain.

[0029] FIG. 6A is a graph showing that an AsLOV2 domain inserted into the ppErk2-specific DARPin pE59 retains its ability to undergo blue light-induced conformational shifts.

[0030] FIG. 6B is a graph showing that an AsLOV2 domain inserted into DARPin pE59 retains its ability to undergo blue light-induced conformational shifts. The AsLOV2-containing pE59 DARPin construct of FIG. 6A is different from the AsLOV2-containing pE59 DARPin construct of FIG. 6B.

[0031] FIGs. 7A-7B are microscopic fluorescence images showing a mammalian cell assay set up to image ligand binding. FIG. 7A shows mCherry is localized to the membrane via a CAAX tag. FIG. 7B shows that a fluorescent nanobody is expressed in the cytoplasm.

[0032] FIGs. 8A-8F show examples of light-responsive nanobodies against mCherry. FIGs. 8A-8B show light-induced binding of a light-responsive nanobody to membrane-localized mCherry. The light-responsive nanobody was constructed with AsLOV2 fused between amino acids A74 and K75, and was fused to iRFP. Images show nanobody localization before and after blue light (in FIGs. 8A and 8B, respectively), as well as quantification of cytoplasmic nanobody levels during binding and unbinding (FIG. 8C). FIGs. 8D-8F show light-induced dissociation of a similarly-constructed light-responsive nanobody with AsLOV2 inserted between amino acids G15 and G16, as well as quantification of cytoplasmic nanobody levels during binding and unbinding (FIG. 8F).

[0033] FIGs. 9A-9F show examples of light-responsive DARPins against eGFP. FIGs. 9A-9D shows that a cycle of application and removal of blue light induces light-responsive DARPin binding and unbinding from the cell membrane. Light exposure is indicated by a

blue border to each image. FIG. 9E shows the membrane localization of the DARPin's target, GFP, obtained by imaging with GFP excitation/emission settings. FIG. 9F shows a graph of the kinetics of binding/unbinding obtained by measuring cytoplasmic light-responsive DARPin fluorescent intensity over time.

[0034] FIGs. 10A-10B show an example of a light-responsive monobody against SH2 domain. FIG. 10A shows binding of the light-responsive monobody to SH2 domain fused to mCherry and localized to the membrane via CAAX tag. FIG. 10B shows light-induced dissociation of the light-responsive monobody from the binding target on the membrane. Blue light exposure is indicated by a blue border around the image.

[0035] FIGs. 11A-11F show results of an *in vitro* binding assay on agarose beads. FIGs. 11A-11C show that a mCherry-specific nanobody with AsLOV2 introduced between amino acids A74 and K75 exhibits light-induced binding *in vitro*. Images show mCherry fluorescence in the absence (FIG. 11A) and presence (FIG. 11B) of light, with quantification shown in a graph in FIG. 11C. FIGs. 11D-11E show that light-induced dissociation was obtained for the mCherry nanobody with AsLOV2 introduced between G15 and G16, with quantification shown in a graph in FIG. 11F.

[0036] FIG. 12 shows a set-up for size exclusion chromatography (SEC) with the column modified by wrapping with blue LEDs.

[0037] FIGs. 13A-13B show graphs of size exclusion chromatography results demonstrating light-induced binding of light-responsive nanobody (Opto-NB) to mCherry (target) in FIG. 13A and light-induced dissociation of light-responsive nanobody (Opto-NB) to mCherry (target) in FIG. 13B.

[0038] FIG. 14 shows representative kinetic traces from OCTET measurements for a nanobody against mCherry. First trace represents binding of mCherry to the nanobody (kon) and second trace represents dissociation of mCherry from the nanobody (koff).

[0039] FIG. 15 shows a gel showing results of screening monobody-LOV fusion proteins for light-dependent binding to the c-Abl SH3 domain. An insertion of AsLOV2 at position 58 (SS58) shows increased monobody-LOV binding in the dark (presence of dark band) compared to light.

[0040] FIGs. 16A-16B illustrate light-assisted protein purification. FIG. 16A illustrates light-induced protein binding and dark-induced elution of protein of interest. FIG. 16B illustrates dark-induced protein binding and light-induced elution of protein of interest.

[0041] FIGs. 17A-17D show light control over Erk signaling. FIGs. 17A-17B show microscopic photographs of light-induced binding of nanobody-SOScat fusion to membrane localized mCherry controlling kinase translocation reporter and FIGs. 17C-17D show microscopic photographs of a light-induced dissociation of nanobody-SOScat fusion from membrane-localized mCherry controlling kinase translocation reporter. FIG. 17A and FIG. 17C show cells kept in the dark, whereas FIG. 17B and FIG 17D show cells illuminated with blue light.

[0042] FIGs. 18A-18C illustrate three ways of influencing cellular responses with light-responsive fusion proteins (also referred to herein as “opto-binders”): within the intracellular environment, if the light-responsive fusion protein is genetically expressed in the host cell (FIG. 18A); from the extracellular environment, if the light-responsive fusion protein is added to the extracellular medium and acts on a cell surface expressed receptor(FIG. 18B); or within the intracellular environment but without genetic encoding, if the light-responsive fusion protein is fused to a cell-penetrating peptide, enters the cell and interacts with its intracellular target (FIG. 18C). In addition, or alternatively, the heterologous peptide component and/or light-responsive domain in the fusion protein can also be modified to render the fusion protein more cell permeable (e.g., obviating the need for a cell-penetrating peptide).

[0043] FIG. 19 illustrates EGFR-specific light-responsive nanobodies useful for light-switchable EGFR inhibition. Image to the left of the arrow shows binding in the dark between a light-responsive nanobody and its EGFR target domain (domain III), leading to inhibition of EGF binding and EGFR signaling activity. Image to the right of the arrow shows light-induced conformational change in the light-responsive nanobody induces dissociation from EGFR, restoring the function of this growth factor pathway.

[0044] FIGs. 20A-20F show microscopic photographs of the experimental set-up for screening the effectiveness of Syn-Notch activity in dark and light conditions. FIGs. 20A-20C represent a positive control where the cells containing both constructs (FIG. 20A) are incubated with mCherry-coated agarose beads (FIG. 20B) leading to activation of SynNotch receptor further promoting BFP expression (FIG. 20C). FIGs. 20D-20F represent a negative control where the same cells as in FIG. 20A (FIG. 20D) are not incubated with the mCherry beads (FIG. 20E), therefore lacking the activation of SynNotch and subsequent expression of BFP (FIG. 20F).

DETAILED DESCRIPTION

[0045] A description of example embodiments follows.

[0046] A first embodiment is a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity of the fusion protein.

[0047] The term “fusion protein” refers to a synthetic, semi-synthetic or recombinant single protein molecule that comprises all or a portion of two or more different proteins and/or peptides. The fusion can be an *N*-terminal fusion (with respect to the heterologous peptide component), a *C*-terminal fusion (with respect to the heterologous peptide component) or an internal fusion (with respect to the light responsive domain and/or the heterologous peptide component). When the fusion protein is an internal fusion protein, the light responsive domain is typically inserted into the heterologous peptide component. *See*, for example, FIG. 2.

[0048] Fusion proteins of the invention can be produced recombinantly or synthetically, using routine methods and reagents that are well known in the art. For example, a fusion protein of the invention can be produced recombinantly in a suitable host cell (*e.g.*, bacteria, yeast, insect cells, mammalian cells) according to methods known in the art. *See, e.g.*, *Current Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., John Wiley & Sons, 1992; and *Molecular Cloning: a Laboratory Manual*, 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press. For example, a nucleic acid molecule comprising a nucleotide sequence encoding a fusion protein described herein can be introduced and expressed in suitable host cell (*e.g.*, *E. coli*), and the expressed fusion protein can be isolated/purified from the host cell (*e.g.*, in inclusion bodies) using routine methods and readily available reagents. For example, DNA fragments coding for different protein sequences (*e.g.*, a light-responsive domain, a heterologous peptide component) can be ligated together in-frame in accordance with conventional techniques. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of nucleic acid fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive nucleic acid fragments that can subsequently be annealed and re-amplified to generate a chimeric nucleic acid sequence (see Ausubel *et al.*, *Current Protocols in Molecular Biology*, 1992).

[0049] The fusion proteins described herein can include other amino acid sequences in addition to the amino acid sequences of the light-responsive domain and the heterologous peptide component. In some aspects, a fusion protein includes a linker amino acid sequence (*e.g.*, positioned between the light-responsive domain and the heterologous peptide component). A variety of linker amino acid sequences are known in the art and can be used in the fusion proteins described herein. In some embodiments, a linker sequence includes one or more amino acid residues selected from Gly, Ser, Ala, Val, Leu, Ile, Thr, His, Asp, Glu, Asn, Gln, Lys and Arg. In some embodiments, a linker sequence includes a polyglycine sequence (*e.g.*, a 6X glycine sequence).

[0050] In some aspects, the fusion protein is isolated. As used herein, “isolated” means substantially pure. For example, an isolated fusion protein makes up at least about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 97%, about 98%, about 99% or about 99.5% by weight of a mixture containing substances (*e.g.*, chemicals, proteins, peptides, other biological matter) other than the fusion protein.

[0051] “Light-responsive domain,” as used herein, refers to a peptide or protein that, upon exposure to at least one particular wavelength of light (more typically, a range of wavelengths of light), undergoes a conformational change which mediates, in turn, a conformational change in the fusion protein. Conformational changes include unfolding, tilting, rotating and multimerizing (*e.g.*, dimerizing, trimerizing), or a combination of any of the foregoing (*e.g.*, unfolding and multimerizing). Accordingly, in some aspects, the conformational change is an allosteric change, such as the allosteric change undergone by AsLOV2 upon exposure to blue light. In some aspects, the conformational change induces multimerization (*e.g.*, dimerization, trimerization) of the fusion protein.

[0052] Typically, the light-responsive domain is an optogenetic activator from the plants, fungi, or bacteria. Non-limiting examples of light responsive domains include light oxygen voltage (LOV) domains (*e.g.*, EL222, YtvA, aureochrome-1, AsLOV2), blue light-using flavin adenine dinucleotide (FAD) (BLUF) domains (*e.g.*, PixD, AppA, BLrP1, PAC, BlsA), cryptochrome domains (*e.g.*, Cry2), fluorescent protein domains (*e.g.*, Dendra, Dronpa, Kohinoor) and phytochromes (*e.g.*, PhyB, CPh1, BphP, Phy1, PixJ, Ac-NEO1). In some aspects, the light-responsive domain is a light oxygen voltage (LOV) domain, *e.g.*, AsLOV2, the LOV2 domain from *Avena sativa* Phototropin 1.

[0053] A light responsive domain, such as “light oxygen voltage 2 domain” or “LOV2 domain”, can be naturally occurring or non-naturally occurring (*e.g.*, engineered). For

example, the LOV domain can be isolated (*e.g.*, from a natural source), recombinant or synthetic. Examples of LOV domains that are suitable for use in the fusion proteins and methods described herein are known in the art and include variants of naturally occurring LOV domains (*e.g.*, variants having at least about 70%, about 75%, about 80%, about 85%, about 90, about 95%, about 96%, about 97%, about 98% or about 99% identity to a naturally occurring LOV domain), such as AsLOV2, the LOV2 domain from *Avena sativa* Phototropin 1. In some embodiments, a LOV domain is a polypeptide having the amino acid sequence of AsLOV2, the LOV2 domain from *Avena sativa* Phototropin 1, assigned UniProt Accession No. O49003 (SEQ ID NO:1), or a variant thereof having at least about 70% (*e.g.*, about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99%) identity to the amino acid sequence of SEQ ID NO:1.

[0054] “Heterologous peptide component,” as used herein, refers to a protein or peptide component that is different from the protein or peptide of the light-responsive domain (whether or not derived from the same organism as the peptide or protein of the light-responsive domain). Typically, however, the heterologous peptide component is not derived from the same organism as the peptide or protein of the light-responsive domain. Examples of heterologous peptide components include nanobodies, monobodies, antibodies, growth factors, designed ankyrin repeat proteins (DARPs) (*e.g.*, E40, pE59), antimicrobial peptides (*e.g.*, LL-37, IDR-1018, IDR-1019, pexiganan, Bac2A, W3), enzymes and therapeutic proteins or peptides.

[0055] A “therapeutic protein or peptide” is a heterologous peptide component useful as a therapeutic agent. Some therapeutic proteins or peptides can bind a cell surface target, such as a cell surface protein or receptor. In some embodiments, the therapeutic protein or peptide is a cytokine (*e.g.*, an interferon), peptide hormone (*e.g.*, insulin), growth factor, (*e.g.*, an epidermal growth factor, a fibroblast growth factor, and platelet-derived growth factor). Examples of growth factors which bind and activate a receptor tyrosine kinase include epidermal growth factor and fibroblast growth factor-7 (also known as keratinocyte growth factor). Other nonlimiting examples of therapeutic proteins and peptides include insulin, monoclonal antibodies (*e.g.*, ipilimumab, infliximab, adalimumab), circulating receptor fusion proteins (*e.g.*, etanercept), thrombolytic proteins (*e.g.*, tissue plasminogen activator), and natural product toxins (*e.g.*, conotoxin).

[0056] In some embodiments, the therapeutic protein or peptide is a nanobody (*e.g.*, which binds and inhibits a cell surface receptor, such as a receptor tyrosine kinase). An

example of a nanobody which binds and inhibits a receptor tyrosine kinase is the 7D12 anti-EGFR nanobody (Creative Biolabs, New York). In some embodiments, the therapeutic protein or peptide is a monobody. Monobodies, examples of which are known in the art, are synthetic binding proteins that are constructed using a fibronectin type III domain (FN3) as a molecular scaffold. Monobodies are an alternative to antibodies and nanobodies for creating a target-binding protein.

[0057] “Activity,” as used herein with respect to proteins (*e.g.*, fusion proteins), encompasses, for example, metabolic activity, enzymatic activity, binding activity and therapeutic (*e.g.*, a biopharmaceutical) activity. In some aspects, the activity is a binding activity, for example, the ability to bind to a target protein in an *in vitro* binding activity assay or an *in vivo* extracellular or intracellular binding activity assay. Binding activity is altered, in the fusion proteins described herein, upon exposure of the fusion protein to light that induces a conformational change in the fusion protein and alters its interaction with the target protein. Typically, binding activity of the fusion proteins described herein is mediated by the heterologous peptide component of the fusion protein, which is made available for binding, or obstructed from binding, depending on the conformational state of the fusion protein.

[0058] “Binding activity” is observed when a fusion protein described herein binds to a target molecule, such as a protein, nucleic acid (*e.g.*, deoxyribonucleic acid (DNA), ribonucleic acid (RNA)), carbohydrate, lipid, organic small molecule, *etc.* A fusion protein “binds to” a target molecule if the dissociation constant (K_d) of the interaction between the two species is less than about 10 μ M, less than about 1 μ M, or less than about 100 nM. Decreased binding (indicated by a larger K_d value) corresponds to decreased binding activity. Increased binding (indicated by a smaller K_d value) corresponds to increased binding activity. In some aspects, exposure of the fusion protein to light increases its binding activity. In some aspects, exposure of the fusion protein to light decreases its binding activity. In some aspects, the conformational change induced by exposing a fusion protein described herein to light results in an increase or decrease of activity (*e.g.*, binding activity) of at least two-fold, at least five-fold, at least ten-fold, and more preferably at least fifty-fold. In some aspects, the fusion protein will not exhibit any measurable degree of activity in at least one conformational state.

[0059] Binding activity can be measured, *e.g.*, by measuring a signal produced by the fusion protein. In some embodiments, the signal is an optical signal, such as luminescence, fluorescence or absorbance. Therefore, the signal produced by the fusion protein can be

measured using optical methods, for example, luminescence, absorbance or fluorescence spectroscopy. Alternatively, low angle static light scattering and particle size analysis can be used to detect complex formation using, for example, a Zetasizer (available from Malvern Instruments, Ltd.). Other methods suitable for measuring the signal produced by a fusion protein include electronic absorption spectroscopy, nuclear magnetic resonance spectroscopy, X-ray crystallography, mass spectrometry, infrared and Raman spectroscopy and cyclic voltammetry.

[0060] *In vitro* binding activity is binding activity occurring outside of the cellular environment, for example, in test tubes, multi-well plates and/or Petri dishes containing no live cells. *In vitro* binding activity includes both intermolecular and intramolecular binding activity.

[0061] *In vivo* extracellular binding activity refers to binding activity occurring in the extracellular environment of live cells. Extracellular binding activity includes binding activity occurring between a fusion protein described herein and a cell surface target, such as a cell surface protein or receptor, as well as intramolecular binding activity of a fusion protein that occurs in the extracellular environment of live cells. For example, a nanobody (*e.g.*, which binds and inhibits a cell surface receptor, such as a receptor tyrosine kinase) exhibits *in vivo* extracellular binding activity. Growth factors (*e.g.*, an epidermal growth factor, a fibroblast growth factor, a growth factor which binds a receptor tyrosine kinase and activates or inhibits the receptor tyrosine kinase) also exhibit *in vivo* extracellular binding activity. *See also* FIG. 3, which depicts a light-activated protein therapeutic linked to a light-deactivated inhibitor. Binding activity between the light-activated protein therapeutic and the light-deactivated inhibitor occurring in the extracellular environment of live cells is also *in vivo* extracellular binding activity.

[0062] *In vivo* intracellular binding activity refers to binding activity occurring in the intracellular environment of live cells. Intracellular binding activity includes binding activity occurring between a fusion protein described herein and an intracellular target, as well as intramolecular binding activity of a fusion protein that occurs in the intracellular environment of live cells. Intracellular binding activity can be assessed using a variety of assays known in the art, including, for example, fluorescence microscopy.

[0063] Because the light-responsive domain and, hence, the fusion protein, is light responsive, exposure to light induces a conformational change that alters an activity of the fusion protein. In some aspects, the conformational change of the fusion protein (typically,

the light-responsive domain of the fusion protein) will be induced by visible light (*e.g.*, from about 400-nm to 700-nm light). In particular aspects, the conformational change will be induced by blue light (*e.g.*, from about 380-nm to about 500-nm light, in particular, about 450-nm light), red light (*e.g.*, from about 620-nm to about 750-nm light) or far-red light (*e.g.*, from about 710-nm to about 850-nm light). In other aspects, the conformational change will be induced by infrared light (*e.g.*, from greater than 700-nm to about 1-mm light). LOV domains, BLUF domains, cryptochromes and fluorescent proteins, for example, are typically responsive to blue light, and phytochromes, for example, are typically responsive to red light and far-red light. The C-terminal J α helix of AsLOV2, in particular, undocks and unfolds upon excitation with blue light (*e.g.*, $\lambda_{\text{max}} = 450$ nm), resulting in a substantial increase in the distance between the N- and C-termini of AsLOV2, which are typically within less than 10 Å of one another in the absence of light.

[0064] In some aspects, the fusion protein is a therapeutic fusion protein.

[0065] In addition to being useful as a therapeutic, the fusion proteins described herein can be useful tools for research or industrial protein purification. For example, a fusion protein wherein the heterologous peptide component is a DNA polymerase could be useful as a light-responsive DNA polymerase in a polymerase chain reaction. The fusion proteins described herein could also facilitate protein purification by enabling light-responsive columns (*e.g.*, columns containing a resin with an immobilized light-responsive substrate). For example, light, instead of harsh reagents otherwise used, can be used to elute a protein of interest. Such purification methods generally are less toxic and have a higher specificity than current approaches. Such purification methods also avoid the need for using harsh elution conditions, such as low pH, which can damage the protein of interest, reducing yields. Furthermore, such purification methods can be done in a “label-free” setup (where the fusion protein directly binds to a protein of interest) or with fusion proteins that are specific for a short peptide tag attached to the protein of interest.

[0066] In some aspects, the fusion protein (*e.g.*, a therapeutic fusion protein) comprises a light-responsive domain, a first heterologous peptide component (*e.g.*, a therapeutic protein or peptide) and a second heterologous peptide component different from the first heterologous peptide component (*e.g.*, an inhibitor, such as a nanobody, of the first heterologous peptide component), wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity of the fusion protein (*e.g.*, a binding activity selected from an *in vitro* binding activity, an *in vivo* extracellular binding

activity and an intracellular binding activity). FIG. 3 depicts a generalizable platform for light-activated therapeutics in accordance with this aspect of the invention. It will be appreciated that exposure of the fusion protein depicted in FIG. 3 to light induces a conformational change in the fusion protein that alters the therapeutic activity of the protein therapeutic depicted in FIG. 3. Exposure of the fusion protein depicted in FIG. 3 to light also alters the binding activity between the protein therapeutic and its target protein and the protein and its inhibitor, both of which are *in vivo* extracellular binding activities, if they occur in the extracellular environment of live cells.

[0067] In some aspects, the fusion protein comprises a cell-penetrating peptide component, allowing for intracellular delivery of the fusion protein. Fusion proteins comprising a cell-penetrating peptide component can be used to contact the outside of a cell, penetrate the cell, and then bind an intracellular target determined by the first heterologous peptide component (e.g., a nanobody, a monobody, or an antibody).

[0068] A “cell-penetrating peptide” is a short peptide that facilitates cellular intake/uptake of a molecular component with which it is associated. The cell-penetrating peptide can be associated with the fusion proteins described herein either through chemical linkage via covalent bonds or through non-covalent interactions. The function of the cell-penetrating peptide is to deliver the fusion protein into cells (e.g., through endocytosis). Examples of suitable cell penetrating peptides for the fusion proteins described herein is the Twin-arginine translocation (Tat) pathway signal sequence (InterPro Accession: IPR006311) and polyarginine sequences.

[0069] Alternatively, the fusion proteins described herein can be resurfaced to improve cell-penetrating ability, for example, as described in Chapman and McNaughton, *Cell Chem Biol.* 2016 May 19;23(5):543-553, the relevant contents of which are incorporated herein by reference.

[0070] Also provided herein is a composition (e.g., a pharmaceutical composition, a cosmetic composition, a nutraceutical composition) comprising a pharmaceutically acceptable carrier and a fusion protein described herein. In some embodiments, the composition is a topical composition (e.g., topical pharmaceutical composition) comprising a fusion protein that comprises an antimicrobial peptide and light-responsive domain.

[0071] Compositions described herein may be administered orally, parenterally (including subcutaneously, intramuscularly, intravenously and intradermally), topically, rectally, nasally, buccally or vaginally. In some embodiments, provided compositions are

administrable intravenously, intraarterially, and/or intraperitoneally. In some embodiments, the pharmaceutical composition is administrable locally (*e.g.*, via buccal, nasal, rectal or vaginal route). In some embodiments, the pharmaceutical composition is administrable systemically (*e.g.*, by ingestion).

[0072] The compositions of the present invention may be administered in an appropriate pharmaceutically acceptable carrier having an absorption coefficient similar to water, such as an aqueous gel. Alternatively, a transdermal patch can be used as a carrier. The pharmaceutical agents of the present invention can be administered in a gel, ointment, lotion, suspension, solution or patch, which incorporate any of the foregoing.

[0073] For other topical applications, the compositions can be formulated in a suitable ointment containing the active component suspended or dissolved in one or more carriers.

[0074] Carriers for topical administration of a pharmaceutical agent described herein include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water and penetration enhancers. Alternatively, compositions can be formulated in a suitable lotion or cream containing the active compound suspended or dissolved in one or more pharmaceutically acceptable carriers. Alternatively, the composition can be formulated with a suitable lotion or cream containing the active compound suspended or dissolved in a carrier with suitable emulsifying agents. In some embodiments, suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

[0075] A second embodiment is a method of altering an activity of a fusion protein comprising a light-responsive domain (*e.g.*, a LOV domain, such as AsLOV2, the LOV2 domain from *Avena sativa* Phototropin 1) and a heterologous peptide component. The method comprises exposing the fusion protein to light that induces a conformational change in the fusion protein, thereby altering an activity of the fusion protein. The conformational change alters an activity of the fusion protein and, in some aspects, the activity is a binding activity selected from an *in vitro* binding activity and an *in vivo* extracellular binding activity.

[0076] A third embodiment is a method for treating a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a therapeutic fusion protein comprising a light-responsive domain and a therapeutic protein or peptide, and delivering a sufficient amount of light (*e.g.*, intensity, duration) to at least a portion of the subject to induce the conformational change, thereby altering the therapeutic efficacy of the fusion

protein exposed to the light and treating the subject. Exposure of the fusion protein to light induces a conformational change in the fusion protein that alters its therapeutic efficacy.

[0077] A “subject” refers to a patient who has, or is at risk for developing, a disease or mental condition treatable by a therapeutic protein or peptide. A skilled medical professional (*e.g.*, physician) can readily determine whether a subject has, or is at risk for developing such a condition. In an embodiment, the subject is a mammal (*e.g.*, human, non-human primate, cow, sheep, goat, horse, dog, cat, rabbit, guinea pig, rat, mouse or other bovine, ovine, equine, canine, feline or rodent organism). In a particular embodiment, the subject is a human.

[0078] “Therapeutic efficacy,” as used herein with respect to a fusion protein, means that, when administered to a subject in a therapeutically effective amount, the fusion protein is capable of achieving a desired therapeutic or prophylactic effect (*e.g.*, a therapeutic effect) under the conditions of administration. The effectiveness of a therapy can be determined by suitable methods known to those of skill in the art.

[0079] As used herein, a “therapeutically effective amount” is an amount that is sufficient to achieve the desired therapeutic or prophylactic (*e.g.*, therapeutic) effect under the conditions of administration. The effectiveness of a therapy can be determined by suitable methods known to those of skill in the art.

[0080] In some aspects, exposure of the fusion protein to light increases its therapeutic efficacy and, hence, delivering a sufficient amount of light increases the therapeutic efficacy of the fusion protein exposed to the light. In some aspects, exposure of the fusion protein to light decreases its therapeutic efficacy and, hence, delivering a sufficient amount of light decreases the therapeutic efficacy of the fusion protein exposed to the light.

[0081] A fourth embodiment is a method of identifying a fusion protein comprising a light-responsive domain (*e.g.*, a LOV2, such as an AsLOV2, domain) and a heterologous peptide component (*e.g.*, a therapeutic protein or peptide), wherein exposure of the fusion protein to light induces a conformational change that alters its ability to bind a target protein. The method comprises providing one or more phages displaying a fusion protein intended to bind a target protein. The fusion protein comprises a light-responsive domain and a heterologous peptide component. The immobilized target protein is exposed to the one or more phages in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein, and one or more phages is eluted in the absence of light, thereby eluting one or more phages displaying a fusion protein that binds the target protein

upon exposure to light and dissociates from the target protein in the absence of light. Alternatively, immobilized target protein is exposed to the one or more phages in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein, and one or more phages is eluted in the presence of light, thereby eluting one or more phages displaying a fusion protein that binds the target protein in the absence of light and dissociates from the target protein upon exposure to light. A fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the fusion protein undergoes a photodependent conformational change that alters its ability to bind the target protein, is thereby identified.

[0082] In some aspects, the fourth embodiment further comprises determining the nucleic acid sequence of the coding sequence of the fusion protein or the amino acid sequence of the fusion protein displayed on the one or more eluted phages.

[0083] In some aspects, the fourth embodiment further comprises generating the one or more phages using random transposon-mediated insertion of the coding sequence of the light-responsive domain into the coding sequence of the heterologous peptide component.

[0084] In some aspects, the fourth embodiment further comprises providing a library of phages, each phage displaying a fusion protein intended to bind a target protein and comprising a light-responsive domain and a heterologous peptide component, and exposing immobilized target protein to the library of phages.

[0085] A fifth embodiment is a method of identifying a fusion protein comprising a light-responsive domain (*e.g.*, a LOV2, such as an AsLOV2, domain) and a heterologous peptide component (*e.g.*, a therapeutic protein or peptide), wherein exposure of the fusion protein to light induces a conformational change that alters its ability to bind a target protein. The method comprises providing one or more cells expressing a fusion protein intended to bind a target protein. The fusion protein comprises a light-responsive domain and a heterologous peptide component. One or more cells is sorted using fluorescence-activated cell sorting (FACS). Immobilized target protein is exposed to the one or more cells in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein, and one or more cells is eluted in the absence of light, thereby eluting one or more cells expressing a fusion protein that binds the target protein upon exposure to light and dissociates from the target protein in the absence of light. Alternatively, immobilized target protein is exposed to the one or more cells in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein, and one or more cells is eluted in the

presence of light, thereby eluting one or more cells expressing a fusion protein that binds the target protein in the absence of light and dissociates from the target protein upon exposure to light. A fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the fusion protein undergoes a photodependent conformational change that alters its ability to bind the target protein, is thereby identified.

[0086] In some aspects, the fifth embodiment further comprises determining the nucleic acid sequence of the coding sequence of the fusion protein or the amino acid sequence of the fusion protein expressed by the one or more cells.

[0087] In some aspects, the fifth embodiment further comprises generating the one or more cells by transforming plasmids generated using random transposon-mediated insertion of the coding sequence of the light-responsive domain into the coding sequence of the biopharmaceutical into the one or more cells.

[0088] In some aspects, the fifth embodiment further comprises providing a library of cells, each cell expressing a fusion protein intended to bind a target protein and comprising a light-responsive domain and a heterologous peptide component, and exposing immobilized target protein to the library of cells.

[0089] A sixth embodiment is a method of purifying a target protein from a cell lysate. The method comprises providing a substrate comprising an immobilized fusion protein intended to bind a target protein, the fusion protein comprising a light-responsive domain and a heterologous peptide component. Exposure of the fusion protein to light induces a conformational change that alters its ability to bind the target protein. The method comprises exposing the substrate to the cell lysate in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and eluting the target protein in the absence of light, wherein the fusion protein binds the target protein upon exposure to light and dissociates from the target protein in the absence of light, thereby purifying the target protein. Alternatively, the method comprises exposing the substrate to the cell lysate in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and eluting the target protein in the presence of light, wherein the fusion protein binds the target protein in the absence of light and dissociates from the target protein upon exposure to light, thereby purifying the target protein.

[0090] In some aspects of the sixth embodiment, the light-responsive domain is a light oxygen voltage (LOV) domain, such as AsLOV2, the LOV2 domain from *Avena sativa* Phototropin 1.

[0091] In some aspects of the sixth embodiment, the target protein comprises a purification tag, wherein the immobilized fusion protein binds the purification tag on the target protein under conditions sufficient to induce binding. In some aspects of the sixth embodiment, the target protein does not possess a purification tag (i.e., tag-less purification).

[0092] In some aspects of the sixth embodiment, the substrate is a resin. In some embodiments, the resin is inside of a purification column.

[0093] In some aspects of the methods described herein, light (e.g., visible light, such as blue light, red light and/or far-red light, infrared light) is delivered using two photon excitation.

[0094] A seventh embodiment is an isolated fusion protein comprising a light-responsive domain, a heterologous peptide component, and a cell-penetrating peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity (e.g., a binding activity) of the fusion protein. The cell-penetrating peptide allows for intracellular delivery of the fusion protein. Fusion proteins comprising a cell-penetrating peptide component can be used to contact the outside of a cell, penetrate the cell, and then bind a target determined by the first heterologous peptide component (e.g., a nanobody, a monobody, or an antibody).

[0095] An eighth embodiment is a method of delivering a fusion protein to a cell, comprising contacting the cell extracellularly with an isolated fusion protein comprising a light-responsive domain, a heterologous peptide component, and a cell-penetrating peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity (e.g., a binding activity) of the fusion protein, wherein the fusion protein is delivered intracellularly.

[0096] An ninth embodiment is a chimeric antigen receptor (CAR), comprising an extracellular antigen-binding domain, a transmembrane domain and an intracellular signaling domain, wherein the extracellular antigen-binding domain comprises a light-responsive domain and a heterologous peptide component, wherein exposure of the CAR to light induces a conformational change in the extra-cellular antigen-binding domain that alters an activity (e.g., an *in vivo* extracellular binding activity) of the extra-cellular antigen-binding domain.

[0097] In some aspects, the present disclosure provides isolated lymphocytes expressing the CAR described herein. In some aspects, the lymphocyte is a T lymphocyte.

[0098] The term “Chimeric Antigen Receptor” or, alternatively, “CAR,” refers to a recombinant polypeptide construct comprising at least an extracellular antigen binding

domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain.

[0099] An “intracellular signaling domain,” as the term is used herein, refers to an intracellular portion of a molecule. It is the functional portion of the protein which acts by transmitting information within the cell to regulate cellular activity via defined signaling pathways by generating second messengers or functioning as effectors by responding to such messengers. The intracellular signaling domain generates a signal that promotes an immune effector function of the CAR containing cell, e.g., a CAR-T cell. Examples of immune effector function, e.g., in a CAR-T cell, include cytolytic activity and helper activity, including the secretion of cytokines.

[00100] A tenth embodiment is a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the heterologous peptide component is selected from the group consisting of a nanobody, a monobody, an antibody, a growth factor, and a designed ankyrin repeat protein (DARPin), wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity (e.g., a binding activity) of the fusion protein.

[00101] An eleventh embodiment is a method of delivering a fusion protein to a cell, comprising contacting the cell with a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the heterologous peptide component is selected from the group consisting of a nanobody, a monobody, an antibody, a growth factor, and a designed ankyrin repeat protein (DARPin), wherein the fusion protein is delivered intracellularly. In some aspects, the fusion protein is delivered by externally contacting the cell with a nucleic acid encoding the fusion protein. In a further aspect, the nucleic acid encoding the fusion protein is in a viral expression vector. Once introduced into a cell, the nucleic acid can be expressed transiently, or subsequent to stable integration into the cell's genome.

[00102] The term “expression vector” or “vector”, used interchangeably herein, refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an *in vitro* expression system. Expression vectors include all those known in the art, including cosmids, plasmids (e.g., naked or contained in liposomes) and

viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

EXEMPLIFICATION

Yeast 2 Hybrid (Y2H) Screening of Light-switchable Protein Binding

[00103] Unless otherwise specified, liquid yeast cultures were grown in 24-well plates, at 30 °C and shaken at 200 rpm, in either YPD or SC-dropout media with 2% glucose.

[00104] When cells were grown under light, blue LED panels (HQRP New Square 12" Grow Light Blue LED 14W) were placed 40 cm from cell cultures. To control light duty cycles, the LED panels were regulated with a Nearpow Multifunctional Infinite Loop Programmable Plug-in Digital Timer Switch (from Amazon).

[00105] Y2H_Report_Ura3: Gal1 promoter driving Ura3 gene was placed into an integration vector that will restore *trp1* auxotrophy (pNH60x series);

[00106] Y2H_Bait_X: Adh1 promoter driving Gal4 binding domain attached to eGFP, mcherry, or any protein therapeutic was placed into an integration vector that will restore His3 auxotrophy (pNH60x series);

[00107] Y2H_Prey_X: Adh1 promoter driving the Sv40 nuclear localization sequence connected to the Gal4 activation domain connected to the nanobody or inhibitor against the bait (see FIG. 1) was placed into a CEN/ARS plasmid that will restore *Leu2* auxotrophy (pRS41x series);

[00108] Y2H_Prey_X was opened at optimally determined amino acid sites in conserved or high likelihood regions by backbone PCR;

[00109] Light-oxygen-voltage domain was added to the Y2H_Prey_X plasmid through Gibson isothermal assembly. This makes Y2H_Prey_LightSense_X plasmids;

[00110] CEN.PK2-1C (*MATa his3Δ1 leu2-3_112 trp1-289 ura3-53*) strains with *GAL4* and *GAL80* knocked out were transformed with Y2H_Report_Ura3, Y2H_Bait_X and Y2H_Prey_LightSense_X plasmids and selected on plates that lacked histidine, leucine, and tryptophan.

[00111] Colonies were picked and grown in SC-Leucine-Ura media overnight under light. Samples were then diluted into 24-well plates to an OD₆₀₀ of 0.1. Samples were then placed in light or tin-foiled. Measurements were taken at 0 hours (H), 9H...43H.

[00112] The exponential phase of the data was identified as the most linear portion of the plot of Log_e(OD) versus time, and then fit to Log_e(OD)=Log_e(OD₀)+μ*t, where OD₀ and μ

are constants, using least squares linear regression. The μ constant from these calculations is taken as the mean specific growth rate.

Directed evolution for better kinetics/activity

[00113] Y2H_Prey_LightSense_X identified with some light-deactivation activity were mutagenized to improve the final inhibitors. Y2H_Prey_LightSense_X was amplified through backbone PCR at the Gal4 activation domain and the terminator. The inhibitor-LOV gene was amplified using GenemorphII random mutagenesis kit from Agilent technologies. These pieces were combined into a Gibson assembly mix to make Y2H_Prey_LightSense_X mutants.

[00114] Y2H_Report_Ura3_BFP: Gal1 promoter driving Ura3 gene and Gal1 promoter driving blue fluorescent protein (BFP) gene was placed into an integration vector that will restore *trp1* auxotrophy (pNH60x series).

[00115] CEN.PK2-1C (*MATa his3 Δ 1 leu2-3_112 trp1-289 ura3-53*) strains with *GAL4* and *GAL80* knocked out were transformed with Y2H_Report_Ura3_BFP, Y2H_Bait_X, and Y2H_Prey_LightSense_X mutants plasmids and selected on plates that lacked histidine, leucine, and tryptophan.

[00116] The new yeast were plated onto SC-Leucine + 5FOA plates while exposed to light. One the colonies that grow have low leakiness (do not bind in the light to the protein therapeutic). 5FOA will kill yeast cells if they have Ura3 protein in them.

[00117] Colonies that grew on previous test were then grown in the dark and sorted using cell sorting based on flow cytometry. This process was repeated and good mutants were identified, sequenced, and tested using other methods.

Library Generation

[00118] The TAGIT plasmid was generated using TAGIT-GFP plasmid as a template (generously provided by Dr. James Gregory) and inserting AsLOV2 in place of GFP using Gibson method. As a first proof-of-principle test, whether the TAGIT system could be used to generate a library of light-switchable fusion proteins (the TAGIT workflow is shown in FIG. 4B) was validated. To simplify subsequent validation, a library of AsLOV2 insertions into the mCherry red fluorescent protein was generated, with the intention of screening for blue light switchable changes in mCherry fluorescence.

[00119] Tn5 transposition reaction was performed *in vitro* using commercially available EZ-Tn5 Transposase (Epicentre) to randomly insert a TAGIT transposon containing AsLOV2 into a bacterial expression vector driving arabinose-inducible mCherry expression.

Transposition was achieved successfully, leading to colonies that were kanamycin resistant, conferred by successful insertion of the TAGIT transposon. Moreover, in-frame insertions were identified by the translation of mCherry-LacZ fusion proteins, resulting in blue colonies in a blue/white selection assay. The DNA from the generated library was then transformed into Strataclone Solopack *E.coli* cells expressing Cre recombinase. Cre recognizes LoxP sites included in the TAGIT transposon to successfully excise the LacZ and KanR genes, leaving a library of mCherry sequences with a randomly inserted, in-frame AsLOV2 domain.

Individual successful insertions were validated by sequencing. Finally, it was verified that AsLOV2 can be properly folded along with the protein of interest by expressing, purifying, and characterizing multiple AsLOV2-mCherry fusion proteins. Using UV-visible spectroscopy, it was confirmed that the AsLOV2 can indeed bind its chromophore and undergo a natural photocycle, and that mCherry can still fluoresce (FIG. 4B). These proof-of-principle experiments demonstrated that the TAGIT transposon system can be used to generate rich construct libraries and generate functional, photoswitchable fusion proteins.

[00120] This approach was further modified for high-throughput library construction of diverse targets. An *in vitro* Tn5 transposase reaction (Epicentre EZ-Tn5 Transposase) was used to randomly insert the AsLOV2-domain at all nucleotide positions within all three coding sequences. The AsLOV2 plasmid includes a LacZ gene lacking a START codon that will only be expressed if fusion is in-frame. By transforming the resulting fusion plasmids into Lac⁻ Ara⁺ BW2581 *E. coli* and growing cultures in lactose, only constructs with in-frame fusion between AsLOV2 and the protein of interest were selected. Finally, the DNA of entire library was purified and transformed into Cre recombinase-expressing bacteria (StrataClone Solopack) to excise the LacZ gene, leading to in-frame expression of AsLOV2 at all amino acid positions. This approach was used to generate libraries where the target proteins included various nanobodies, DARPs, and biotherapeutics of interest. Additionally, this approach was used to generate the libraries for yeast-two-hybrid screening, phage display, and pull-down assays, where the appropriate plasmid with the target gene was used in the transposition reaction to then smoothly transition into the desired screening platform. For example, for phage display screening, pJSC plasmid was used along with the TAGIT plasmid in the transposition reaction.

Phage Display for Screening

[00121] This idea is very like the yeast 2 hybrid approach. The phage display method was used by putting a protein of interest (e.g., a nanobody) into the phage display plasmid. The

binding partner of the protein of interest was then immobilized on a column and the selection done by eluting in the light and in the dark. The method was altered using “A twin-arginine translocation (Tat)-mediated phage display system” (Paschke et al 2004), which is needed to express an active LOV domain.

Day 1: This is to grow up the phagemid library and then infect it with helper phage overnight, resulting in phage which display the VHH library on their surface as fusions to pIII.

1. Inoculate 100ml SOC + Amp in 500mL Erlenmeyer flask with 200 μ L-1mL of thawed library stock (ideally want a starting OD₆₀₀ = 0.1-0.2). *Amp is for plasmid selection. Glucose in SOC suppresses expression of VHH to prevent growth bias in library.*
2. Grow culture at 37 °C with shaking until OD₆₀₀ is 0.5-0.7.
3. Add VCMS13 helper phage (around 1mL of a 10¹³ pfu/mL stock is typically used).
4. Incubate inoculated culture 30 minutes (min) at 37 °C without shaking.
5. Incubate 1H 30min at 37 °C with shaking.
6. Split culture into 2 x 50 mL conical tubes and spin down 10min at 8000 rpm at 4 °C.
7. Resuspend pellets in 100mL total 2YT /0.1% glucose/100ug/mL Amp/70 μ g/mL Kan in 500mL Erlenmeyer flask. *Amp if for plasmid selection, kan is for helper phage selection.*
8. Grow overnight at 30 °C with shaking.

****start 100mL overnight culture of ER2738 in 2YT/Tet and grow at 37 °C with shaking in preparation for panning. This strain is available from NEB and has a tet-inducible pili through which the phage infects the cell. Alternatively, you can use TGIs for this step.**

Day 2: This is where the panning of the phage library generated overnight against the immobilized antigen is performed.

1. Split phage culture into 2 x 50 mL conical tubes and spin down 10min at 8000 rpm 4 °C.
2. Pour supernatant to two clear centrifuge bottles and add 10mL 20% PEG/2.5M NaCl, (1/5th volume) to each. Incubate 2h at 4°C on ice. *Using clear bottles will make it easier to see the phage pellet after centrifuging.*
3. Spin down 20min at 8000 rpm at 4°C. Mark orientation so the location of the pellet is known.
4. Pour off supernatant. Resuspend phage pellet (*will appear white and fluffy, may be streaked down side of the tube*) in 4mL PBS. Split this between 4 epi tubes (1mL in

each), and add 200 μ L of the above PEG solution (1/5th volume) to each. Incubate 1H at 4°C on ice.

5. Spin down 10min at 13,000 rpm at 4°C, and remove supernatant. Spin again for another minute and remove any residual liquid.
6. Resuspend the four phage pellets in 1mL PBS total volume.
7. Spin resuspended material 5min at 4 °C at 13,000rpm. You want to keep the supernatant this time, as this is now your phage library stock. *Transfer supernatant to a fresh tube, and calculate concentration by measuring the A296 and A320 (you can do this using the protein measurement tool on the nanodrop).*

$$\text{Phage concentration: \#pfu/mL} = \frac{6000 \times 10^{14} \times (A_{296} - A_{320})}{7200\text{bp}}$$

Panning Round 2

1. Add 2 μ L of pre-cleared phage to each of the protein-biotin-strep tubes + 1mL 1% BSA/PBS and incubate for 15 min at room temperature (RT) with inversion.
2. Aspirate supernatant, wash beads 15 x 1mL with PBS/0.1% Tween (PBST).
3. For final wash, add 500 μ L PBST and incubate at 37 °C for 1H with inversion
4. Add 500 μ l saturated ER2738 bacteria to each tube, incubate 15min at 37 °C with inversion.
 - a. *On the previous day, 100mL overnight culture of ER2738 in 2YT/Tet will have been started and grown at 37 °C with shaking.*
5. Remove cells and store as “Elution 1.”
6. Add 500 μ l 0.2M glycine, pH 2.2 and incubate 10min at RT with inversion.
 - a. *1.5g glycine for 100mL, use HCl to adjust pH to 2.2.*
7. Remove glycine and add to 75 μ L 1M Tris pH 9.1 to neutralize, save as “E2.”
 - a. *12.12g for 100ml, use HCl to adjust pH to 9.1.*
8. Pool elutions E1 and E2, and incubate for 15 min at 37 °C.
9. Remove 100 μ l pooled elutions to titer library for individual colonies, plate the remainder on “big” 2YT/2% glucose/Tet/Amp plates.
10. Grow all overnight at 37 °C and determine titers.
 - a. *Titer by making 10-fold serial dilutions in PBS, and plating 100 μ L of each on “small” 2YT/2% glucose/Tet/Amp plates.*

Day 3

1. Scrape down plates with 2mL SOC + Amp per plate, pool together and add 50% sterile glycerol to 15% final concentration. Aliquot into cryotubes and store at -80 °C as “second round panning” library.
2. Pick individual colonies off of titer plates into 96 well plates, using 200µl SOC+Amp+Tet per well. Cover with airpore sheet (Qiagen) to allow for oxygen exchange. Grow overnight at 37 °C with agitation. This will be the “Master Plate” for bug soup.

Protein Expression and Purification***Wild-type AsLOV2 Expression and Purification***

[00122] The codon optimized gene encoding C-terminal LOV2 domain of *Avena sativa* phototropin 1 (AsLOV2) containing residues 404-546 in pet15b vector (generously provided by Professor Peter Tonge) was sub-cloned into pBAD vector containing residues 404-546 or residues 408-543 using in-fusion method. The resulting plasmid was transformed into Top10 *E. coli* cells for protein expression and a single colony was used to inoculate a 5 mL culture of LB media supplemented with Ampicillin (Amp) at 100µg/mL. After incubating the culture at 37 °C and 250 rpm overnight, it was used to inoculate 250 mL of 2x YT/Amp media in a 1 L flask. The 1 L flask was shaken at 37 °C and 250 rpm until the OD₆₀₀ reached approximately 0.8. The temperature was decreased to 20 °C followed by addition of 0.5% arabinose to induce protein expression overnight (approximately 16 h) in the dark. Cells were harvested by centrifugation at 4 °C and 5000 rpm and stored at -80 °C.

[00123] The cell pellet resulting from a 250 mL culture was thawed and resuspended in 40 mL of buffer A (20mM Tris, 150mM NaCl pH 8.0) supplemented with protease inhibitor cocktail tablet (cOmplete, Roche). The cells were then lysed using a sonicator with about 8 cycles of 15 seconds on and 1 minute off. Cell debris was removed by centrifugation (40,000 rpm for 90 min). The supernatant was incubated with FMN (0.25 mg/mL) for approximately 30 min on ice and in the dark to ensure a homogeneous population of protein bound chromophore followed by loading it onto a column with Ni-NTA resin previously equilibrated with buffer A. The column was washed with buffer A containing increasing concentrations of imidazole (0 mM, 10 mM and 20 mM) until AsLOV2 eluted at 250 mM imidazole. The protein was dialyzed overnight against buffer A and its purity was assessed by SDS-PAGE.

DARPin and DARPIn-AsLOV2 fusions

[00124] Protocol for expression and purification of wild-type AsLOV2 was used as described above.

Nanobodies and Nanobody-AsLOV2 fusions

[00125] The DNA for the desired nanobody was synthesized through IDT and subcloned into pGEX6P1, pET22b, and pBAD vector using in-fusion method. The resulting plasmid was transformed into BL21(DE3) *E. coli* cells or Shuffle T7Express *E. coli* cells for protein expression and a single colony was used to inoculate a 5 mL culture of LB media supplemented with Ampicillin (Amp) or Carbenicillin (Carb) at 100µg/mL. After incubating the culture at 37 °C and 250 rpm overnight, it was used to inoculate 250 mL of 2x YT/Amp media in a 1 L flask. The 1 L flask was shaken at 37 °C and 250 rpm until the OD₆₀₀ reached approximately 0.8. The temperature was decreased to 20 °C followed by addition of 0.8 mM IPTG or 0.2% Arabinose to induce protein expression overnight (approximately 16 h) in the dark. Cells were harvested by centrifugation at 4 °C and 5000 rpm and stored at -80 °C.

[00126] The purification was conducted as described for wild-type AsLOV2.

UV-Vis Spectroscopy

[00127] Absorption spectra of all protein samples were obtained using a UV-visible spectrometer. The concentration of the protein samples was kept at approximately 50 µM (20mM Tris, 150mM NaCl pH 8.0). Dark-adapted spectra were obtained first and then in order to acquire a light state spectra, samples were illuminated with blue LED for 1 minute. Scanning kinetics were used to record the recovery of the dark state from the light state for the light-sensitive proteins. Single exponential decay equation was used to fit the absorbance at 447 nm over time to acquire the rate of the recovery.

Mammalian cell assay screen for assessing protein binding in intracellular environment for a light-responsive fusion protein (comprising heterologous peptide components, including Nanobodies/Darpins/Monobodies and other tight binders such as affibodies, antibodies etc.)

[00128] The goal of this screening platform was to express candidate light-responsive fusion proteins fused to a fluorescent protein in the cytoplasm of a mammalian cell, along with the binding target expressed on the cell membrane. Then, light can be used to switch binding on and off, leading to a change in the fusion protein's localization from cytosol to

membrane. This assay comprises a screening platform for individual fusion protein variants. This approach was used to screen nanobody, monobody, and DARPIn heterologous peptide components against targets of interest.

[00129] Necessary DNA was cloned into PHR vectors using standard Gibson assembly-based cloning. Constructs containing either the light-responsive-nanobody fusion, light-responsive-DARPIn fusion, or light-responsive-monobody fusion (fused to fluorescent protein such as iRFP for visualization purposes) or target were introduced into NIH3T3 cells using lentiviral transduction. The target (i.e. mCherry) was localized to the membrane using a CAAX tag, with the binding partner (ie. nanobody, DARPIn, monobody) expressed in the cytoplasm (FIGs. 7A-7B). In this example, the nanobody is membrane-localized due to its interaction with mCherry, which can then be screened for light sensitivity. The interaction between the light-responsive fusion protein and target was imaged over time, either in the absence or presence of blue light. The mammalian cells were kept at 37°C with 5% CO₂ for the duration of all imaging experiments. Imaging was done using Nikon Eclipse Ti microscope with a Prior linear motorized stage, a Yokogawa CSU-X1 spinning disk, an Agilent laser line module containing 405, 488, 561 and 650 nm lasers, an iXon DU897 EMCCD camera, and a 60X oil immersion objective lens. Also, a 450nm LED light source was used for photo excitation with blue light, which was delivered through a Polygon400 digital micro-mirror device (DMD; Mightex Systems).

[00130] With this assay, light-induced binding as well as light-induced dissociation from specific nanobody-LOV domain fusion proteins was observed (FIGs. 8A-8F). Notably, the site at which a LOV domain is inserted was sufficient to switch between light-induced binding and light-induced dissociation. Similar results have also been obtained from the other classes of protein binders: DARPins (FIGs. 9A-9F) and monobodies (FIGs. 10A and 10B).

Bacterial production and purification of opto-binders for *in vitro* protein binding assays

[00131] In addition to expression within mammalian cells, protein binders can be used *in vitro* as extracellular therapeutics or in the context of protein biochemistry (for protein purification, crystallography, etc). For the expression of light-responsive-nanobody fusion proteins, constructs were cloned into pBAD plasmid with N-terminal His tag. Shuffle T7 Express cells (NEB) were transformed by the plasmid containing the light-responsive-nanobody fusion. The rest of the expression and purification follow the previously described protocol herein.

***In vitro* binding of light-responsive fusion proteins from targets in purified protein solutions**

[00132] To demonstrate the light dependent binding *in vitro*, a binding assay with purified light-responsive fusion proteins was set up, which was validated using the light-responsive-nanobody fusion protein hits from FIG. 7 and FIG. 8. A purified light-responsive-nanobody was fused to Ni-NTA agarose beads via its His-tag, and the purified target with no tag was added to the solution with the beads. The results of binding and dissociation are shown in FIGs. 11A-11F.

[00133] The binding and dissociation of the target was monitored by imaging the fluorescent target (e.g., mCherry) binding on and off the bead. A microscopy imaging system with 20x objective lens was used to image the beads. Blue light was delivered through DMDs either illuminating the entire bead or with a spatial pattern (e.g. by illuminating half of the agarose bead in FIG. 11B), which demonstrated good spatial control of binding/unbinding only in the illuminated region.

Light-switchable changes in elution rate using size exclusion chromatography

[00134] To further characterize light-controlled binding size exclusion chromatography (SEC) was performed using purified protein samples. The premise behind SEC is that when dissolved molecules of various sizes flow through the column, the larger molecules elute faster while smaller molecules enter a larger fraction of the column's pores and take longer to elute (Nagy and Vekey, 2008). Thus, over a run the molecules are sorted by size. The chromatography column was modified with blue light LEDs for light delivery (FIG. 12). The ligand and target complex was incubated for approximately 30 minutes either in the light or dark and then run on the column either in the light or dark. The results show either light-induced binding or dissociation based on the light-responsive fusion protein (FIGs. 13A and 13B). The column also represents a proof-of-principle basic prototype of a light-controlled purification column: a column with a resin inside that can be modified with the correct light input for separating proteins based on their light sensitivity.

[00135] This purification column allows the rate of elution of a protein of interest to be controlled with light.

Quantification of light-induced change in binding affinities using OCTET

[00136] Techniques have also been developed to quantify the binding affinity in the lit and dark states for a light-controlled fusion protein. Crucially, point mutations are available that

lock LOV domains in their lit and dark states. By using these mutant LOV domains in the appropriate fusion constructs, binding experiments need not be performed in the presence of specific light conditions. However, we can also measure the light state binding and dissociation using the OCTET biomolecular interaction assay (ForteBio) by inserting a light plate into the instrument during the measurement.

[00137] Light-induced change in affinity was quantified using the OCTET biomolecular interaction assay (ForteBio). His-tag was kept on all ligand samples and the tag was cleaved off of the target. In order to adequately measure the affinities of the ligands towards their target in the dark as well as under blue light conditions, point mutations were inserted into the photoactive protein which lead to the protein being locked in the dark or in the light position simulating the actual light inputs. Ni-NTA Biosensors were used to bind the ligand and the measurements were conducted based on the standard protocols provided by ForteBio (FIG. 14). Association rate constants as well as dissociation rate constants were measured and used to calculate the affinity constants listed in Table 1.

Sample	k_{on} (1/Ms)	k_{off} (1/s)	Kd
LaM8	7.52×10^4	0.02	322 nM
LaM8 GG15 C450V	5.13×10^5	0.02	378 nM
LaM8 GG15 I539E	4.97×10^5	0.06	1.24 μ M
LaM8 AK74 C450V	2.39×10^5	0.41	17.2 μ M
LaM8 AK74 I539E	3.86×10^5	0.12	3.15 μ M

Table 1. Kinetic parameters for mCherry specific nanobodies. LaM8 denotes the type of a nanobody; GG15 and AK74 denote the insertion of the AsLOV2 domain in the nanobody. Mutants that lack the protein in the dark (C450V) or the light state (I539E) were used to allow for measurements using this instrumental setting.

Light-Dependent Binding Partner Screening Method for Light-Assisted Protein Purification

[00138] In addition to the cell-based screens described herein, screens were developed based on directly measuring light-dependent binding between a light-responsive fusion protein and substrate *in vitro*. This screening platform was applied to monobody-LOV domain fusions, where monobodies are another protein binding domain where the activity can be switched on and off. Results for a screen of light-responsive-monobody fusions is shown in FIG. 15

[00139] A binding target was expressed as a 6xHis-YFP-protein fusion (6xHis: Histidine tag; YFP: yellow fluorescent protein; binding targets include, e.g., SUMO tag and SH2 tag). The fusion protein was expressed and grown in 500mL of autoinduction media + kanamycin for 16 hours. Monobody and Monobody-LOV fusions/chimeras were grown in 250mL of autoinduction media + kanamycin for 16 hours. For each test, 1 monobody control and 3 Monobody-LOV fusions/chimeras were tested.

[00140] Cells were harvested at 7,000 rpm for 25 minutes at 4°C and supernatant was discarded. Pellets were resuspended in 8mL (for the 6xHis-YFP-binder) or 3mL (for the monobodies or monobody chimeras) of wash buffer (100mM Tris, pH 8.0, 150mM NaCl, 1% Glycerol, 5mM Imidazole) with additional 1mM PMSF and 0.5mg/mL lysozyme. Resuspended cells were broken apart using the CryoMill with liquid nitrogen. Broken cells were melted and 4mL of wash buffer + 1x PMSF were added to the 6xHis-YFP-binder while 2mL of wash buffer + 1x PMSF were added to the monobodies or monobody chimeras. Lysates were then spun down at 14,000 rpm for 30 minutes at 4°C. The 6xHis-YFP-binder supernatant was then run through a Co-NTA column and washed with wash buffer. The beads (now with 6xHis-YFP-binder immobilized) were then resuspended in wash buffer to a total volume of 13.5mL. This mixture was then divided into 9 15mL conical tubes. 4 of these tubes were labeled for experimentation under blue light. 4 of these tubes were labeled for experimentation in the dark (red light was used for visualization). The remaining tube was left as a control.

[00141] Monobody and Monobody-LOV fusion/chimera supernatants were then split in half with half added to the tubes designated for light experimentations and the other half added to tubes designated for dark experimentations. Tubes were then placed into 4°C under 20 rpm for 45 minutes for binding under respective light conditions. Tubes were then allowed to settle at 4°C under respective light conditions for 30 minutes. Supernatants were removed

while attempting to retain as much resin as possible. 10mL of wash buffer was added to each tube and rotated at 20 rpm for 45 minutes. This was again allowed to settle at 4°C under respective light conditions for 30 minutes and supernatant was again discarded. Washing was repeated 3 times. All light conditions were held constant with careful experimentation to minimize light exposure for the dark samples. After the last dumping of the supernatant, proteins were eluted with elution buffer (100mM Tris, pH 8.0, 150mM NaCl, 1% Glycerol, 500mM Imidazole).

[00142] Wash buffer = 100mM Tris, pH 8.0, 150mM NaCl, 1% Glycerol, 5mM Imidazole

[00143] Elution buffer = 100mM Tris, pH 8.0, 150mM NaCl, 1% Glycerol, 500mM Imidazole

Light-assisted protein purification with or without common purification tags

[00144] One biotechnological application of the endogenous-protein light-responsive fusion proteins described herein is in the purification of proteins of interest. Optogenetic control can be used over nanobodies, monobodies and DARPins (or other protein binding domains) to bind to their specific target within a crude mixture of proteins. Then, after appropriate washing to remove unbound proteins, the target protein of interest can be eluted by changing illumination conditions (either exposing the purification system to light or darkness). This is particularly useful because it obviates the need for toxic or caustic elution steps (which are often performed under conditions that can alter the activity of the target, or require substantial use of expensive or toxic reagents). It also enhances the options for tag-less purification, e.g., of therapeutic antibodies which typically cannot be used in humans if fused to standard purification tags. An optogenetic Protein A or anti-antibody binder can be used for highly specific light-based binding and elution of any antibody in a protein purification method.

[00145] Light-assisted protein purification can be used in, for example, three distinct modalities:

Light-responsive fusion proteins against a specific protein target for tag-less purification;

Light-responsive fusion proteins against commonly used purification tags to purify tagged proteins with light, eliminating toxic, poorly-selective and/or expensive wash steps; and

Light-responsive fusion proteins derived from commonly-used purification proteins based on affinity against targets with widespread therapeutic or industrial value (e.g. Protein A, Protein L, and Protein G, which target the constant domains of antibodies).

[00146] As an example of this approach, a light-based elution assay is described (FIGs. 16A and 16B). First, the light-responsive fusion proteins (e.g., light-responsive nanobody fusion, light-responsive DARPin fusion, light-responsive monobody fusion, light-responsive antibody fusion, etc.) is immobilized on the resin (creating an opto-resin) and placed in a purification column that is modified with blue LEDs. The target protein (protein to be purified) is expressed and lysed with the conditions best suited for the specific target. The lysate is cleared by ultracentrifugation at 30,000 rpm for 1 hour at 4°C. The supernatant is equilibrated with the opto-resin at 4°C under 20 rpm for 45 minutes for binding under respective light conditions. Tubes are then allowed to settle at 4°C under respective light conditions for 30 minutes. The column is washed with multiple column volumes of lysis buffer under the binding light conditions and then eluted either in the dark or in the light, depending on the specific activity of the fusion protein. This method can be optimized with the buffer conditions and equilibration times based on the target protein being purified.

Engineering light control over cell signaling pathways, including light-induced binding and light-induced dissociation, using light-responsive fusion proteins

[00147] Light-responsive fusion proteins can be genetically expressed in cells and made to hetero-dimerize to their target protein in either the presence or absence of light. This makes them capable of recapitulating all the functions of existing light-gated heterodimerization systems, including the Phy/PIF, iLID-SSPB, and Cry2-CIB systems that have become staples of modern cell biology.

[00148] To demonstrate this capability, a currently used optogenetic tool, the OptoSOS system, was modified to be controlled by the association between a light-responsive fusion protein (i.e., opto-binder) of the present disclosure and its target. The following DNA constructs were generated, representing a nanobody/target directed OptoSOS system (construct a) and the Erk-specific Kinase Translocation Reporter (KTR) fused to iRFP (construct b):

A membrane bound target + cytosolic opto-binder:

pHR SFFVp (opto-binder)-iRFP-SOScat-P2A-(target)-CAAX

A biosensor of SOS-induced Erk kinase activity:

pHR SFFVp KTR-iRFP

[00149] Both constructs were introduced into NIH3T3 cells using lentiviral transduction. Note that expression of construct a is less than that of construct b, so irFP imaging can be used to assess KTR localization, which exits the nucleus when the biological pathway (Erk signaling) is activated (through SOScat being localized to the membrane and activating Ras). FIGs. 17A and 17B, show light-induced activation of Erk (FIG. 17A: before light; FIG. 17B: after light) using a fusion between SOScat and the LaM8-AK74 light-responsive nanobody fusion. FIGs. 17C and 17D, show light-induced inactivation of Erk using an OptoSOS-fused LaM8-GG15 light-responsive-nanobody fusion, which dissociates upon photo-stimulation.

[00150] In addition to recapitulating known OptoSOS results using prior systems without light-responsive fusion proteins (Toettcher et al, Cell 2013), this embodiment described demonstrates that the response can be inverted by using a light-dissociable fusion protein. Because Erk activity directly controls cell proliferation in a number of systems, this also offers a two-hybrid screening platform for testing light-responsive fusion protein variants for light-switchable activity, using cell proliferation as a functional readout.

Light-switchable inhibition of endogenous cellular proteins using light-responsive fusion proteins

[00151] In the example above, a method to use light-responsive fusion proteins as an alternative for engineered hetero-dimerization was described, such as controlling the membrane localization of the Erk-activating SOScat construct. The same binders can be used for binding to, and modulating the activity of, naturally-occurring proteins in the host cell. In doing so, light-responsive fusion proteins fundamentally expand the toolbox of light-controlled protein binding reactions.

[00152] Because many of the protein domains used in light-responsive fusion proteins (such as, e.g., nanobodies or monobodies) bind to naturally-occurring, endogenous proteins, they can also be used to bind to these endogenous proteins after being made photoswitchable. Thus, embodiments of the invention can be used for light-controlled binding to endogenous proteins in various modes (FIG. 18), including inside the cell using a genetically-encoded light-responsive fusion protein (FIG. 18A) or outside of the cell (FIG. 18B, 18C) using a light-responsive fusion protein that is added to cells' external environment, and which then binds to a naturally-occurring target protein on the cell surface in a light-switchable manner (FIG. 18B). Further, embodiments can utilize a light-responsive fusion protein that is fused to a cell-penetrating peptide (FIG. 18C), and which can be added outside of cells, penetrate them, and bind to their target.

[00153] Binding is often able to modulate a target protein's activity. This is achieved by blocking access to a particular patch of the protein's surface to out-compete binding by natural proteins (steric control), or by inducing a conformational change in the target protein that alters its natural function (allosteric control). The ability to target endogenous proteins and regulate their activity in a light-controllable way introduces an extraordinary benefit to metabolic engineering.

[00154] Extracellular light-controlled protein inhibition is schematized in Figure 19 using the example of a light-responsive-nanobody that binds to domain III of the EGF receptor, thereby inhibiting its activity.

[00155] Thus, light-responsive fusion proteins offer extracellular and intracellular methods for inhibiting natural protein function in a light-switchable manner. Additional applications include, e.g., local light stimulation to prevent EGFR inhibition by anti-cancer nanobodies at sites of side-effects (using light-dissociated fusion proteins), or, conversely, local light stimulation to enhance anti-cancer activity at the site of a tumor (using light-induced fusion proteins).

Mammalian cell assay screen for assessing protein binding in extracellular environment for light-responsive fusion proteins with future applications in T-Cell therapy

[00156] Chimeric antigen receptor expressing T cells (CAR-T cells) are frequently used to treat cancers that express specific antigens to which the chimeric antigen receptor (CAR) can be targeted. Notably, CARs are often designed as a fusion between an extracellular single-chain antibody (scFv) and intracellular signaling domains; the scFv can be swapped out for other binding domains that target particular cellular antigens. In an embodiment, CARs are modified with a light-responsive fusion protein as its extracellular targeting domain, leading to light-controllable CAR-T activation *in vivo*. Such light-based control can be used to limit CAR-T activation at undesired sites (e.g., sites of high auto-immune reaction or sites distant from a primary tumor) or enhance CAR-T activation at known tumor sites or sites of co-stimulation (e.g., by local injection of other immunomodulatory compounds).

[00157] A reduced, idealized model was generated to enable cell surface expression of a light-responsive fusion protein whose activity can be transduced to an intracellular response (thereby mimicking the response of a CAR-T cell). As a basis for this approach, the SynNotch technology developed in the laboratory of Wendell Lim (Morsut et al. Cell, 2016; Roybal et al. Cell, 2016) was utilized. Cells expressing the following were engineered:

a transmembrane protein that is cleaved into a functional transcription factor upon binding, and where binding is achieved by a light-regulated opto-binder: pHR SFFVp Opto-binder-Notch transmembrane-VP16-Gal4; and

a Gal4-responsive gene cassette that expresses the BFP blue fluorescent protein in response to the SynNotch receptor's activation, as well as iRFP to mark expressing cells: pHR SFFVp UAS-BFP (term.) PGKp iRFP, where "term." is a transcription terminator sequence.

[00158] The light-responsive-nanobody target (e.g., mCherry) was supplied as a purified protein fused to agarose beads in order to activate binding to the Opto-binder-Notch construct, thereby inducing cleavage and expressing BFP in responsive cells (FIG. 20). In further embodiments, BFP expression can be enhanced under specific illumination conditions for individual light-responsive fusion proteins.

[00159] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[00160] While example embodiments have been particularly shown and described, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the embodiments encompassed by the appended claims.

CLAIMS

What is claimed is:

1. An isolated fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity of the fusion protein, wherein the activity is a binding activity selected from an *in vitro* binding activity and an *in vivo* extracellular binding activity.
2. The fusion protein of claim 1, wherein the light-responsive domain is a light oxygen voltage (LOV) domain.
3. The fusion protein of claim 2, wherein the LOV domain is the LOV2 domain from *Avena sativa* Phototropin 1.
4. The fusion protein of claim 1, 2 or 3, wherein the peptide component is interferon.
5. The fusion protein of claim 1, 2 or 3, wherein the peptide component is ipilimumab.
6. The fusion protein of claim 1, 2 or 3, wherein the peptide component is conotoxin.
7. The fusion protein of claim 1, 2 or 3, wherein the peptide component is a nanobody, monobody or an antibody.
8. The fusion protein of claim 1, 2 or 3, wherein the peptide component is a growth factor.
9. The fusion protein of claim 1, 2 or 3, wherein the peptide component is a designed ankyrin repeat protein (DARPin).
10. The fusion protein of claim 1, 2 or 3, wherein the peptide component is an antimicrobial peptide.
11. The fusion protein of claim 1, 2 or 3, wherein the peptide component is an enzyme.
12. The fusion protein of claim 1, 2 or 3, wherein the peptide component is a therapeutic protein or peptide.
13. The fusion protein of any one of claims 1-12, wherein the conformational change is an allosteric change.

14. The fusion protein of any one of claims 1-12, wherein the conformational change induces multimerization of the fusion protein.
15. The fusion protein of any one of claims 1-14, wherein exposure of the fusion protein to light increases its binding activity.
16. The fusion protein of any one of claims 1-14, wherein exposure of the fusion protein to light decreases its binding activity.
17. The fusion protein of claim 15 or 16, wherein the light is visible light.
18. The fusion protein of claim 17, wherein the light is blue light.
19. The fusion protein of claim 15 or 16, wherein the light is infrared light.
20. An isolated fusion protein comprising a light-responsive domain, a heterologous peptide component, and a cell-penetrating peptide component, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters an activity of the fusion protein, wherein the activity is a binding activity.
21. A composition comprising a pharmaceutically acceptable carrier and a fusion protein of any one of the preceding claims.
22. A method of delivering a fusion protein to a cell, comprising contacting the cell extracellularly with the isolated fusion protein of claim 20, wherein the fusion protein is delivered intracellularly.
23. A method of altering an activity of a fusion protein comprising a light-responsive domain and a heterologous peptide component, the method comprising:

exposing the fusion protein to light that induces a conformational change in the fusion protein, wherein the conformational change alters an activity of the fusion protein, wherein the activity is a binding activity selected from an *in vitro* binding activity and an *in vivo* extracellular binding activity,

thereby altering an activity of the fusion protein.
24. The method of claim 23, wherein the photoactivatable domain is a light oxygen voltage (LOV) domain.
25. The method of claim 24, wherein the LOV domain is the LOV2 domain from *Avena sativa* Phototropin 1.

26. A method for treating a subject in need thereof, comprising:

administering to the subject a therapeutically effective amount of a therapeutic fusion protein of any one of claims 1-20, wherein exposure of the fusion protein to light induces a conformational change in the fusion protein that alters its therapeutic efficacy; and

delivering a sufficient amount of light to at least a portion of the subject to induce the conformational change, thereby altering the therapeutic efficacy of the fusion protein exposed to the light,

thereby treating the subject in need thereof.

27. The method of claim 26, wherein exposure of the fusion protein to light increases its therapeutic efficacy and, hence, delivering a sufficient amount of light increases the therapeutic efficacy of the fusion protein exposed to the light.
28. The method of claim 26, wherein exposure of the fusion protein to light decreases its therapeutic efficacy and, hence, delivering a sufficient amount of light decreases the therapeutic efficacy of the fusion protein exposed to the light.
29. The method of claim 26 or 27, wherein light is delivered using two photon excitation.
30. The method of any one of claims 26-29, wherein the light is visible light.
31. The method of claim 30, wherein the light is blue light.
32. The method of any one of claims 26-29, wherein the light is infrared light.
33. A method of identifying a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change that alters its ability to bind a target protein, the method comprising:
- providing one or more phages displaying a fusion protein intended to bind a target protein, the fusion protein comprising a light-responsive domain and a heterologous peptide component; and
- exposing immobilized target protein to the one or more phages in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and

eluting one or more phages in the absence of light, thereby eluting one or more phages displaying a fusion protein that binds the target protein upon exposure to light and dissociates from the target protein in the absence of light; or

exposing immobilized target protein to the one or more phages in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and

eluting one or more phages in the presence of light, thereby eluting one or more phages displaying a fusion protein that binds the target protein in the absence of light and dissociates from the target protein upon exposure to light,

thereby identifying a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the fusion protein undergoes a photodependent conformational change that alters its ability to bind the target protein.

34. The method of claim 33, wherein the light-responsive domain is a light oxygen voltage (LOV) domain.
35. The method of claim 34, wherein the LOV domain is the LOV2 domain from *Avena sativa* Phototropin 1.
36. The method of claim 33, 34 or 35, wherein the peptide component is a therapeutic protein or peptide.
37. The method of any one of claims 33-36, further comprising determining the nucleic acid sequence of the coding sequence of the fusion protein or the amino acid sequence of the fusion protein displayed on the one or more eluted phages.
38. The method of any one of claims 33-37, further comprising generating the one or more phages using random transposon-mediated insertion of the coding sequence of the light-responsive domain into the coding sequence of the heterologous peptide component.
39. The method of any one of claims 33-38, comprising providing a library of phages, each phage displaying a fusion protein intended to bind a target protein and comprising a light-responsive domain and a heterologous peptide component, and exposing immobilized target protein to the library of phages.

40. A method of identifying a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change that alters its ability to bind a target protein, the method comprising:

providing one or more cells expressing a fusion protein intended to bind a target protein, the fusion protein comprising a light-responsive domain and a heterologous peptide component;

sorting the one or more cells using fluorescence-activated cell sorting (FACS);
and

exposing immobilized target protein to the one or more cells in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and

eluting one or more cells in the absence of light, thereby eluting one or more cells expressing a fusion protein that binds the target protein upon exposure to light and dissociates from the target protein in the absence of light; or

exposing immobilized target protein to the one or more cells in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein; and

eluting one or more cells in the presence of light, thereby eluting one or more cells expressing a fusion protein that binds the target protein in the absence of light and dissociates from the target protein upon exposure to light,

thereby identifying a fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the fusion protein undergoes a photodependent conformational change that alters its ability to bind the target protein.

41. The method of claim 40, wherein the light-responsive domain is a light oxygen voltage (LOV) domain.
42. The method of claim 41, wherein the LOV2 domain is the LOV2 domain from *Avena sativa* Phototropin 1.

43. The method of claim 40, 41 or 42, wherein the peptide component is a therapeutic protein or peptide.
44. The method of any one of claims 40-43, further comprising determining the nucleic acid sequence of the coding sequence of the fusion protein or the amino acid sequence of the fusion protein expressed by the one or more cells.
45. The method of any one of claims 40-44, further comprising generating the one or more cells by transforming plasmids generated using random transposon-mediated insertion of the coding sequence of the light-responsive domain into the coding sequence of the biopharmaceutical into the one or more cells.
46. The method of any one of claims 40-45, comprising providing a library of cells, each cell expressing a fusion protein intended to bind a target protein and comprising a light-responsive domain and a heterologous peptide component, and exposing immobilized target protein to the library of cells.
47. A method of purifying a target protein from a cell lysate, the method comprising:
 - providing a substrate comprising an immobilized fusion protein intended to bind a target protein, the fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein exposure of the fusion protein to light induces a conformational change that alters its ability to bind the target protein; and
 - exposing the substrate to the cell lysate in the presence of light under conditions sufficient to induce binding of the target protein and the fusion protein;
 - and
 - eluting the target protein in the absence of light, wherein the fusion protein binds the target protein upon exposure to light and dissociates from the target protein in the absence of light, thereby purifying the target protein; or
 - exposing the substrate to the cell lysate in the absence of light under conditions sufficient to induce binding of the target protein and the fusion protein;
 - and

eluting the target protein in the presence of light, wherein the fusion protein binds the target protein in the absence of light and dissociates from the target protein upon exposure to light, thereby purifying the target protein.

48. The method of claim 47, wherein the light-responsive domain is a light oxygen voltage (LOV) domain.
49. The method of claim 48, wherein the LOV domain is the LOV2 domain from *Avena sativa* Phototropin 1.
50. The method of any one of claims 47-49, wherein the target protein comprises a purification tag and wherein the immobilized fusion protein binds the purification tag on the target protein under conditions sufficient to induce binding.
51. The method of any one of claims 47-50, wherein the substrate comprises the immobilized fusion protein conjugated to a resin.
52. The method of claim 51, wherein the resin is in a purification column.
53. A chimeric receptor (CR), comprising an extracellular antigen-binding domain, a transmembrane domain and an intracellular signaling domain, wherein the extracellular binding domain comprises a light-responsive domain and a heterologous peptide component, wherein exposure of the CR to light induces a conformational change in the extracellular binding domain that alters an activity of the extracellular binding domain, wherein the activity is an *in vivo* extracellular binding activity.
54. An isolated cell expressing the CR of claim 53.
55. The isolated cell of claim 54, wherein the cell is a lymphocyte.
56. The isolated lymphocyte of claim 55, where the lymphocyte is a T lymphocyte.
57. A fusion protein comprising a light-responsive domain and a heterologous peptide component, wherein the heterologous peptide component is selected from the group consisting of a nanobody, a monobody, an antibody, a designed ankyrin repeat protein (DARPin),), a cytokine, a growth factor, a peptide hormone, an antimicrobial peptide, and a peptide toxin, wherein exposure of the fusion protein to light induces a

conformational change in the fusion protein that alters an activity of the fusion protein, wherein the activity is a binding activity.

58. A method of delivering a fusion protein to a cell, comprising contacting the cell with the fusion protein of claim 57, wherein the fusion protein is delivered intracellularly.
59. A method of delivering a fusion protein to a cell, comprising externally contacting the cell with a nucleic acid encoding the fusion protein of claim 57.
60. The method of claim 59, wherein the nucleic acid encoding the fusion protein is in a viral expression vector.

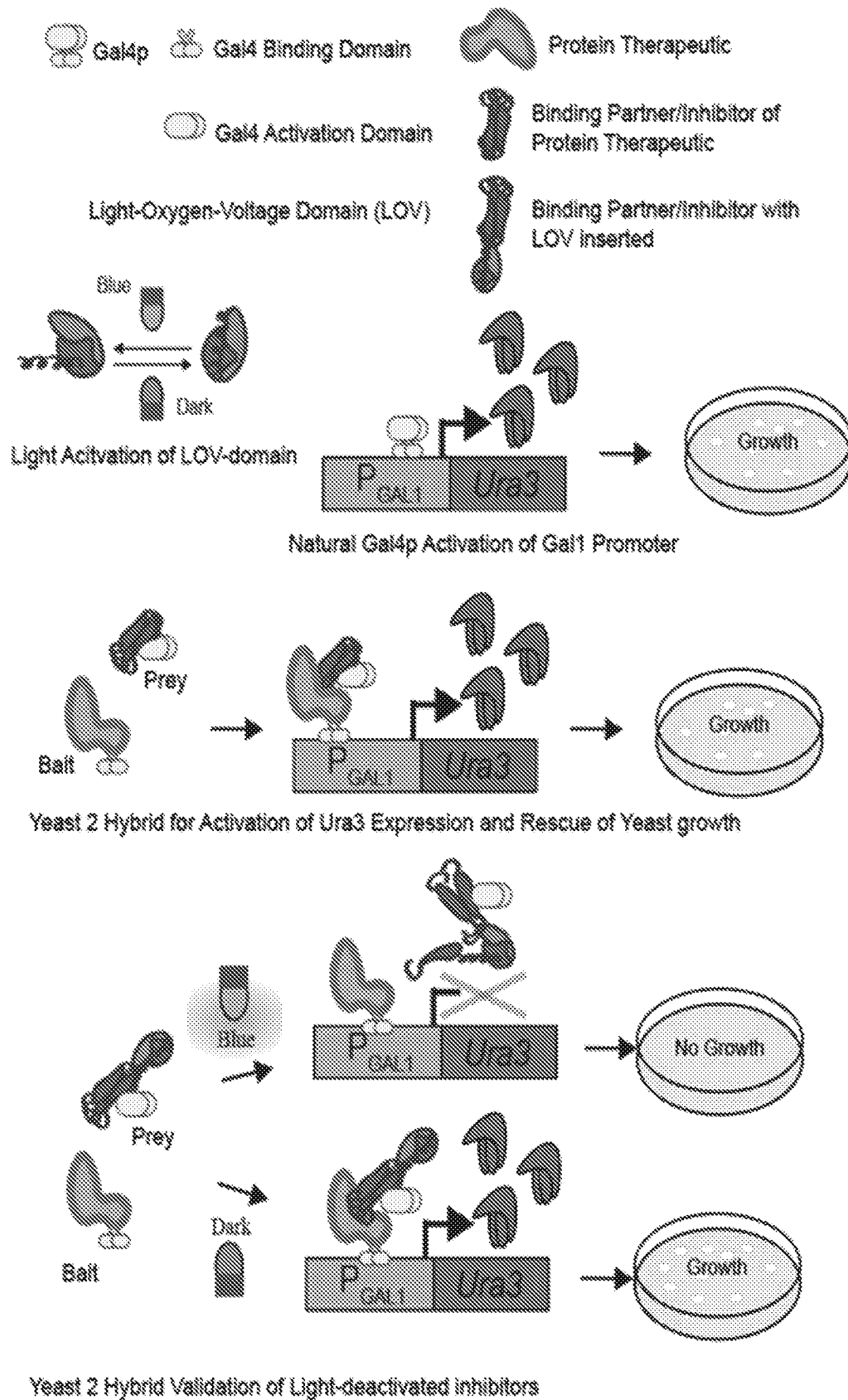


FIG. 1

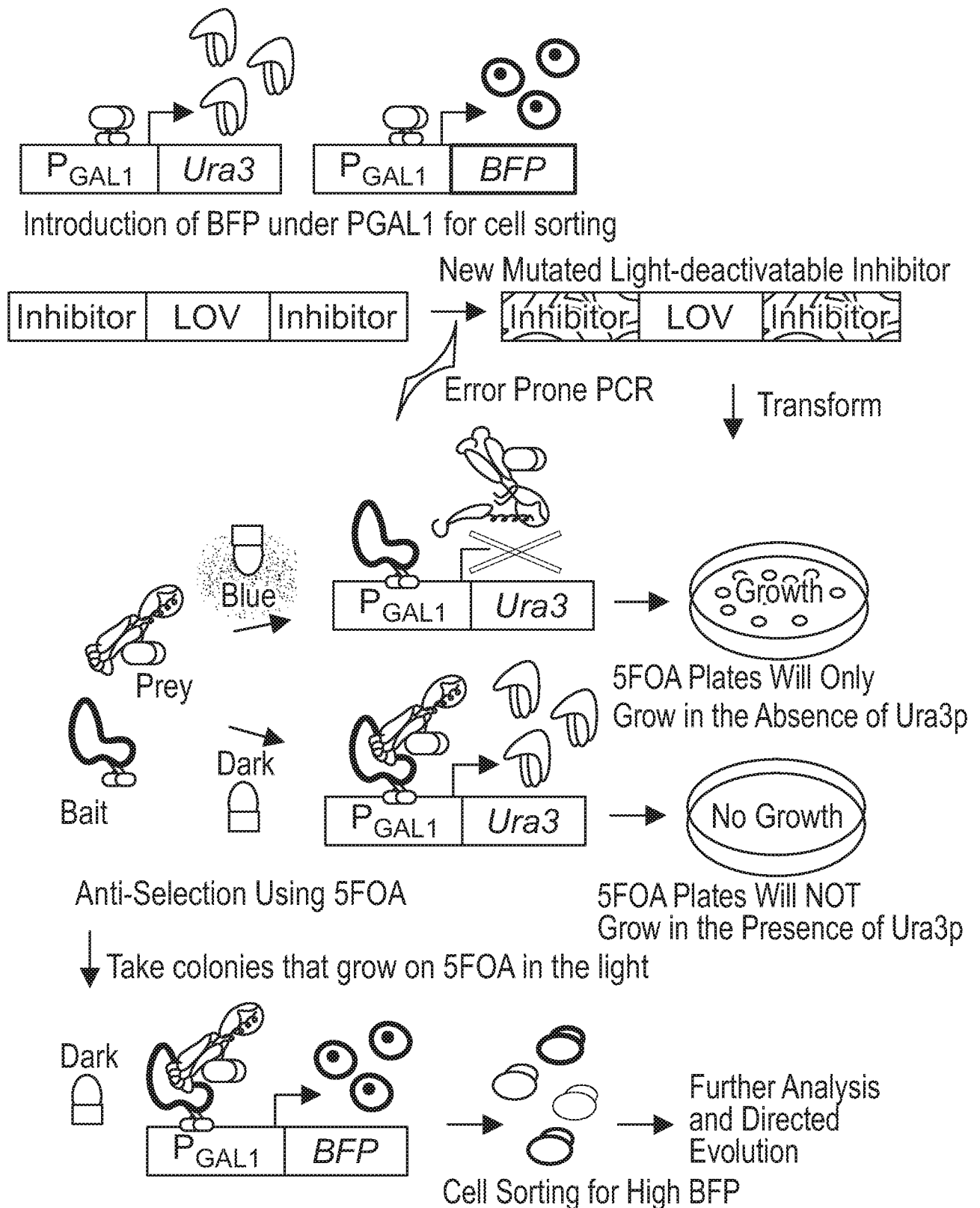


FIG. 2

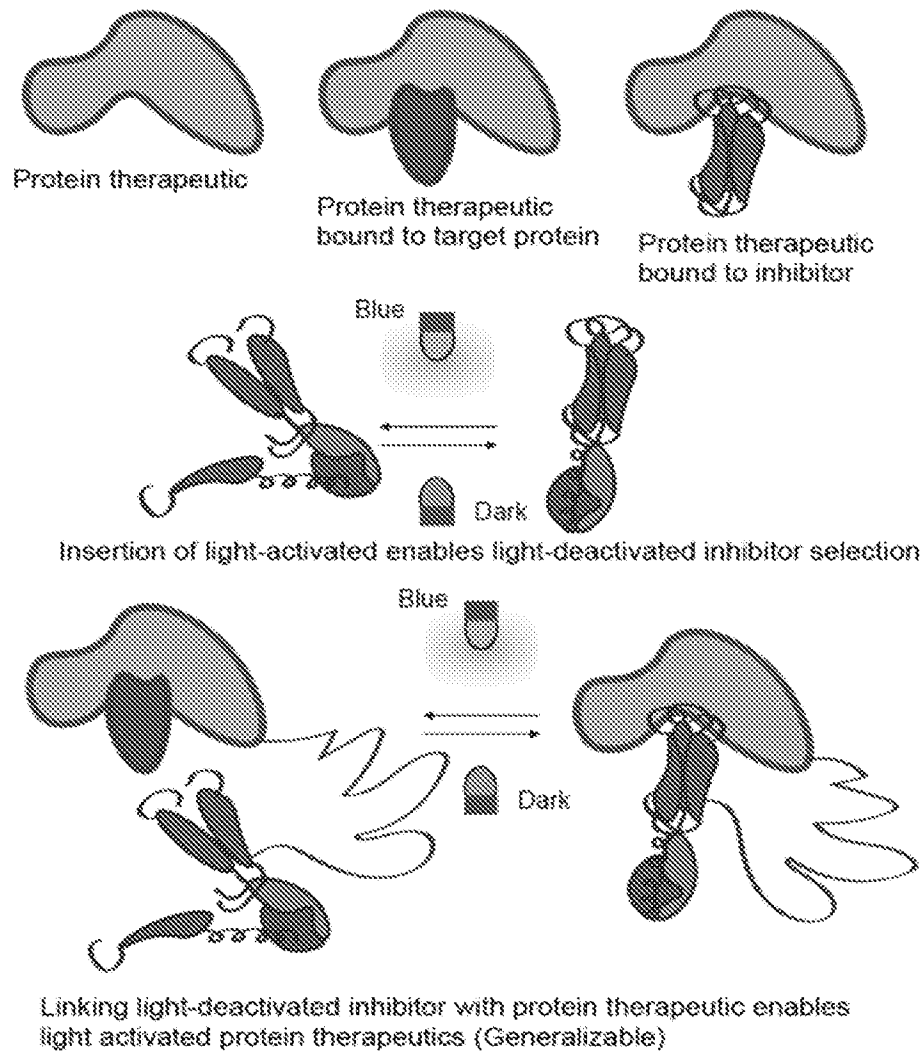


FIG. 3

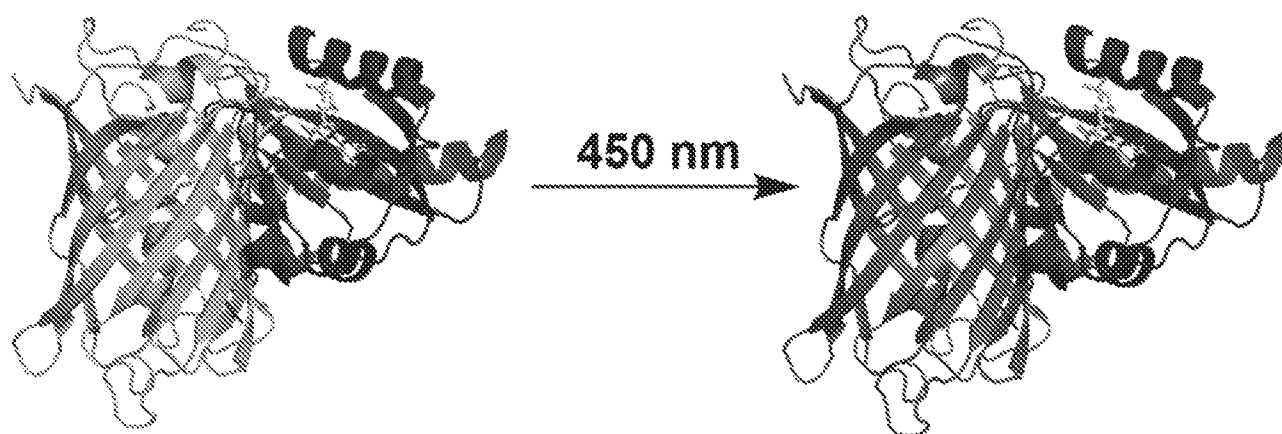


FIG. 4A

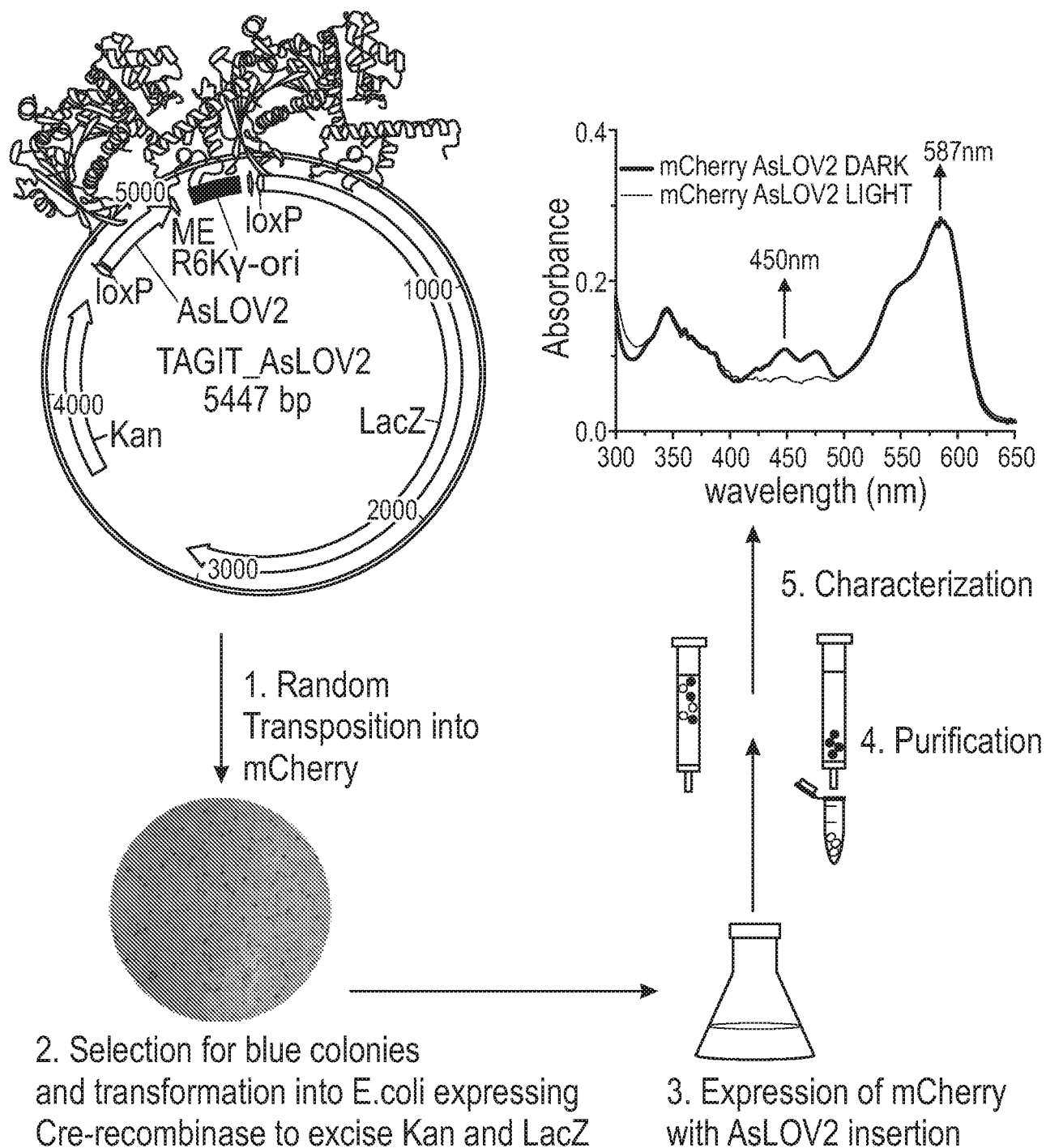


FIG. 4B

6/20

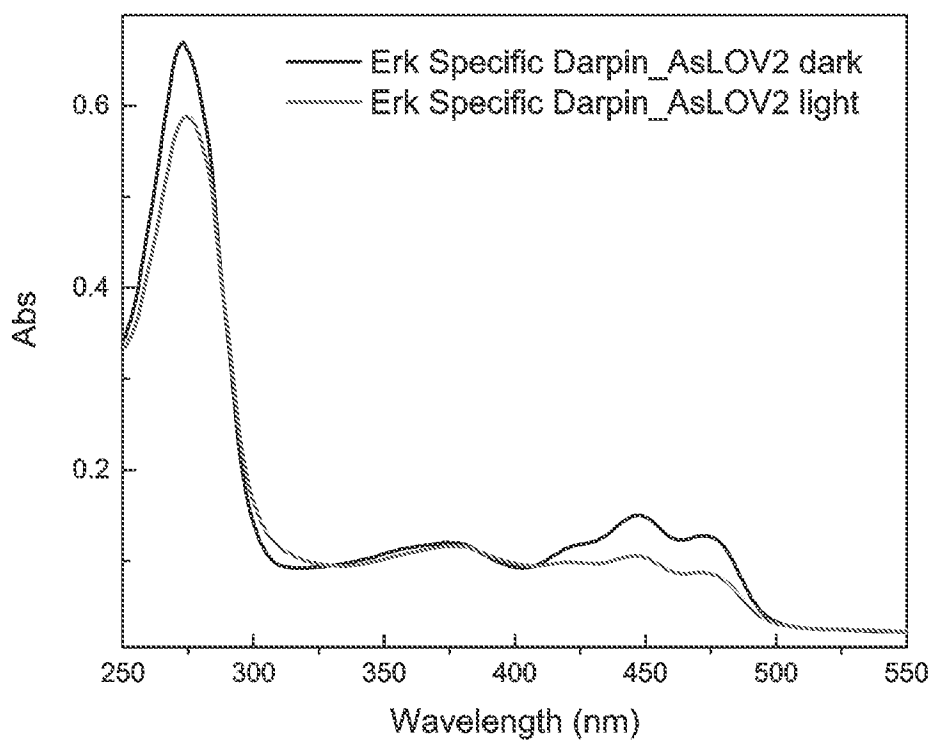


FIG. 5A

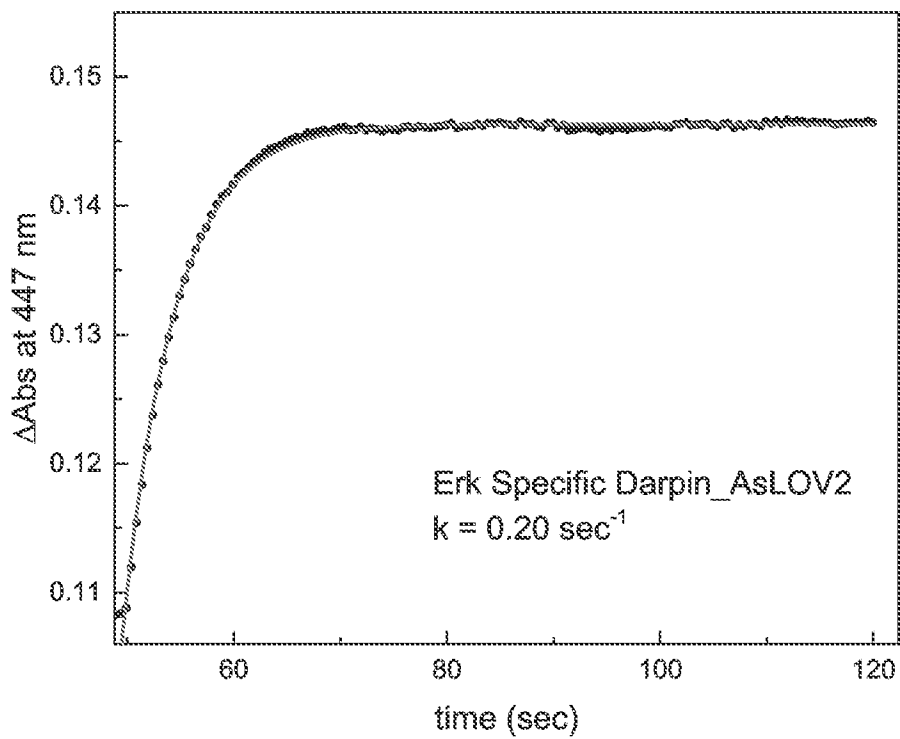


FIG. 5B

7/20

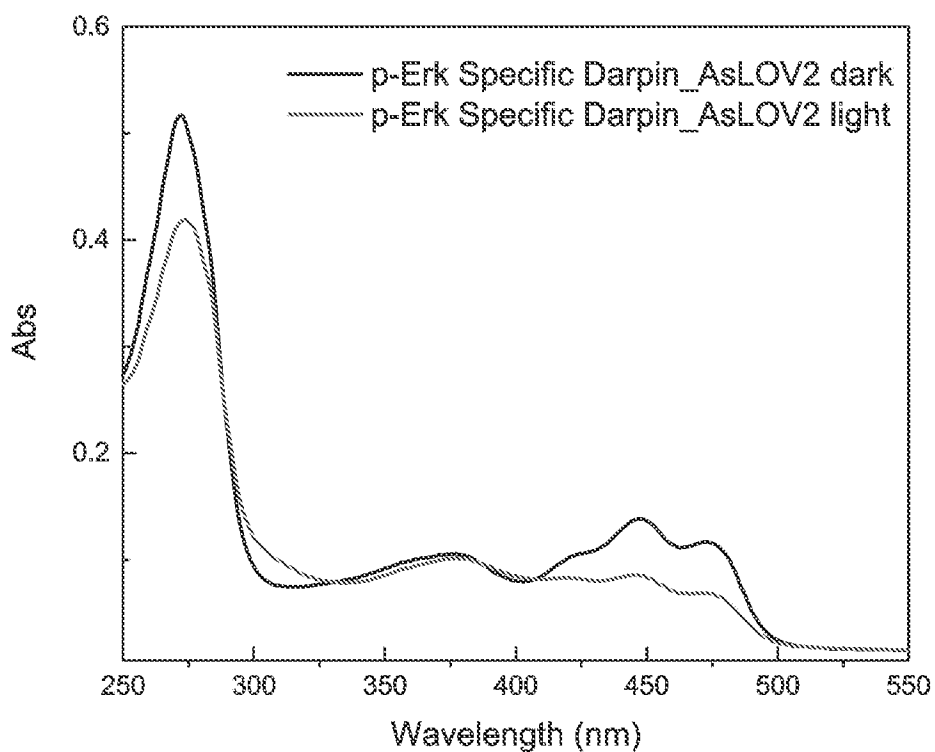


FIG. 6A

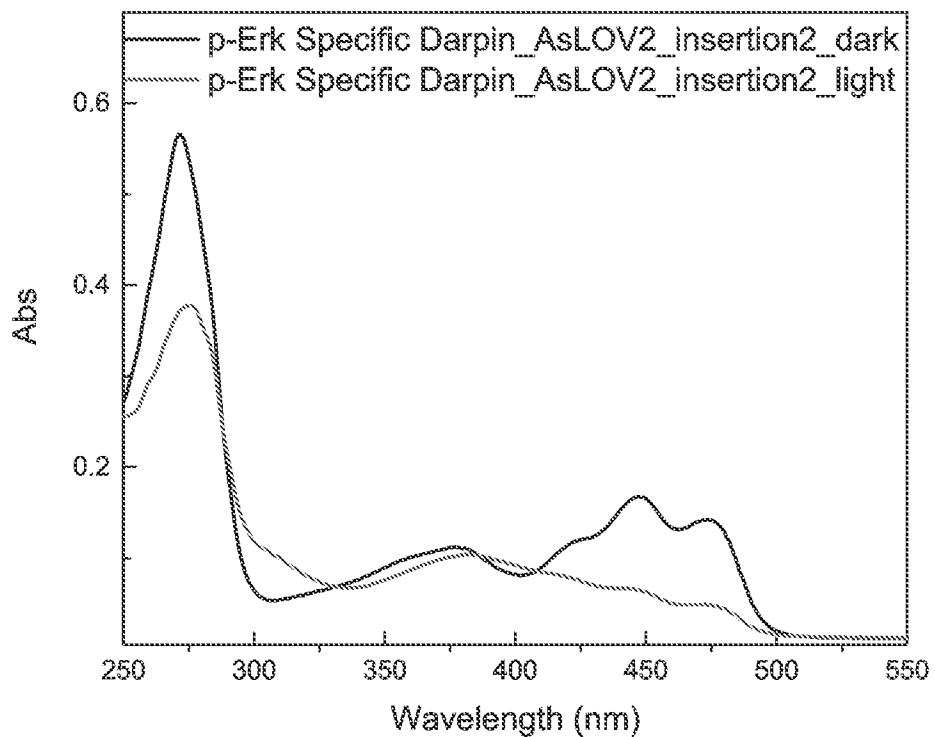


FIG. 6B

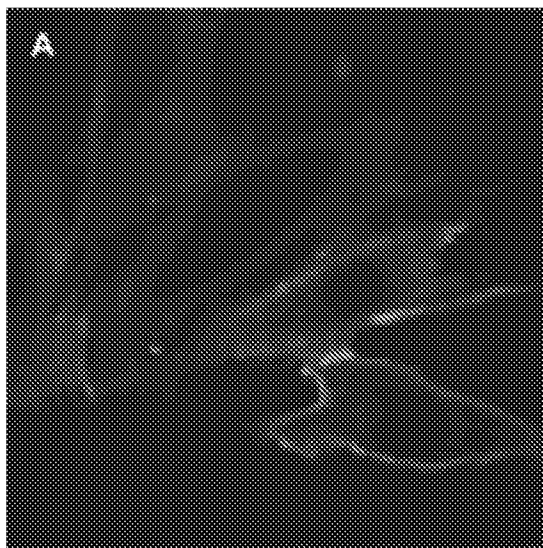


FIG. 7A

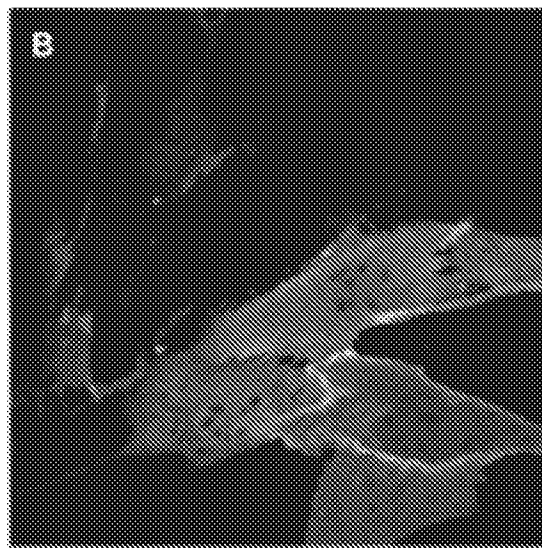


FIG. 7B

FIGS. 7A-7B

Light Induced Binding

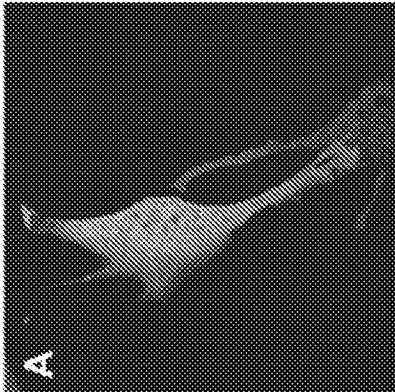


FIG. 8A

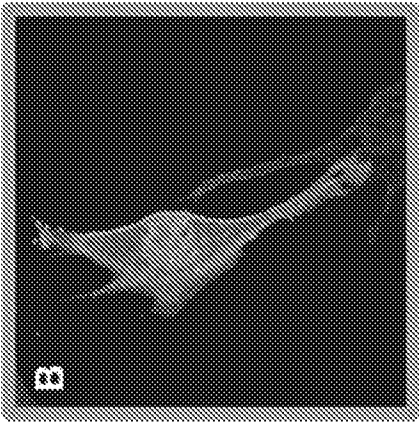


FIG. 8B

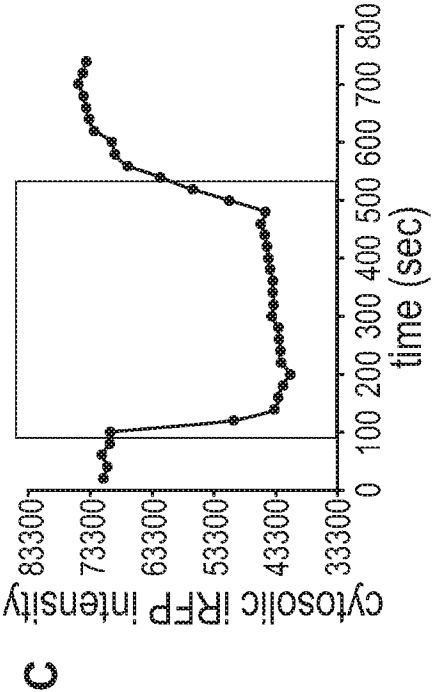


FIG. 8C

Light Induced Dissociation

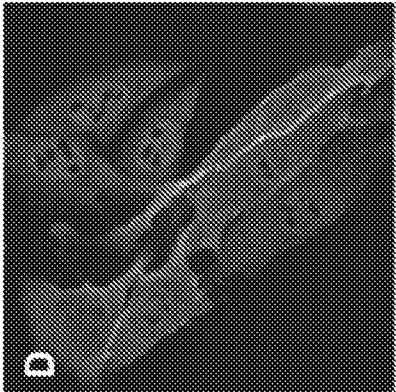


FIG. 8D

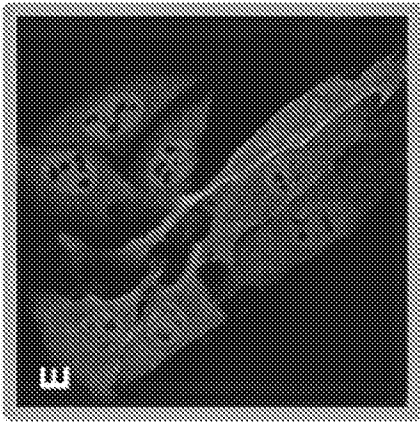


FIG. 8E

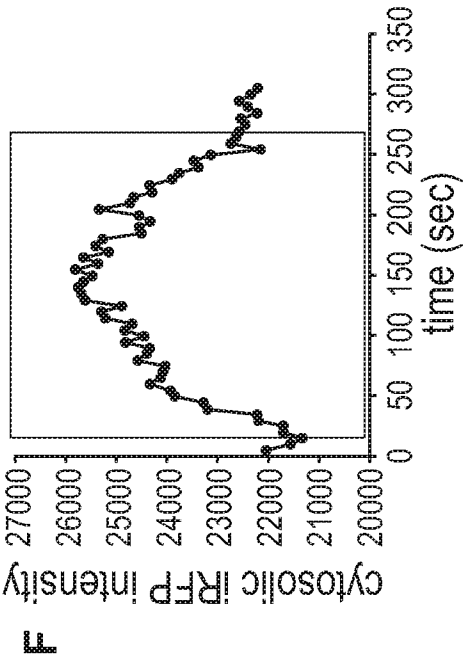
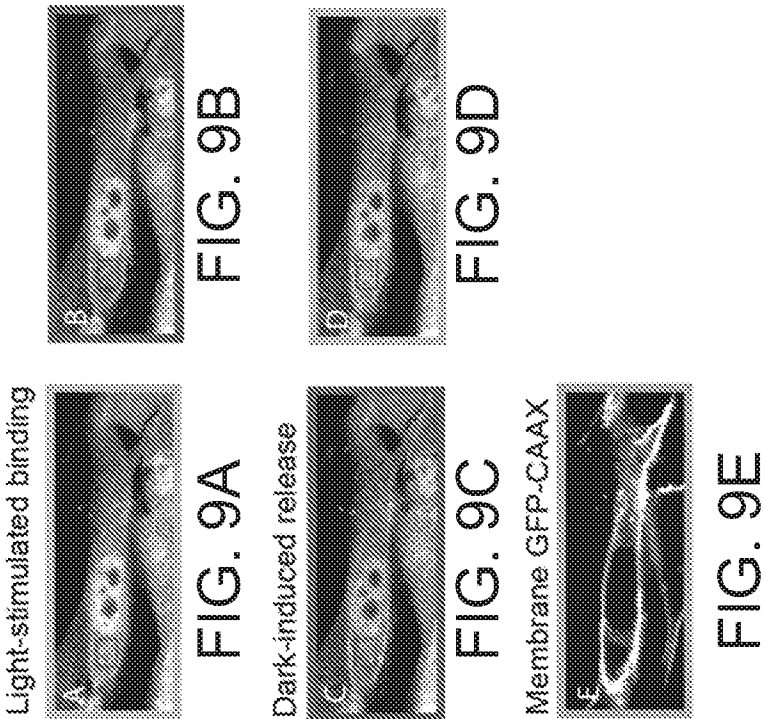


FIG. 8F

FIGS. 8A-8F



F

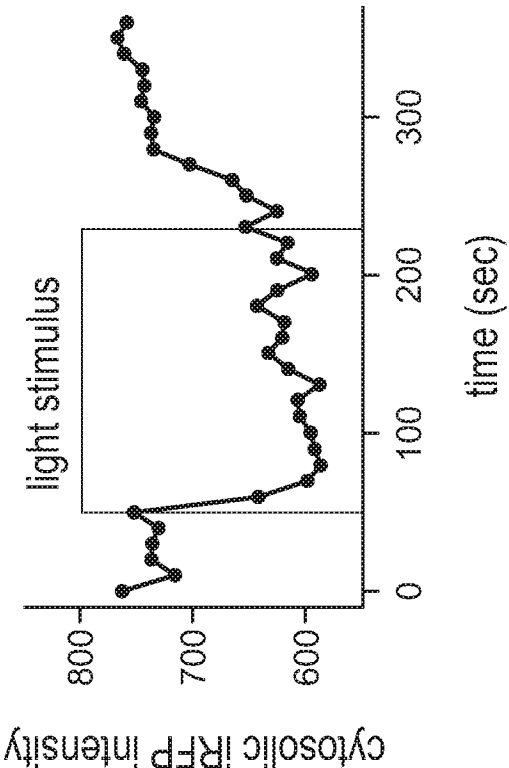


FIG. 9F

FIGS. 9A-9F

Light Induced Dissociation

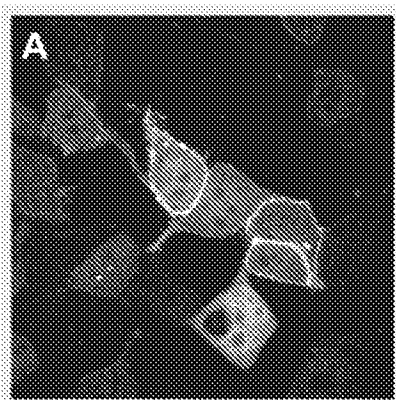


FIG. 10A

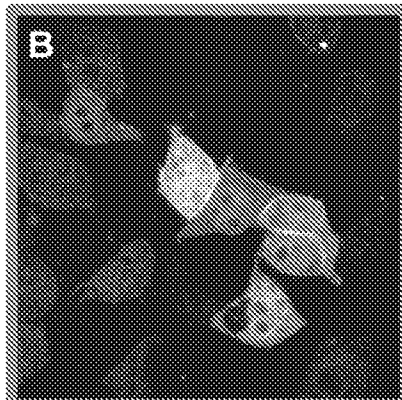


FIG. 10B

FIGS. 10A-10B

Light Induced Binding

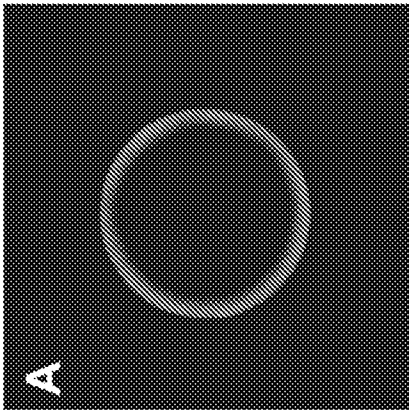


FIG. 11A

Light Induced Dissociation

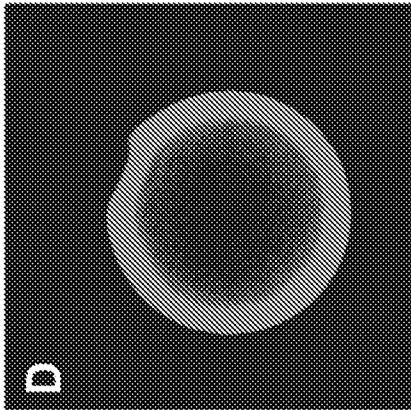


FIG. 11D

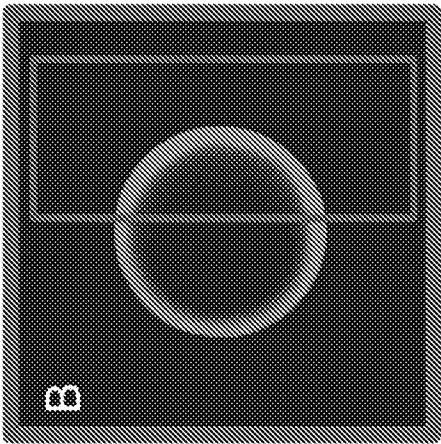


FIG. 11B

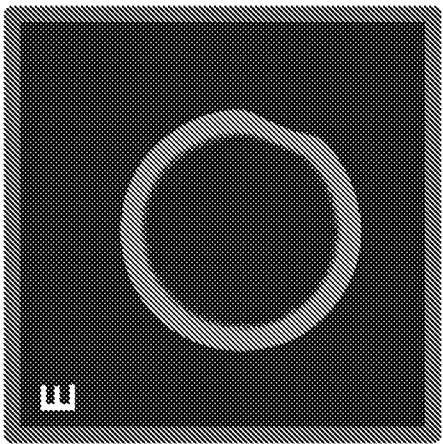


FIG. 11E

FIGS. 11A-11F

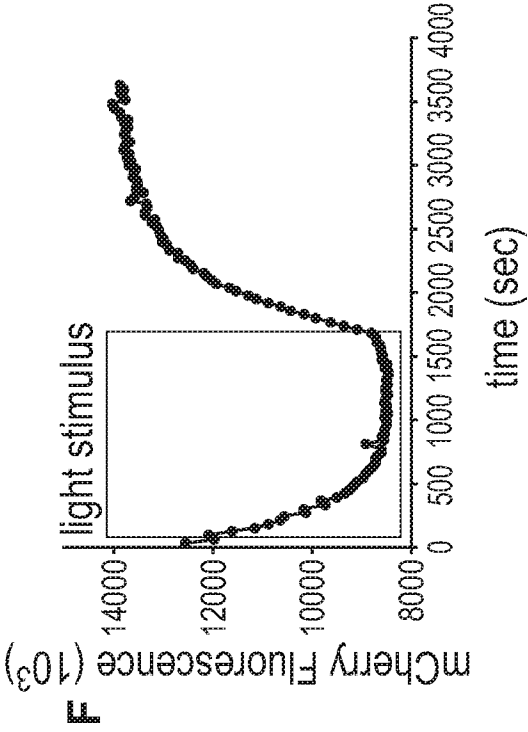
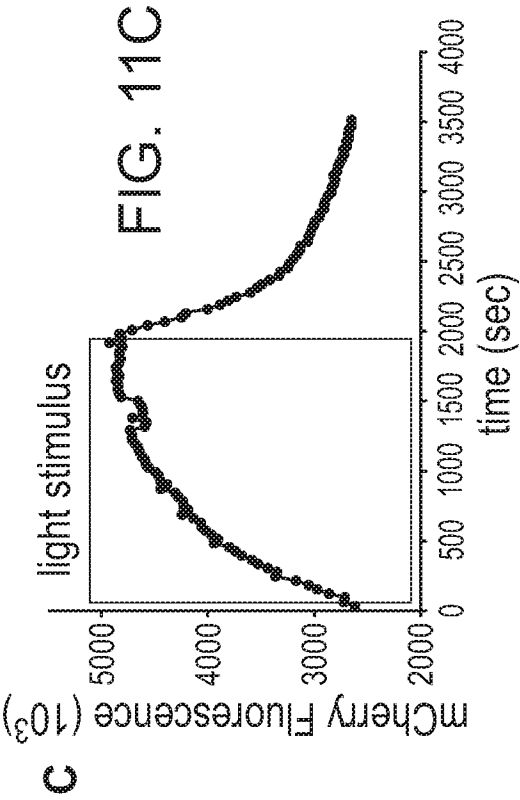




FIG. 12

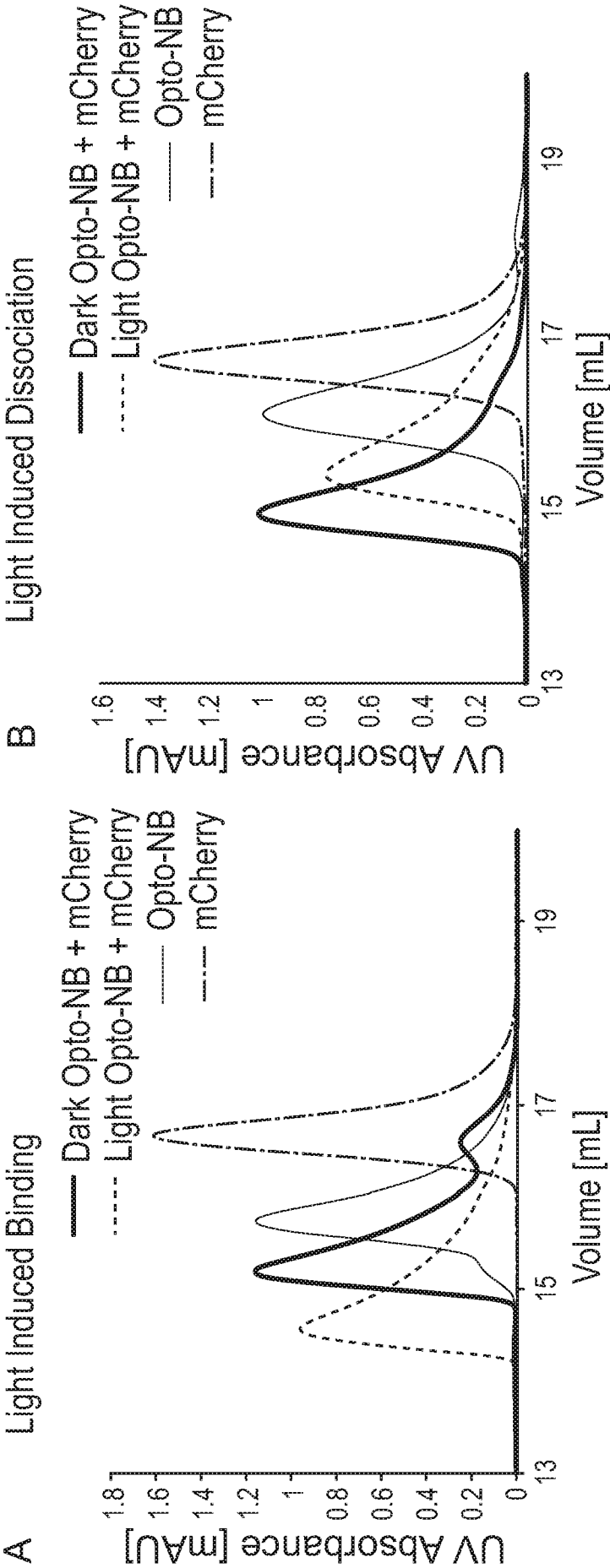


FIG. 13B

FIG. 13A

FIGS. 13A-13B

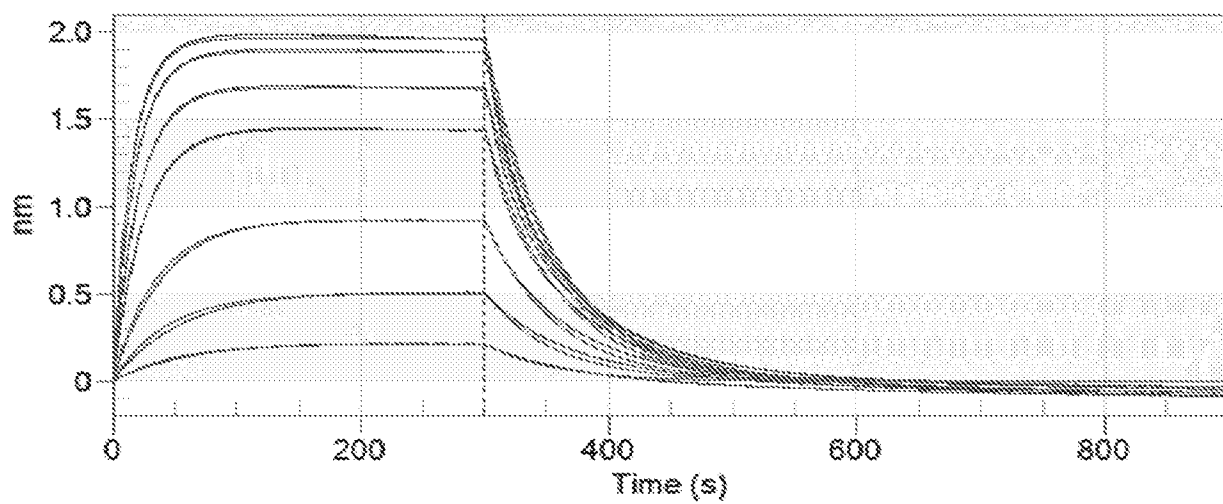


FIG. 14

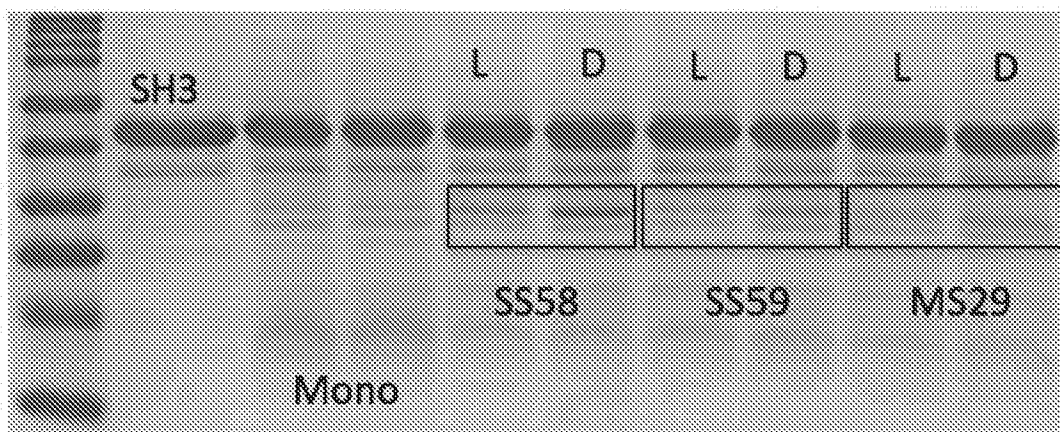


FIG. 15

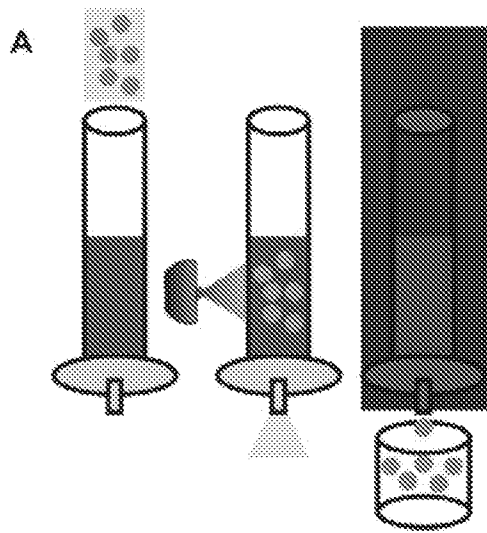


FIG. 16A

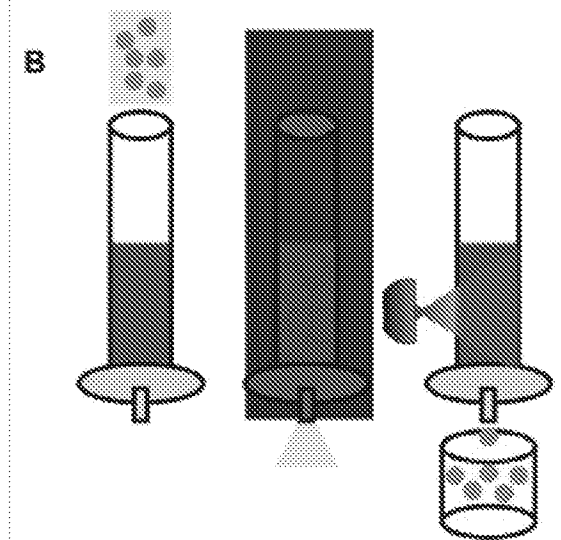


FIG. 16B

FIGS. 16A-16B

Light Induced Binding

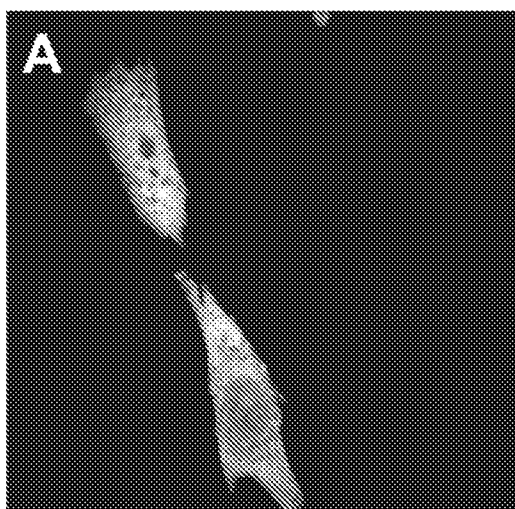


FIG. 17A

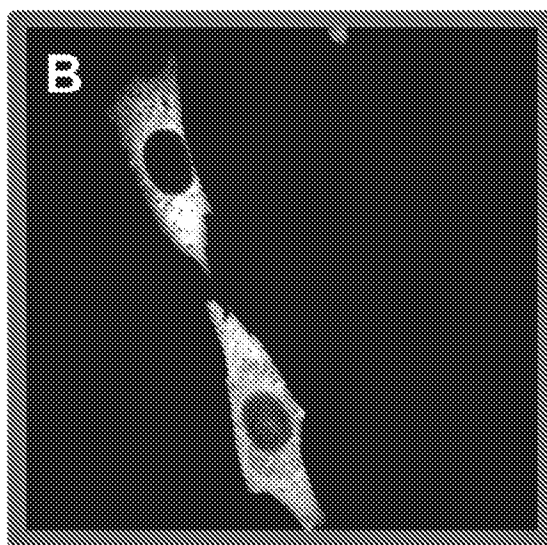


FIG. 17B

Light Induced Dissociation

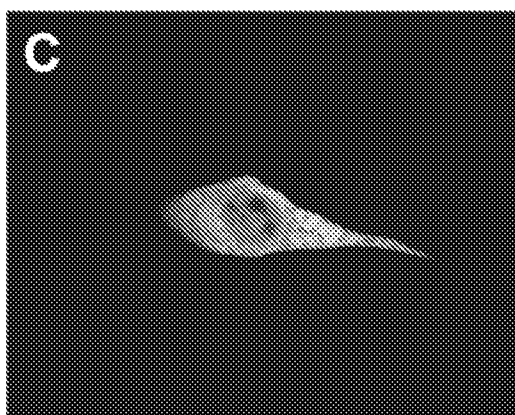


FIG. 17C

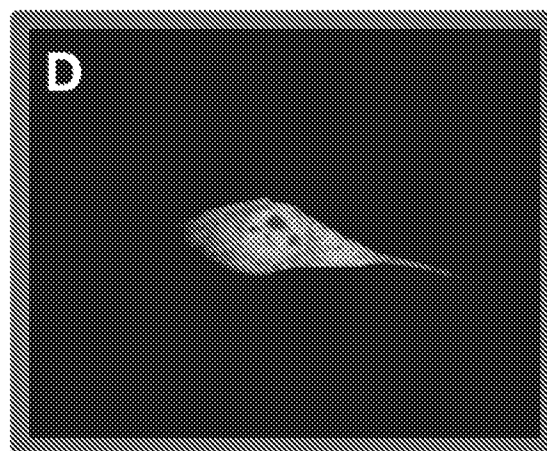


FIG. 17D

FIGS. 17A-17D

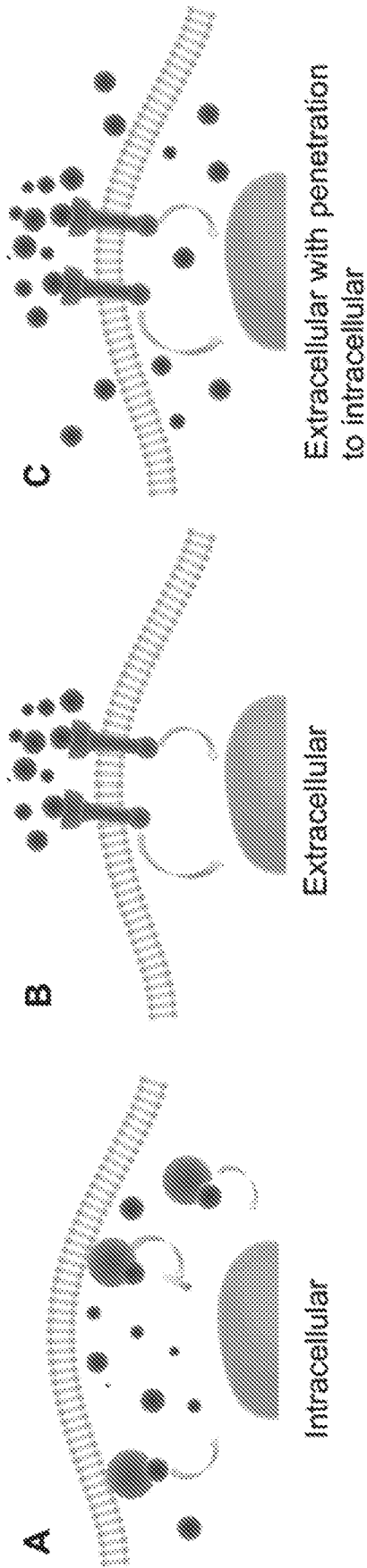


FIG. 18A

FIG. 18B

FIG. 18C

FIGS. 18A-18C

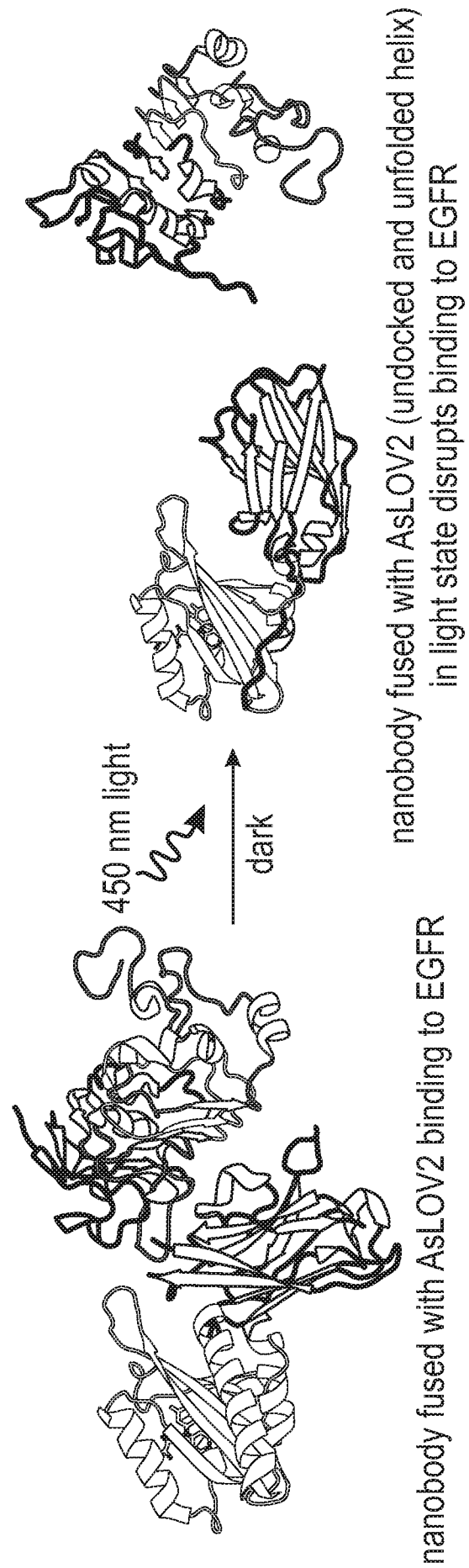


FIG. 19

FIG. 20A

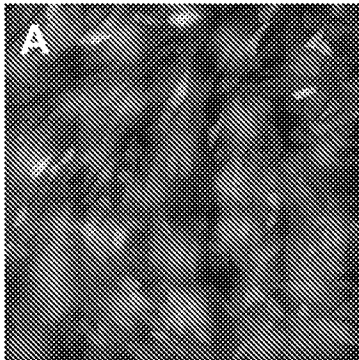


FIG. 20B

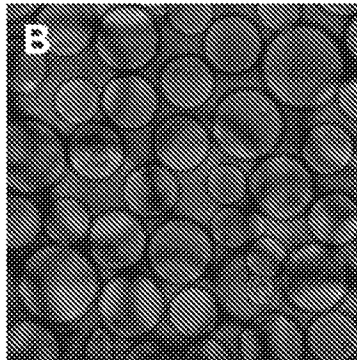
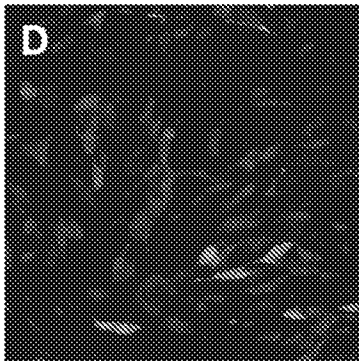
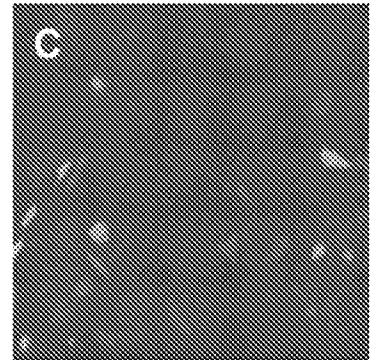
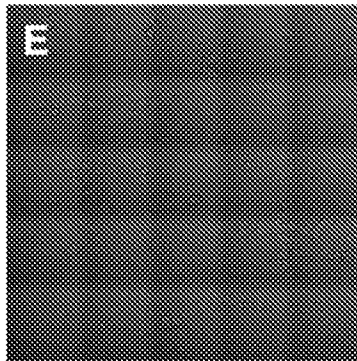


FIG. 20C



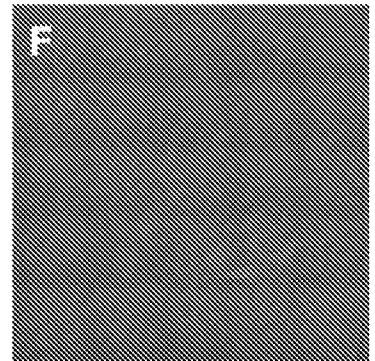
Cells containing:
Gal4_UAS_tBFP_PGK_tRFP
+
NB_synNotch_Gal4VP64

FIG. 20D



+/- mCherry Beads

FIG. 20E



incubate → check tBFP expression

FIG. 20F

FIGS. 20A-20F

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/063447

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K14/555 C07K16/00 C07K16/28 C07K7/08 C07K14/00 C07K14/415 C07K14/435 C07K14/47 C07K14/475 C12N9/00 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/002977 A2 (UNIV NORTH CAROLINA [US]; HAHN KLAUS [US]; WU YI [US]; KUHLMAN BRIAN []) 6 January 2011 (2011-01-06) paragraphs [0007], [0014], [0018], [0022], [0023], [0072] - [0081], [0096], [0110], [0124]; claims 1-102; figure 1 -----	1-3, 12-15, 18,21
X	TOETTCHER J.E. ET AL.: "Light-based feedback for controlling intracellular signaling dynamics", NATURE METHODS, vol. 8, no. 10, October 2011 (2011-10), pages 837-839, -Methods, XP002789197, DOI: 10.1038/NMETH.1700 the whole document -----	1-3, 12-15, 18,21
☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 25 February 2019		Date of mailing of the international search report 06/05/2019
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Galli, Ivo

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/063447

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-3, 12-15, 18, 21(all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-3, 12-15, 18, 21(all partially)

An isolated protein comprising a "light switch" and a heterologous peptide component, preferably a (generic) therapeutic peptide or protein, wherein the "light switch" responds to blue light and causes a conformational change that alters the activity of the heterologous peptide, said activity being in vitro binding activity.

2. claims: 1-3, 12-15, 18, 21(all partially)

An isolated protein comprising a "light switch" and a heterologous peptide component, preferably a (generic) therapeutic peptide or protein, wherein the "light switch" responds to blue light and causes a conformational change that alters the activity of the heterologous peptide, said activity being in vivo extracellular binding activity.

3. claims: 20, 22

An isolated protein comprising a "light switch" and a heterologous peptide component, preferably a (generic) therapeutic peptide or protein, wherein the "light switch" responds to blue light and causes a conformational change that alters the activity of the heterologous peptide, said activity being in vivo intracellular binding activity, achieved by intracellular delivery via a vector or via a cell-penetrating peptide incorporated in the fusion protein.

4. claims: 23-25

A method of altering an activity of a fusion protein comprising a "light switch" and a heterologous peptide component.

5. claims: 26, 27, 29, 31

A method of treating a subject using a fusion protein comprising a "light switch" and a therapeutic component.

6. claims: 33-39

A method of identifying a fusion protein comprising a "light switch" and a heterologous peptide component using a phage library

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. claims: 40-46

A method of identifying a fusion protein comprising a "light switch" and a heterologous peptide component using a cell surface expression library

8. claims: 47-52

A method of purifying a target protein from a cell lysate by fusing it to a light switch and having it bind.

9. claims: 53-56

A regulatable CAR, wherein the extracellular antigen-binding domain is fused to a "light switch" to make binding regulatable. Corresponding cells (CAR-T).

10. claims: 4(completely); 1-3, 12-15, 18, 21, 57-60(partially)

As ITEM 1 or 2, but wherein the heterologous peptide is a cytokine and in particular interferon

11. claims: 5(completely); 1-3, 12-15, 18, 21(partially)

As ITEM 1 or 2, but wherein the heterologous peptide is ipilimumab

12. claims: 6(completely); 1-3, 12-15, 18, 21, 57-60(partially)

As ITEM 1 or 2, but wherein the heterologous peptide is a peptide toxin, and in particular conotoxin

13. claims: 7(completely); 1-3, 12-15, 18, 21, 57-60(partially)

As ITEM 1 or 2 or 3, but wherein the heterologous peptide is a nanobody, a monobody or an antibody

14. claims: 8(completely); 1-3, 12-15, 18, 21, 57-60(partially)

As ITEM 1 or 2 or 3, but wherein the heterologous peptide is a growth factor

15. claims: 9(completely); 1-3, 12-15, 18, 21, 57-60(partially)

As ITEM 1 or 2 or 3, but wherein the heterologous peptide is a designed DARPIn

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

16. claims: 10(completely); 1-3, 12-15, 18, 21, 57-60(partially)

As ITEM 1 or 2 or 3, but wherein the heterologous peptide is
an antimicrobial peptide

17. claims: 11(completely); 1-3, 12-15, 18, 21(partially)

As ITEM 1 or 2, but wherein the heterologous peptide is an
enzyme

18. claims: 1-3, 12-15, 18, 21, 57-60(all partially)

As ITEM 1 or 2 or 3, but wherein the heterologous peptide is
a peptide hormone

19. claims: 16, 28

As ITEM 1 or 2, but wherein exposure to light decreases the
activity

20. claims: 17, 30

As ITEM 1 or 2 or 5, but wherein the light is visible light.

21. claims: 19, 32

As ITEM 1 or 2 or 5, but wherein the light is infrared
light.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2018/063447

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011002977 A2	06-01-2011	US 2012165204 A1 WO 2011002977 A2	28-06-2012 06-01-2011
