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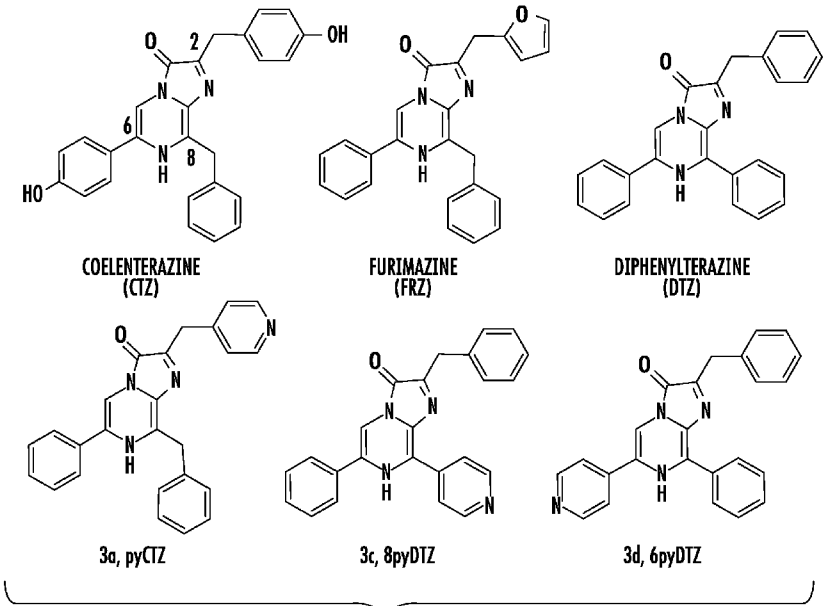

FIG. 1

(57) Abstract: Provided herein are chemically modified luciferase substrates for spectrally shifted emission and enhanced water solubility. Provided herein are engineered luciferases. Moreover, provided herein are new ATP-independent bioluminescent reporters which have improved biochemical and photophysical properties and are expected to have broad applications. Finally, provided herein are spectral-resolved triple-color bioluminescent systems, suitable for flexible and convenient approaches to monitor multiple biological events in either qualitative or quantitative manners.

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DESCRIPTION

ATP-INDEPENDENT BIOLUMINESCENT REPORTER VARIANTS TO IMPROVE *IN VIVO* IMAGING

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Application Serial No. 62/811,129, filed February 27, 2019, the disclosure of which is incorporated herein by reference in its entirety.

GRANT STATEMENT

This invention was made with government support under Grant Nos. R01GM118675 and R01GM129291 awarded by the National Institute of General Medical Sciences of the National Institutes of Health, and Grant No. CHE1750660 awarded by the National Science Foundation. The government has certain rights in the invention.

TECHNICAL FIELD

The presently disclosed subject matter relates to ATP-independent bioluminescent reporter variants to improve *in vivo* imaging. In some embodiments, methods, kits, and compositions for enhanced bioluminescent imaging are provided.

BACKGROUND

In the past few decades, fluorescence imaging has evolved quickly and become a dominant visualization method for live-cell studies.[1] However, fluorescence imaging has several limitations, such as photobleaching, phototoxicity, and poor tissue penetration, largely due to the need for light excitation. Unlike fluorescence, bioluminescence produces photons *via* enzyme-catalyzed biochemical reactions in which luciferases oxidize their corresponding small-molecule substrates (also referred to as luciferins) to generate excited-state emitters. As a result, bioluminescence signals glow essentially on dark background, leading to excellent signal-to-background ratios. Moreover, even though the spatiotemporal resolution of bioluminescence imaging (BLI) is usually worse than that of fluorescence imaging, the emitted photons can escape through several centimeters of tissue.[2] BLI is thus especially suited for diverse, noninvasive *in vivo* imaging applications.[3-5]

Photinus pyralis firefly luciferase (FLuc) and D-luciferin (λ_{max}: 563 nm) constitute the most widely used luciferase-luciferin pair for *in vivo* BLI. Recently, research has been performed to develop FLuc and D-luciferin derivatives for brighter and more red-shifted emission. In particular, an Akaluc-AkaLumine luciferase-luciferin pair with near-infrared (NIR) emission (λ_{max}: 650 nm) was reported for highly sensitive deep-tissue *in vivo* BLI.[6] Despite the progress, AkaLumine has been shown to induce cytotoxicity.[7-9] Moreover, FLuc, Akaluc, and other insect luciferases consume ATP for photon production; the bioluminescence reaction between FLuc and D-luciferin reduced the intracellular ATP-to-ADP ratio of live mammalian cells from greater than 40:1 to about 20:1,[8] suggesting metabolic disruption by all ATP-dependent luciferases. Because ATP is required for the activation of the luciferin substrates, this metabolic disruption issue cannot be addressed by simply improving insect luciferases and the corresponding substrates.

In contrast to insect luciferases, a large family of marine luciferases and photoproteins, such as *Renilla* luciferase (RLuc), *Gaussia* luciferase (GLuc), *Oplophorus* luciferase (OLuc), and aequorin, are ATP-independent and use coelenterazine (CTZ, Figure 1) as their native substrate for bioluminescence production.[10] The 19kDa catalytic domain of OLuc[11] was recently engineered into NanoLuc, which has a fast enzyme turnover and produces intense blue bioluminescence (λ_{max}: 456 nm) in the presence of a synthetic CTZ analog, furimazine (FRZ, Figure 1).[12] To expand the color palette, NanoLuc was further engineered into teLuc, which emits red-shifted photons (λ_{max}: 502 nm) when paired with a synthetic substrate diphenylterazine (DTZ, Figure 1).[7] Since biological tissues significantly absorb and scatter short-wavelength photons,[13] NanoLuc and teLuc have been fused to fluorescent proteins, resulting in Antares, Antares2, and enhanced Nano-Lanterns for further red-shifted emission *via* bioluminescence resonance energy transfer (BRET).[7, 14, 15] The water solubility of CTZ, FRZ, and DTZ is adequate for protein- and cell-based assays, because their solubility is already higher than typical substrate concentrations in these *in vitro* assays. However, *in vivo* applications of existing ATP-independent bioluminescent reporters are greatly hindered by the low solubility of CTZ, FRZ, and DTZ. Small animals, such as mice, can only tolerate a small injection volume. To enhance bioluminescence brightness by delivering more luciferin substrate, hydroxypropyl-β-cyclodextrin, polyethylene glycols (PEGs), or other organic cosolvents are typically used to formulate CTZ, FRZ, or DTZ for *in vivo* administration.[7, 14, 16, 17] These formulation ingredients are not biologically inert and can cause irritation or biotoxicity. It is also practically difficult to

intravenously (i.v.) inject these highly viscous solutions into small animals. Furthermore, it is still of great interest to further red-shift marine luciferases for enhanced tissue penetration.

Taken together, there is a long-felt need for new and improved ATP-independent bioluminescent reporters, which have improved biochemical and photophysical properties and broad applicability. Such needs are address by the present disclosure.

SUMMARY

This summary lists several embodiments of the presently disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently disclosed subject matter, whether listed in this summary or not. To avoid excessive repetition, this summary does not list or suggest all possible combinations of such features.

In some embodiments, provided herein are bioluminescent proteins, comprising a substituted luciferase polypeptide comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 2 with amino acid substitutions at one or more of positions corresponding to positions 4, 18, 19, 27, 28, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2. In some embodiments, the bioluminescent proteins comprise amino acid substitutions at positions corresponding to positions 18, 19, 27 and 28, and further comprise one or more amino acid substitutions at one or more of positions corresponding to positions 4, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2. In some embodiments, the bioluminescent protein comprise an amino acid sequence having at least 95% homology to SEQ ID NO: 2 with amino acid substitutions at least eight positions selected from positions corresponding to positions 4, 18, 19, 27, 28, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2. In some embodiments, a luciferase polypeptide is substituted at positions corresponding to positions 4, 18, 19, 27, 28, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2. In some embodiments, the luciferase polypeptide can comprise SEQ ID NO: 3.

In some embodiments, bioluminescent proteins disclosed herein can comprise a fluorescent protein connected to the substituted luciferase polypeptide. The fluorescent protein can be connected to the substituted luciferase polypeptide so as to allow

bioluminescence resonant energy transfer (BRET) between the substituted luciferase polypeptide and the fluorescent protein. A substituted luciferase polypeptide can comprise an amino acid sequence having at least 90% homology to SEQ ID NO: 5, or can comprise the amino acid sequence of SEQ ID NO: 5.

Provided herein are bioluminescent proteins comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, or SEQ ID NO: 12. Nucleic acids encoding these bioluminescent proteins are also provided. Vectors encoding the nucleic acids are provided, as are expression vectors encoding the nucleic acids and functionally connected to a promoter. In some aspects, cell lines containing the expression vector and expressing the bioluminescent protein are provided. Additionally, non-human cells transfected with the expression vector and expressing the bioluminescent protein are provided.

Combinations comprising a bioluminescent protein and a luciferin are provided. The luciferin can comprise a luciferin selected from pyCTZ, 6pyDTZ, and 8pyDTZ. Methods of producing luminescence, comprising reacting a luciferin with a disclosed bioluminescent protein, are also provided. In such methods the bioluminescent protein can further comprise a fluorescent protein connected to the substituted luciferase polypeptide so as to allow BRET activity between the substituted luciferase polypeptide and the fluorescent protein.

Provided herein are luciferin compounds comprising an analog of pyridyl coelenterazine (CTZ), pyridyl furimazine (FRZ) and/or a pyridyl diphenylterazine (DTZ). The compounds comprise a water solubility increased by at least about 4-fold as compared to CTZ and/or DTZ, optionally by at least about 10-fold as compared to CTZ and/or DTZ. The compounds can in some aspects comprise a water solubility ranging from about 1,000 μM to about 1,800 μM. The luciferin compounds can comprise a bioluminescence ranging from about 450 λ_{max} (nm) to about 550 λ_{max} (nm), and can be compatible with any luciferase, optionally any ATP-independent luciferase, including for example luciferases comprising a polypeptide comprising SEQ ID NO: 3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

Methods of making a luciferin compound are provided herein, and can include making one or more pyridyl isomer substitutions at a C-2, C-6 and C-8 position of an imidazopyrazinone core.

In some embodiments, provided herein are bioluminescent reporter systems, such systems comprising at least two bioluminescent proteins selected from RLuc8, OpyLuc and Akaluc, and at least two luciferin compounds select from pyOMeCTZ, pyDTZ and AkaLumine. In some aspects, the system comprises bioluminescent proteins consisting of RLuc8, OpyLuc and Akaluc, and luciferin compounds consisting of pyOMeCTZ, pyDTZ and AkaLumine. In some aspects, the bioluminescent proteins and luciferin compounds are provided in pairs as follows: RLuc8 with pyOMeCTZ, OpyLuc with pyDTZ, and Akaluc with AkaLumine. The pairs of bioluminescent proteins and luciferins produce distinct colors of emission across the visible spectrum. In some aspects, RLuc8 paired with pyOMeCTZ yields a bioluminescence of about 380-470 (nm), OpyLuc paired with pyDTZ yields a bioluminescence of about 480-600 (nm), and Akaluc paired with AkaLumine yields a bioluminescence of about 600-750 (nm). In such reporter systems, RLuc8 can comprise a polypeptide sequence comprising SEQ ID NO: 14, OpyLuc can comprise a polypeptide sequence comprising SEQ ID NO: 6, and Akaluc can comprise a polypeptide sequence comprising SEQ ID NO: 16. Nucleic acids, vectors, expression vectors and cell lines encoding and/or expressing the reporter systems are also provided.

In some embodiments, provided herein are methods of monitoring bioluminescence in a subject, the methods comprising providing a subject, establishing in the subject a luciferase expressing cell, wherein the luciferase is selected from the group consisting of LumiLuc, teLuc, RLuc8 and OpyLuc, administering to the subject a luciferin selected from the group consisting of pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ and 8pyDTZ, and measuring bioluminescence in the subject. In some aspects, the subject comprises a tumor or a cancer cell. In some aspects, the luciferase expressing cell comprises a tumor or a cancer cell. In some embodiments, the subject comprises an *in vivo* tumor or cancer model. In some embodiments, the subject is a non-human animal. In some embodiments, the bioluminescence is measured in a tumor or cancer cell in the subject.

Provided herein are cells containing an expression vector for expressing a bioluminescent protein, wherein the bioluminescent protein is selected from the group consisting of LumiLuc, teLuc, RLuc8 and OpyLuc, wherein the expression vector comprises a responsive promoter element, wherein the responsive promoter element is responsive to a stimuli of a cell signaling pathway. Such cells can be cultured *in vitro*.

Provided herein are cell signaling assay systems, the systems comprising cells containing an expression vector for expressing a bioluminescent protein, and a luciferin selected from the group consisting of pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ and 8pyDTZ, wherein the assay is configured to indicate activation of a cell signaling pathway by bioluminescence.

Methods for monitoring a cell signaling pathway are provided, where the methods comprise culturing cells containing an expression vector for expressing a bioluminescent protein, exposing the cell to a luciferin selected from the group consisting of pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ and 8pyDTZ, exposing the cell to a compound of interest, and measuring bioluminescence in the cell, wherein an increase in bioluminescence in the cell is indicative of an activation of the cell signaling pathway in the cell. The compound of interest can comprise a drug candidate compound.

Provided herein are stable cell lines integrated with a series of nucleic acids disclosed herein and expressing the bioluminescent proteins.

Accordingly, it is an object of the presently disclosed subject matter to provide engineered luciferases, modified luciferase substrates and related methods. This and other objects are achieved in whole or in part by the presently disclosed subject matter. Further, an object of the presently disclosed subject matter having been stated above, other objects and advantages of the presently disclosed subject matter will become apparent to those skilled in the art after a study of the following description, Figures, and Examples.

BRIEF DESCRIPTION OF THE FIGURES

The presently disclosed subject matter can be better understood by referring to the following figures. The components in the figures are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the presently disclosed subject matter (often schematically). In the figures, like reference numerals designate corresponding parts throughout the different views. A further understanding of the presently disclosed subject matter can be obtained by reference to an embodiment set forth in the illustrations of the accompanying drawings. Although the illustrated embodiment is merely exemplary of systems for carrying out the presently disclosed subject matter, both the organization and method of operation of the presently disclosed subject matter, in general, together with further objectives and advantages thereof, can be more easily understood by reference to the

drawings and the following description. The drawings are not intended to limit the scope of this presently disclosed subject matter, which is set forth with particularity in the claims as appended or as subsequently amended, but merely to clarify and exemplify the presently disclosed subject matter.

Like numbers refer to like elements throughout. In the figures, the thickness of certain lines, layers, components, elements or features can be exaggerated for clarity. Where used, broken lines illustrate optional features or operations unless specified otherwise.

For a more complete understanding of the presently disclosed subject matter, reference is now made to the below drawings.

Figure 1 includes schematic illustration of the chemical structures of coelenterazine (CTZ), furimazine (FRZ), diphenylterazine (DTZ), pyCTZ (3a in Table 2), 8pyDTZ (3c in Table 2) and 6pyDTZ (3d in Table 2).

Figures 2A and 2B show chemiluminescence (Fig. 2A) and bioluminescence (Fig. 2B) spectra for synthetic CTZ and DTZ analogs described herein. Chemiluminescence was initiated with peroxymonocarbonate generated *in situ*. Bioluminescence was determined with 1 nM teLuc in PBS.

Figures 3A through 3D show the relative bioluminescence intensities of various luciferases. Total signals were integrated for the first 10 min after injection of indicated substrates (the final concentrations were 25 μM) in the presence of 1 nM purified (Fig. 3A) teLuc and (Fig. 3B) NanoLuc, or 10 nM (Fig. 3C) RLuc8 and (Fig. 3D) aequorin in PBS. Values were normalized to the intensity of CTZ in each group.

Figures 4A through 4D show results of efforts to engineer a LumiLuc luciferase. Figure 4A is a schematic of a procedure to derive LumiLuc from teLuc. Figure 4B is an illustration of the putative substrate-binding site and LumiLuc mutations from teLuc. CTZ is shown as spheres and mutated residues are presented in sticks. Figure 4C shows the bioluminescence of pyCTZ, 6pyDTZ, or 8pyDTZ in the presence of either teLuc or LumiLuc. Figure 4D shows normalized bioluminescence emission spectra of pyCTZ, 6pyDTZ, and 8pyDTZ in the presence of LumiLuc.

Figure 5 shows alignments of primary polypeptide sequences of NanoLuc (SEQ ID NO. 1), teLuc (SEQ ID NO. 2), and LumiLuc (SEQ ID NO. 3). teLuc mutations are highlighted. Likewise, all LumiLuc mutations are highlighted. Residues in this figure are numbered according to Protein Date Bank (PDB) ID 5B0U.

Figures 6A through 6C show the comparison of luciferase-luciferin pairs in enzyme-based assays. Figure 6A shows the determination of apparent Michaelis constants (K_M) by substrate titrations (teLuc-DTZ: 9.9 ± 0.9 μM; NanoLuc-FRZ: 9.1 ± 0.6 μM; LumiLuc-8pyDTZ: 4.6 ± 0.6 μM; LumiLuc-pyCTZ: 13.1 ± 0.8 μM; LumiLuc-6pyDTZ: 11.0 ± 1.2 μM). Final concentrations of all enzymes were 20 pM. Substrate concentrations varied from 0.78 to 50 μM, and peak bioluminescence intensities at individual substrate concentrations were used to fit the Michaelis-Menten equation. Figure 6B shows the total bioluminescence over the first 10 min post addition of corresponding luciferins. Final concentrations for luciferins were 10 μM and final concentrations for luciferases were 50 pM. Data are normalized to the intensity of Akaluc-AkaLumine and shown as mean and s.d. from three independent measurements. Under this condition, LumiLuc-8pyDTZ produced about 1200-fold higher photon flux than Akaluc-AkaLumine. Assays in panels a and b were all performed in PBS. Figure 6C shows the bioluminescence kinetic of LumiLuc-8pyDTZ in PBS or in a formulated assay buffer containing 1 mM CDTA, 0.5% Tergitol NP-40, 0.05% Antifoam 204, 150 mM KCl, 100 mM MES, pH 6.0, 1 mM DTT, and 35 mM thiourea.

Figure 7 shows the comparison of luciferase-luciferin pairs in live mammalian cells. Measurements were performed with 5000 HEK 293T cells in PBS. Luciferase genes were introduced by transient transfection (about 70% transfection efficiency). Final concentrations for FRZ, DTZ, and 8pyDTZ were 20 μM, and final concentrations for AkaLumine and D-luciferin were 100 μM. Signals were integrated over the first 10 min post injection of substrates. Data are presented as mean and s.d. from three independent measurements. Under this condition, LumiLuc-8pyDTZ produced about 300-fold higher photon flux than Akaluc-AkaLumine.

Figures 8A and 8B show the bioluminescence of teLuc- and LumiLuc-expressing HEK 293T cells. Images were acquired (Fig. 8A) without or (Fig. 8B) with a 600-700 nm bandpass filter. Values for relative brightness were normalized to teLuc in the presence of 6.25 μM DTZ.

Figures 9A and 9B show bioluminescence characterizations of HeLa cells stably expressing luciferases. (Fig. 9A) Bioluminescence intensities integrated over the first 10 mins post injection of substrates. The y-axis is shown in a logarithmic scale. Under this condition, LumiLuc-8pyDTZ produced about 190-fold higher photon flux than Akaluc-AkaLumine. (Fig. 9B) Decay kinetics. Assays were performed with 20 μM substrates and 500 HeLa cells. Data are presented as mean and s.d. from three independent measurements.

Figures 10A and 10B show the tracking of tumor growth in a xenograft mouse model with various luciferase-luciferin pairs. (Fig. 10A) BLI (n = 4) on day 1, 3, 5, and day 7. 104 luciferase-expressing HeLa cells were injected to the left and right dorsolateral trapezius regions and 105 cells were injected to the left and right dorsolateral thoracolumbar regions of NU/J mice. For i.v. administration of substrates, AkaLumine-HCl (3 μmol/mouse) and 8pyDTZ (0.2 μmol/mouse) were dissolved in normal saline, and DTZ (0.3 μmol/mouse) was formulated with a mixture of organic cosolvents. (Fig. 10B) Comparison of luciferase-luciferin pairs at tumor sites inoculated with 104 cells. (*$p < 0.05$ for LumiLuc-8pyDTZ and teLuc-DTZ, and for LumiLuc-8pyDTZ and Akaluc-AkaLumine; **$p < 0.05$ for LumiLuc-8pyDTZ and Antares2-DTZ).

Figures 11A-11C show tracking of tumor growth in a xenograft mouse model with various luciferase-luciferin pairs. (Fig. 11A) HeLa cells stably expressing indicated luciferase were injected at four sites of each female NU/J mouse. 10^4 cells were injected to the left and right dorsolateral trapezius regions and 10^5 cells were injected to the left and right dorsolateral thoracolumbar regions. BLI were obtained on days 1, 3, 5, 7, 14, and 28 (n = 4). Pseudocolored images are presented on a scale of $5x10^5$ to $5x10^7$ p/sec/cm^2/sr. Images are identical to Figure 4, except for that data on days 14 and 28 are presented here and that a different scale is used for pseudocoloring. (Fig. 11B) Comparison of luciferase-luciferin pairs at tumor sites inoculated with 10^5 cells (*$p<0.05$ for LumiLuc-8pyDTZ and Akaluc-AkaLumine; **$p<0.05$ for LumiLuc-8pyDTZ and teLuc-DTZ; ***$p<0.05$ for LumiLuc-8pyDTZ and teLuc-8pyDTZ). (Fig. 11C) Bioluminescence decay kinetics for i.v.-injected luciferins (1.5 μmol for AkaLumine, 0.2 μmol for 8pyDTZ, and 0.3 μmol for DTZ) in a xenograft NU/J mouse model. Measurements were done 5 days after tumor implantation. Quantifications were made at sites initially inoculated with 10^5 cells. Data are presented as mean and s.d. from four replicates.

Figures 12A-12F show the engineering and characterization of BRET-based LumiScarlet and teScarlet. (Fig. 12A) Libraries screened for high BRET. Each "X" represents an amino acid residue randomized with the NNK codon, in which N = A/C/G/T and K = G/T. (Fig. 12B) Bioluminescence spectra of constructs selected from each library in the presence of 8pyDTZ. (Fig. 12C) Normalized fluorescence excitation and emission spectra of mScarlet-I and bioluminescence emission of LumiLuc-8pyDTZ, showing excellent spectral overlap for BRET. (Fig. 12D) Comparison of LumiLuc and LumiScarlet (100 pM purified enzymes) for bioluminescence integrated over the first 10 min post

injection of 20 μM 8pyDTZ. (Fig. 12E) Schematic diagram of teScarlet, a genetic fusion of mScarlet-I and teLuc. (Fig. 12F) Bioluminescence emission of teScarlet in the presence of DTZ, showing significant emission longer than 600 nm.

Figures 13A-13D show BRET-based LumiScarlet for deep tissue BLI. (Fig. 13A) Schematic diagram of LumiScarlet, a genetic fusion of mScarlet-I and LumiLuc. (Fig. 13B) Bioluminescence emission of LumiScarlet in the presence of pyCTZ, 6pyDTZ, or 8pyDTZ, showing significant emission longer than 600 nm. (Fig. 13C) Comparison of LumiLuc-8pyDTZ, LumiScarlet-8pyDTZ, and Akaluc-AkaLumine in NU/J mice (n = 4) at 4 h post i.v. injection of 106 luciferase-expressing HeLa cells. (Fig. 13D) Quantitative analysis of bioluminescence from the regions around the lungs in panel c (n.s.: not significant).

Figures 14A-14C show the chemical structures and maximum bioluminescence (BL) emission wavelength of (Fig. 14A) pyOMeCTZ, (Fig. 14B) pyDTZ, and (Fig. 14C) AkaLumine in the presence of its corresponding luciferase.

Figures 15A-15C show engineering of OpyLuc luciferase for pyDTZ selectivity over pyOMeCTZ. (Fig. 15A) The schematic representation of directed evolution to derive OpyLuc. (Fig. 15B) Illustration of the putative substrate-binding site and OpyLuc mutations. CTZ is shown as spheres and mutated residues near the binding site are highlighted in yellow. (Fig. 15C) Normalized bioluminescence activity ratio of pyCTZ/pyOMeCTZ in the presence of either teLuc or OpyLuc.

Figure 16 shows the alignments of primary polypeptide sequences of OpyLuc (SEQ ID NO. 6) and teLuc (SEQ ID NO. 2). OpyLuc mutations are highlighted in magenta background. Residues in this figure are numbered according to Protein Date Bank (PDB) ID 5B0U.

Figures 17A and B show the results of the analysis of orthogonal luciferase-luciferin pairs. (Fig. 17A) Schematic representation of spectral-resolved and orthogonal luciferase-luciferin pairs. (Fig. 17B) Normalized bioluminescence emission spectra of RLuc8-pyOMeCTZ (purple), OpyLuc-pyDTZ (green), and Akaluc-AkaLumine pairs (red).

Figures 18A and 8B show the determination of apparent Michaelis constants (K_M) by substrate titrations. (Fig. 18A) OpyLuc-pyDTZ: 9.8 ± 1.7 μM. (Fig. 18B) RLuc8-pyOMeCTZ: 6.3 ± 0.5 μM. Final concentrations of all enzymes were 100 pM. Substrate concentrations varied from 0.78 to 50 μM, and 10 min integration of total bioluminescence intensities at individual substrate concentrations were used to fit the Michaelis-Menten equation.

Figures 19A-19D show bioluminescence imaging of (Figs. 19A-B) purified recombinant RLuc8, OpyLuc, and Akaluc and (Figs. 19C-D) luciferase-expressing HEK 293T cells. (Fig. 19A) Images were acquired without a filter or with either a 360-500 nm, 495-580 nm, or 600-700 nm bandpass filter. (Fig. 19B) Quantitative values for each tested luciferase-luciferin pair. Final concentrations were 10 nM for RLuc8 and OpyLuc; 100 nM for Akaluc; 30 μM corresponding luciferin. (Fig. 19C) Live HEK293T cells were transfected with either RLuc8, Akaluc, or OpyLuc. 5000 cells per well for RLuc8 and OpyLuc; 30,000 cells per well for Akaluc. Images were acquired without a filter after addition of 1: 10 μM pyDTZ, 2: 100 μM AkaLumine, or 3: 25 μM pyOMeCTZ. (Fig. 19D) Quantitative analysis of BL signals gained from (Fig. 19C).

Figure 20A shows results of the triple luciferase assay in live HEK293T after co-transfection of SRE-RLuc8, ARE-OpyLuc, and NFκB-Akaluc plasmids. Figure 20B shows the BL signals from each luciferase were acquired from intact cells after adding its corresponding luciferin. The cells were induced by 1: 10 ng/mL TNFα; 2: 20% FBS; 3: 50 μM tBHQ; 4: 20% FBS + 50 μM tBHQ; 5: 20% FBS + 10 ng/mL TNFα, 6: 50 μM tBHQ + 10 ng/mL TNFα; 7: 20% FBS + 50 μM tBHQ + 10 ng/mL TNFα for 16 h post PEI transfection.

Figures 21A-21I show results of triple luciferase assay in live HEK293T after co-transfection of SRE-RLuc8, ARE-OpyLuc, and NFκB-Akaluc plasmids. (Fig. 21A) Schematic of triple luciferase assay in live HEK293T after co-transfection of SRE-RLuc8, ARE-OpyLuc, and NFκB-Akaluc plasmids. The emission spectra were acquired from intact cells after adding Optimal Mix solution. The cells were (Fig. 21B) non-treated, induced by (Fig. 21C) 20% FBS, (Fig. 21D) 50 μM tBHQ, (Fig. 21E) 10 ng/mL TNFα, (Fig. 21F) 20% FBS + 10 ng/mL TNFα, (Fig. 21G) 20% FBS + 50 μM tBHQ, (Fig. 21H) 50 μM tBHQ + 10 ng/mL TNFα, (Fig. 21I) 20% FBS + 50 μM tBHQ 10 ng/mL + TNFα for 16 h post PEI transfection.

Figures 22A-22C show bioluminescent Ca^{2+} biosensors. (Fig. 22A) Schematic diagrams of LumiCameleon1 and LumiCameleon2. (Fig. 22B) Bioluminescence emission of LumiCameleon1 in the presence of pyCTZ, showing large dynamic range in response to Ca^{2+}. (Fig. 22C) Bioluminescence emission of LumiCameleon2 in the presence of DTZ.

DETAILED DESCRIPTION

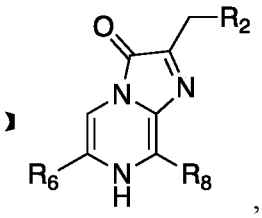
The presently disclosed subject matter now will be described more fully hereinafter, in which some, but not all embodiments of the presently disclosed subject matter are described. Indeed, the presently disclosed subject matter can be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements.

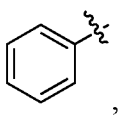
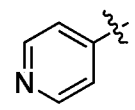
I. GENERAL CONSIDERATIONS

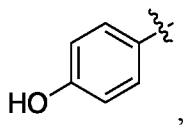
Provided herein are chemically modified luciferase substrates, namely coelenterazine (CTZ) and diphenylterazine (DTZ), for spectrally shifted emission and enhanced water solubility. Concurrently, teLuc was engineered into a LumiLuc luciferase, which is highly active toward the new modified substrates for intense blue, teal, and yellow emission. Moreover, provided herein is a new reporter, LumiScarlet, with significant emission longer than 600 nm. The disclosed multipronged approach yielded a new family of ATP-independent bioluminescent reporters, which have improved biochemical and photophysical properties and are expected to have broad applications.

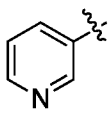
To elaborate, a series of pyridyl CTZ and DTZ analogs, or luciferin compounds, with diverse emission profiles were prepared. The water solubility of these synthetic analogs generally increased by about 10-fold from their ancestors. Surprisingly, these substrate analogs can not only be paired with the new luciferases engineered herein, but also existing ATP-independent reporters, such as RLuc and aequorin.

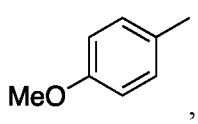
Such luciferin compounds, as described further herein, can include the following structure:

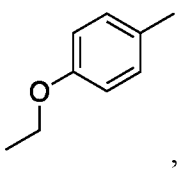


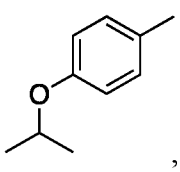
, wherein R6 is selected from  , 

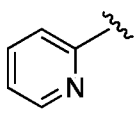
, 

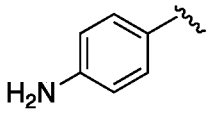
, 

, 

, 

, 

, 

, 

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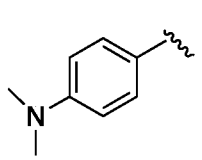
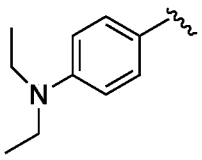
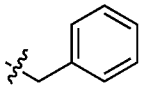
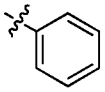
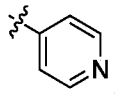
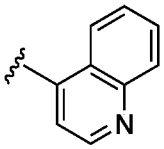
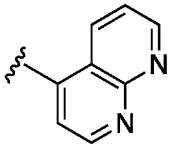
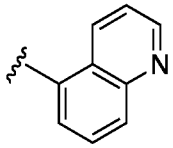
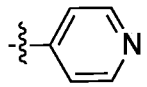
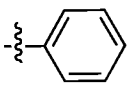
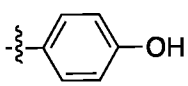
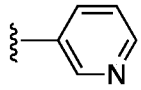
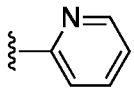
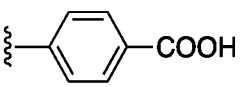
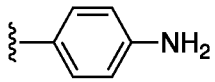
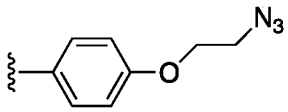

, or 
; wherein R8 is selected from 
, 
, 
, 
, 
, or 
; and wherein R2 is selected from 
, 
, 
, 
, 
, 
, 
or 
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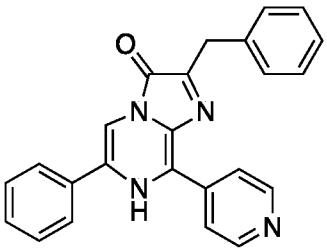
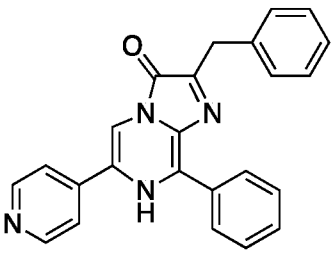
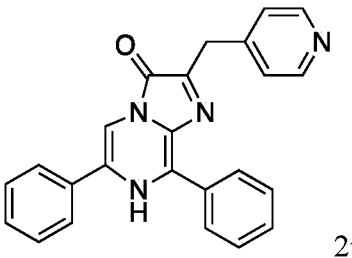
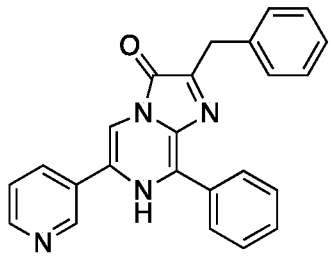
More particularly, the luciferin compounds include:

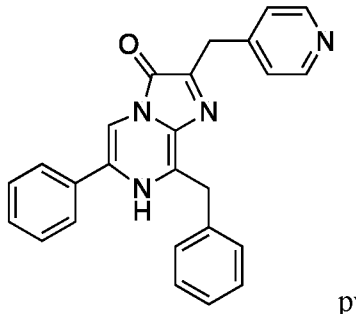

8pyDTZ;


6pyDTZ;

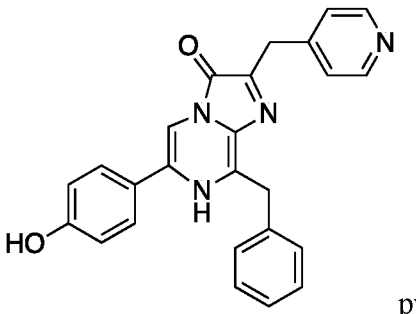

2pyDTZ;



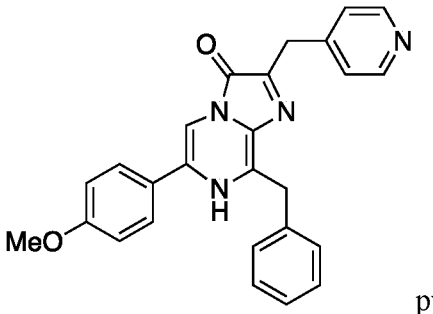
6opyDTZ;



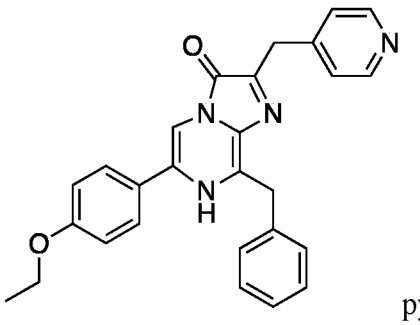
pyCTZ;



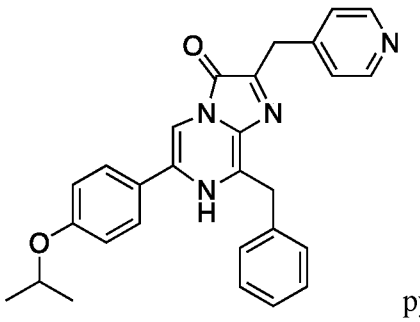
pyOHCTZ;



pyOMeCTZ;

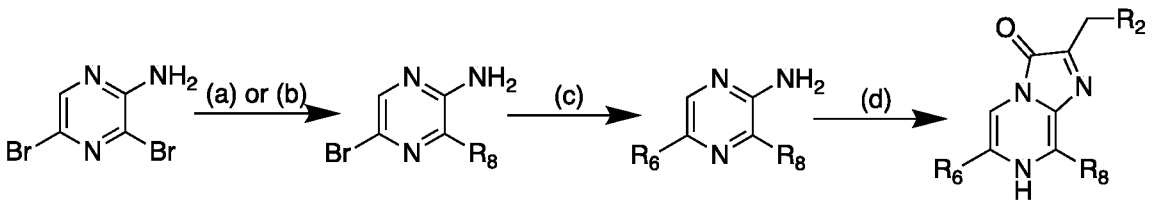


pyOEtCTZ; and



pyiPrCTZ.

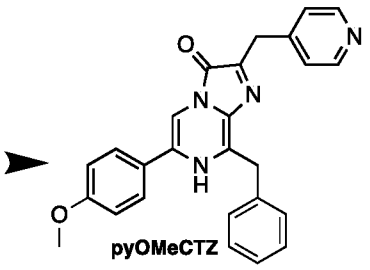
Methods of making luciferin compounds are provided herein, and comprise making one or more pyridyl isomer substitutions at a C-2, C-6 and C-8 position of an imidazopyrazinone core according to the following chemical synthetic route:



,

wherein step (a) comprises Suzuki coupling, comprising $Pd(PPh_3)_4$, Na_2CO_3, R_8-$B(OH)_2$, and/or EtOH; step (b) comprises Negishi coupling, comprising $PhCH_2MgCl$, $ZnCl_2$, $(PPh_3)_2PdCl_2$, and/or THF; step (c) comprises Suzuki coupling, comprising XPhos-Pd-G2, Na_2CO_3, R_6-$B(OH)_2$, and/or EtOH; and step (d) comprises acid-catalyzed ring closing, comprising corresponding α-ketoacetal, HCl, and/or dioxane.

Disclosed herein are luciferin compounds consisting of pyOMeCTZ, pyDTZ and AkaLumine, which comprise the following chemical structures:



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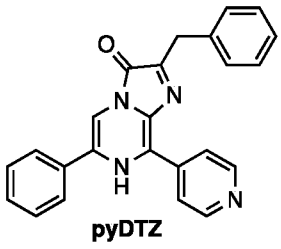
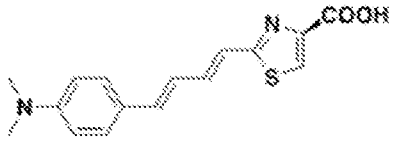


; and



AkaLumine .

Moreover, the LumiLuc luciferase provided herein can in some embodiments comprise a substituted teLuc luciferase, including up to twelve substitutions, as discussed further hereinbelow. In some aspects, a fluorescent protein can be connected to the substituted luciferase polypeptides so as to allow bioluminescence resonant energy transfer (BRET) between the substituted luciferase polypeptide and the fluorescent protein.

Engineered luciferases provided herein, also referred to as bioluminescent proteins, include LumiLuc (SEQ ID NO. 3), LumiScarlet (SEQ ID NOs. 4 and 5), OpyLuc (SEQ ID NO. 6), teScarlet (SEQ ID NOs. 7 and 8), LumiCameleon1 (SEQ ID NOs. 9 and 10), and LumiCameleon2 (SEQ ID NOs. 11 and 12). Additional luciferases are also provided as follows: NanoLuc (SEQ ID NO. 1), teLuc (SEQ ID NO. 2), RLuc8 (SEQ ID NOs. 13 and 14), Akaluc (SEQ ID NOs. 15 and 16), NanoKAZ (SEQ ID NO. 17), and yeLuc (SEQ ID NO. 18).

In addition, provided herein are engineered mutually orthogonal luciferase-luciferin pairs for multiplexed cell-based bioluminescence (BL) assays. Disclosed triple-color BL systems feature the selectivity of synthetic substrates and production of well separated

emission spectra from about 400 nm to about 650 nm. The disclosed spectral-resolved triple-color BL systems provide flexible and convenient approaches to monitor multiple biological events in either qualitative or quantitative manners.

New bioluminescent Ca^{2+} biosensors were also developed based on the modified luciferase compounds disclosed herein. These bioluminescent Ca^{2+} biosensors showed large bioluminescence increase in response to Ca^{2+}, making them well suited for *in vivo* and *in vitro* applications.

The disclosed luciferase-luciferin pairs can also be used for *in vivo* monitoring of tumor models. For example, bioluminescence can be monitored in a tumor model by administering to that model, or otherwise establishing in the model, a luciferase expressing cell (e.g. a tumor cell expressing LumiLuc, teLuc, RLuc8 and OpyLuc), and administering to the model a luciferin (e.g. pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ or 8pyDTZ). Using these luciferase-luciferin pairs as reporter systems the bioluminescence can be used as a tool to monitor the tumor.

The present disclosure provides for the use of the new luciferase-luciferin pairs for assays of cell signaling pathways. By way of example and not limitation, the disclosed luciferase-luciferin pairs can allow for the monitoring of particular cell signaling pathways, which can be implemented to for candidate compound and drug screening applications.

II. DEFINITIONS

While the following terms are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently disclosed subject matter.

All technical and scientific terms used herein, unless otherwise defined below, are intended to have the same meaning as commonly understood by one of ordinary skill in the art. Mention of techniques employed herein are intended to refer to the techniques as commonly understood in the art, including variations on those techniques or substitutions of equivalent techniques that would be apparent to one of skill in the art. Thus, unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the presently disclosed subject matter. Although any compositions, methods, kits, and means for communicating information similar or equivalent to those described herein can

be used to practice the presently disclosed subject matter, particular compositions, methods, kits, and means for communicating information are described herein. It is understood that the particular compositions, methods, kits, and means for communicating information described herein are exemplary only and the presently disclosed subject matter is not intended to be limited to just those embodiments.

Following long-standing patent law convention, the terms "a", "an", and "the" refer to "one or more" when used in this application, including the claims. Thus, in some embodiments the phrase "a peptide" refers to one or more peptides.

The term "about", as used herein to refer to a measurable value such as an amount of weight, time, dose, etc., is meant to encompass in some embodiments variations of ± 20%, in some embodiments ± 10%, in some embodiments ± 5%, in some embodiments ± 1%, in some embodiments ± 0.5%, in some embodiments ± 0.1%, and in some embodiments ± 0.01% from the specified amount, as such variations are appropriate to perform the disclosed methods.

As used herein, the term "and/or" when used in the context of a list of entities, refers to the entities being present singly or in any and every possible combination and subcombination. Thus, for example, the phrase "A, B, C, and/or D" includes A, B, C, and D individually, but also includes any and all combinations and subcombinations of A, B, C, and D. It is further understood that for each instance wherein multiple possible options are listed for a given element (i.e., for all "Markush Groups" and similar listings of optional components for any element), in some embodiments the optional components can be present singly or in any combination or subcombination of the optional components. It is implicit in these forms of lists that each and every combination and subcombination is envisioned and that each such combination or subcombination has not been listed simply merely for convenience. Additionally, it is further understood that all recitations of "or" are to be interpreted as "and/or" unless the context clearly requires that listed components be considered only in the alternative (e.g., if the components would be mutually exclusive in a given context and/or could not be employed in combination with each other).

As used herein, the term "subject" refers to an individual (e.g., human, animal, or other organism) to be treated by the methods or compositions of the present invention. Subjects include, but are not limited to, mammals (e.g., murines, simians, equines, bovines, porcines, canines, felines, and the like), and includes humans. In the context of the invention, the term "subject" generally refers to an individual who will receive or who has received

treatment for a condition characterized by the presence of bacteria (e.g., *Bacillus anthracis* (e.g., in any stage of its growth cycle), or in anticipation of possible exposure to bacteria. As used herein, the terms "subject" and "patient" are used interchangeably, unless otherwise noted.

As used herein, the terms "effective amount" and "therapeutically effective amount" are used interchangeably and refer to the amount that provides a therapeutic effect, e.g., an amount of a composition that is effective to treat or prevent pathological conditions, including signs and/or symptoms of disease, associated with a pathogenic organism infection (e.g., spore germination, bacterial growth, toxin production, etc.) in a subject.

As used herein, the term "adjuvant" as used herein refers to an agent which enhances the pharmaceutical effect of another agent.

The expression "amino acid" as used herein is meant to include both natural and synthetic amino acids, and both D- and L- amino acids. "Standard amino acid" means any of the twenty standard L-amino acids commonly found in naturally occurring peptides. "Nonstandard amino acid residue" means any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or derived from a natural source. As used herein, "synthetic amino acid" also encompasses chemically modified amino acids, including but not limited to salts, amino acid derivatives (such as amides), and substitutions. Amino acids contained within the peptides of the present invention, and particularly at the carboxy- or amino-terminus, can be modified by methylation, amidation, acetylation or substitution with other chemical groups which can change the peptide's circulating half-life without adversely affecting their activity. Additionally, a disulfide linkage can be present or absent in the peptides of the invention.

The term "amino acid" is used interchangeably with "amino acid residue", and can refer to a free amino acid and to an amino acid residue of a peptide. It will be apparent from the context in which the term is used whether it refers to a free amino acid or a residue of a peptide.

The term "antibody", as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen. Antibodies can be derived from natural sources or from recombinant sources and can be intact immunoglobulins or immunoreactive portions of intact immunoglobulins (for example, a fragment or derivative of an antibody that includes an antigen-binding site or a paratope). Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention can exist

in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and $F(ab)_2$, as well as single chain antibodies and humanized antibodies (see e.g., Harlow & Lane (1999) Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, United States of America; Harlow & Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, United States of America; Houston et al. (1988) Proc Natl Acad Sci U S A 85:5879-5883; Bird et al. (1988) Science 242:423-426; each of which is incorporated herein by reference in its entirety).

The term "synthetic antibody" as used herein refers to an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage or a host cell. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

As used herein, the term "antisense oligonucleotide" means a nucleic acid polymer, at least a portion of which is complementary to a nucleic acid which is present in a normal cell or in an affected cell. The antisense oligonucleotides of the invention include, but are not limited to, phosphorothioate oligonucleotides and other modifications of oligonucleotides. Methods for synthesizing oligonucleotides, phosphorothioate oligonucleotides, and otherwise modified oligonucleotides are well known in the art (see e.g., U.S. Patent No. 5,034,506 to Summerton and Weller; Nielsen et al. (1991) Science 254:1497-1500). The term "antisense" refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule encoding a protein, or to a sequence which is substantially homologous to the non-coding strand. As defined herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a protein. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence can be complementary to regulatory sequences specified on the coding strand of a DNA molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

As used herein, the term "biologically active fragments" or "bioactive fragment" of a polypeptide encompasses natural or synthetic portions of the full-length protein that are

capable of specific binding to their natural ligand or of performing the function of the protein.

"Complementary" refers to the broad concept of sequence complementarity between regions of two nucleic acid strands or between two regions of the same nucleic acid strand. It is known that an adenine residue of a first nucleic acid region is capable of forming specific hydrogen bonds ("base pairing") with a residue of a second nucleic acid region which is antiparallel to the first region if the residue is thymine or uracil. As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (i.e., a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T", is complementary to the sequence "T-C-A."

The term "complex", as used herein in reference to proteins, refers to binding or interaction of two or more proteins. Complex formation or interaction can include such things as binding, changes in tertiary structure, and modification of one protein by another, such as phosphorylation.

A "compound", as used herein, refers to any type of substance or agent that is commonly considered a chemical, drug, or a candidate for use as a drug, as well as combinations and mixtures of the above. The term compound further encompasses molecules such as peptides and nucleic acids.

As used herein, a "derivative" of a compound refers to a chemical compound that can be produced from another compound of similar structure in one or more steps, as in replacement of H by an alkyl, acyl, or amino group. Similarly, a "derivative" of a peptide (or of a polypeptide) is a compound that can be produced from or has a biological activity similar to a peptide (or a polypeptide) but that differs in the primary amino acid sequence of the peptide (or the polypeptide) to some degree. By way of example and not limitation, a derivative of a subject peptide of the presently disclosed subject matter is a peptide that has a similar although not identical primary amino acid sequence as the subject peptide (for example, has one or more amino acid substitutions) and/or that has one or more other modifications (e.g., N-terminal, C-terminal, and/or internal modifications) as compared to the subject peptide. Thus, the term "derivative" compasses the term "modified peptide" and vice versa, in the context of peptides. In some embodiments, a derivative of a peptide is a C-terminal amidated peptide.

As used herein, a "detectable marker" or a "reporter molecule" is an atom or a molecule that permits the specific detection of a compound comprising the marker in the

presence of similar compounds without a marker. Detectable markers or reporter molecules include, e.g., radioactive isotopes, antigenic determinants, enzymes, nucleic acids available for hybridization, chromophores, fluorophores, chemiluminescent molecules, electrochemically detectable molecules, and molecules that provide for altered fluorescence-polarization or altered light-scattering.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate.

In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA can include introns.

As used herein, an "essentially pure" preparation of a particular protein or peptide is a preparation wherein at least about 95%, and preferably at least about 99%, by weight, of the protein or peptide in the preparation is the particular protein or peptide.

A "fragment" or "segment" is a portion of an amino acid sequence, comprising at least one amino acid of the amino acid sequence, or a portion of a nucleic acid sequence comprising at least one nucleotide. The terms "fragment" and "segment" are used interchangeably herein.

As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property or activity by which it is characterized. A functional enzyme, for example, is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

The terms "formula" and "structure" are used interchangeably herein.

The term "identity" as used herein relates to the similarity between two or more sequences. Identity is measured by dividing the number of identical residues by the total number of residues and multiplying the product by 100 to achieve a percentage. Thus, two copies of exactly the same sequence have 100% identity, whereas two sequences that have amino acid deletions, additions, or substitutions relative to one another have a lower degree of identity. Those skilled in the art will recognize that several computer programs, such as those that employ algorithms such as BLAST (Basic Local Alignment Search Tool, Altschul et al. (1993) J Mol Biol 215:403-410) are available for determining sequence identity.

In some embodiments, "identity" can be expressed as a "percent identity". As used herein, the phrase "percent identity" in the context of two nucleic acid or polypeptide sequences, refers to two or more sequences or subsequences that have in some embodiments 60%, in some embodiments 70%, in some embodiments 75%, in some embodiments 80%, in some embodiments 85%, in some embodiments 90%, in some embodiments 92%, in some embodiments 94%, in some embodiments 95%, in some embodiments 96%, in some embodiments 97%, in some embodiments 98%, in some embodiments 99%, and in some embodiments 100% nucleotide or amino acid residue identity, respectively, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. The percent identity exists in some embodiments over a region of the sequences that is at least about 50 residues in length, in some embodiments over a region of at least about 100 residues, and in some embodiments, the percent identity exists over at least about 150 residues. In some embodiments, the percent identity exists over the entire length of the sequences.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm disclosed in Smith & Waterman (1981) 2 Adv Appl Math 482-489; by the homology alignment algorithm disclosed in Needleman & Wunsch (1970) 48 J Mol Biol 443-453; by the search for similarity method disclosed in Pearson & Lipman (1988) Proc Natl Acad Sci U S A 85:2444-2448; by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the GCG® WISCONSIN PACKAGE®, available from Accelrys, Inc., San Diego, California, United States of America), or by visual inspection. See generally, Altschul et al. (1990) 215 J Mol Biol 403-410; Ausubel et al. (2002) Short Protocols in Molecular Biology, Fifth ed. Wiley, New York, New York, United States of America; and Ausubel et al. (2003) Current Protocols in Molecular Biology, John Wylie & Sons, Inc, New York, New York, United States of America.

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al. (1990) 215 J Mol Biol 403-410. Software for performing BLAST analysis is publicly available through the website of the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. See generally, Altschul et al. (1990) 215 J Mol Biol 403-410. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, $M = 5$, $N = 4$, and a comparison of both strands. For amino acid

sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. See Henikoff & Henikoff (1992) 89 Proc Natl Acad Sci U S A 10915-10919.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see e.g., Karlin & Altschul (1993) 90 Proc Natl Acad Sci U S A 5873-5877). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is in some embodiments less than about 0.1, in some embodiments less than about 0.01, and in some embodiments less than about 0.001.

The term "inhibit", as used herein, refers to the ability of a compound or any agent to reduce or impede a described function or pathway. For example, inhibition can be by at least 10%, by at least 25%, by at least 50%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 97%, by at least 99%, or more.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the peptide of the invention in the kit for effecting alleviation of the various diseases or disorders recited herein. Optionally, or alternately, the instructional material can describe one or more methods of alleviating the diseases or disorders in a cell or a tissue of a mammal. The instructional material of the kit of the invention can, for example, be affixed to a container which contains the identified compound invention or be shipped together with a container which contains the identified compound. Alternatively, the instructional material can be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

An "isolated" compound/moiety is a compound/moeity that has been removed from components naturally associated with the compound/moiety. For example, an "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also

applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, e.g., RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

The term "modulate", as used herein, refers to changing the level of an activity, function, or process. The term "modulate" encompasses both inhibiting and stimulating an activity, function, or process.

The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as used herein refers to a compound that is greater than 90% pure.

As used herein, the term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in an animal. In some embodiments, a pharmaceutically acceptable carrier is pharmaceutically acceptable for use in a human.

The term "polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer.

The term "protein" typically refers to large polypeptides (e.g., a polypeptide of in some embodiments at least 50 amino acids, in some embodiments at least 75 amino acids, in some embodiments at least 100 amino acids, in some embodiments at least 200 amino acids, in some embodiments at least 300 amino acids, in some embodiments at least 500 amino acids, and in some embodiments more than 500 amino acids).

A peptide encompasses a sequence of 2 or more amino acids wherein the amino acids are naturally occurring or synthetic (non-naturally occurring) amino acids.

The term "linked" or like terms refers to a connection between two entities. The linkage can comprise a covalent, ionic, or hydrogen bond or other interaction that binds two compounds or substances to one another.

As used herein the term "peptidomimetic" refers to a chemical compound having a structure that is different from the general structure of an existing peptide, but that functions in a manner similar to the existing peptide, e.g., by mimicking the biological activity of that peptide. The term "modified peptide" encompasses a peptidomimetic. Peptidomimetics typically comprise naturally-occurring amino acids and/or unnatural amino acids, but can also comprise modifications to the peptide backbone. For example, a peptidomimetic can include one or more of the following modifications:

1. Peptides wherein one or more of the peptidyl –C(O)NR– linkages (bonds) have been replaced by a non-peptidyl linkage such as a –CH_2-carbamate linkage (–$CH_2OC(O)NR$–), a phosphonate linkage, a –CH_2-sulfonamide (–CH_2-$S(O)_2NR$–) linkage, a urea (–NHC(O)NH–) linkage, a –CH_2-secondary amine linkage, an azapeptide bond (CO substituted by NH), or an ester bond (e.g., depsipeptides, wherein one or more of the amide (–CONHR–) bonds are replaced by ester (COOR) bonds) or with an alkylated peptidyl linkage (–C(O)NR–) wherein R is C_1-C_6 alkyl;

2. Peptides wherein the N-terminus is derivatized to a –NRR1 group, to a –NRC(O)R group, to a –NRC(O)OR group, to a –$NRS(O)_2R$ group, to a –NHC(O)NHR group where R and R1 are hydrogen or C_1-C_6 alkyl with the proviso that R and R1 are not both hydrogen;

3. Peptides wherein the C terminus is derivatized to –C(O)R2 where R2 is selected from the group consisting of C_1-C_6 alkoxy, and –NR3R4 where R3 and R4 are independently selected from the group consisting of hydrogen and C_1-C_4 alkyl;

4. Modification of a sequence of naturally-occurring amino acids with the insertion or substitution of a non-peptide moiety, e.g., a retroinverso fragment.

The term "permeability", as used herein, refers to transit of fluid, cell, or debris between or through cells and tissues.

A "sample", as used herein, refers preferably to a biological sample from a subject, including, but not limited to, normal tissue samples, diseased tissue samples, biopsies, blood, saliva, feces, semen, tears, and urine. A sample can also be any other source of material obtained from a subject which contains cells, tissues, or fluid of interest. A sample can also be obtained from cell or tissue culture.

By the term "specifically binds", as used herein, is meant a compound which recognizes and binds a specific protein, but does not substantially recognize or bind other molecules in a sample, or it means binding between two or more proteins as in part of a cellular regulatory process, where said proteins do not substantially recognize or bind other proteins in a sample.

The term "standard", as used herein, refers to something used for comparison. For example, it can be a known standard agent or compound which is administered or added to a control sample and used for comparing results when measuring said compound in a test sample. Standard can also refer to an "internal standard", such as an agent or compound which is added at known amounts to a sample and is useful in determining such things as purification or recovery rates when a sample is processed or subjected to purification or extraction procedures before a marker of interest is measured.

The term "symptom", as used herein, refers to any morbid phenomenon or departure from the normal in structure, function, or sensation, experienced by the patient and indicative of disease. In contrast, a sign is objective evidence of disease. For example, a bloody nose is a sign. It is evident to the patient, doctor, nurse and other observers.

As used herein, the term "treating" includes prophylaxis of the specific disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs.

As used herein an "amino acid modification" refers in some embodiments to a substitution, addition, or deletion of an amino acid, and includes substitution with, or

addition of, any of the 20 amino acids commonly found in human proteins, as well as unusual or non-naturally occurring amino acids such as but not limited to D-amino acids. Commercial sources of unusual amino acids include Sigma-Aldrich (Milwaukee, Wisconsin, United States of America), ChemPep Inc. (Miami, Florida, United States of America), and Genzyme Pharmaceuticals (Cambridge, Massachusetts, United States of America). Unusual amino acids can be purchased from commercial suppliers, synthesized de novo, or chemically modified or derivatized from naturally occurring amino acids. Amino acid modifications include linkage of an amino acid to a conjugate moiety, such as a hydrophilic polymer, acylation, alkylation, and/or other chemical derivatization of an amino acid. The term "modified peptide" encompasses any amino acid modification as described herein.

Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivatization of polypeptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; e.g., by exposing the polypeptide to enzymes which affect glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

Substitutions can be designed based on, for example, the model of Dayhoff et al. (in Atlas of Protein Sequence and Structure 1978, National Biomedical Research Foundation, Washington D.C., United States of America).

In some embodiments, an amino acid substitution is a conservative amino acid substitution. As used herein, the term "conservative amino acid substitution" is defined in some embodiments as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro, Gly;

II. Polar, charged residues and their amides: Asp, Asn, Glu, Gln, His, Arg, Lys;

III. Large, aliphatic, nonpolar residues: Met Leu, Ile, Val, Cys

IV. Large, aromatic residues: Phe, Tyr, Trp

Conservative substitutions are likely to be phenotypically silent. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr. Guidance concerning which amino acid changes are likely to be phenotypically silent are found in Bowie et al. (1990) Science 247:1306-1310.

For example, the hydropathic index of amino acids may be considered (Kyte & Doolittle (1982) J Mol Biol 157:105-132). The relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte & Doolittle (1982) J Mol Biol 157:105-132), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5). In making conservative substitutions, the use of amino acids whose hydropathic indices are within +/-2 is preferred, within +/-1 are more preferred, and within +/- 0.5 are even more preferred.

Amino acid substitution may also take into account the hydrophilicity of the amino acid residue (e.g., U.S. Patent No. 4,554,101). Hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0); glutamate (+3.0); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5.+-0.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). Replacement of amino acids with others of similar hydrophilicity is preferred.

Other considerations include the size of the amino acid side chain. For example, in some embodiments an amino acid with a compact side chain, such as glycine or serine, would not be replaced with an amino acid with a bulky side chain, e.g., tryptophan or

tyrosine. The effect of various amino acid residues on protein secondary structure is also a consideration. Through empirical study, the effect of different amino acid residues on the tendency of protein domains to adopt an alpha-helical, beta-sheet, or reverse turn secondary structure has been determined and is known in the art (see e.g., Chou & Fasman (1974) Biochemistry 13:222-245; Chou & Fasman (1978) Ann Rev Biochem 47: 251-276; Chou & Fasman (1979) Biophys J 26:367-384).

Based on such considerations and extensive empirical study, tables of conservative amino acid substitutions have been constructed and are known in the art. By way of example and not limitation, the following substitutions can be made: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine. Alternatively, Table 1 lists exemplary conservative amino acid substitutions.

Table 1

Exemplary Conservative Amino Acid Substitutions

Amino Acid	Possible Substitution(s)	Amino Acid	Possible Substitution(s)
Ala (A)	Leu, Ile, Val	Leu (L)	Val, Met, Ala, Phe, Ile
Arg (R)	Gln, Asn, Lys	Lys (K)	Gln, Asn, Arg
Asn (N)	His, Asp, Lys, Arg, Gln	Met (M)	Phe, Ile, Leu
Asp (D)	Asn, Glu	Phe (F)	Leu, Val, Ile, Ala, Tyr
Cys (C)	Ala, Ser	Pro (P)	Ala
Gln (Q)	Glu, Asn	Ser (S)	Thr
Glu (E)	Gln, Asp	Thr (T)	Ser
Gly (G)	Ala	Trp (W)	Phe, Tyr
His (H)	Asn, Gln, Lys, Arg	Tyr (Y)	Trp, Phe, Thr, Ser
Ile (I)	Val, Met, Ala, Phe, Leu	Val (V)	Ile, Leu, Met, Phe, Ala

As used herein, amino acids are represented by the full name thereof, by the three letter code corresponding thereto, or by the one-letter code corresponding thereto, as indicated in the following table (Table 1A):

Table 1A.

Full Name	**3-Letter Code**	**1-Letter Code**	**Functionally Equivalent Codons**
Aspartic Acid	Asp	D	GAC GAU
Glutamic Acid	Glu	E	GAA GAG
Lysine	Lys	K	AAA AAG

Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Histidine	His	H	CAC CAU
Tyrosine	Tyr	Y	UAC UAU
Cysteine	Cys	C	UGC UGU
Asparagine	Asn	N	AAC AAU
Glutamine	Gln	Q	CAA CAG
Serine	Ser	S	ACG AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Glycine	Gly	G	GGA GGC GGG GGU
Alanine	Ala	A	GCA GCC GCG GCU
Valine	Val	V	GUA GUC GUG GUU
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Isoleucine	Ile	I	AUA AUC AUU
Methionine	Met	M	AUG
Proline	Pro	P	CCA CCC CCG CCU
Phenylalanine	Phe	F	UUC UUU
Tryptophan	Trp	W	UGG

In some embodiments, another consideration for amino acid substitutions include whether or not the residue is located in the interior of a protein or is solvent exposed. For interior residues, conservative substitutions can include in some embodiments: Asp and Asn; Ser and Thr; Ser and Ala; Thr and Ala; Ala and Gly; Ile and Val; Val and Leu; Leu and Ile; Leu and Met; Phe and Tyr; Tyr and Trp. For solvent exposed residues, conservative substitutions can include in some embodiments: Asp and Asn; Asp and Glu; Glu and Gln; Glu and Ala; Gly and Asn; Ala and Pro; Ala and Gly; Ala and Ser; Ala and Lys; Ser and Thr; Lys and Arg; Val and Leu; Leu and Ile; Ile and Val; Phe and Tyr. Various matrices have been constructed to assist in selection of amino acid substitutions, such as the PAM250 scoring matrix, the Dayhoff matrix, the Grantham matrix, the McLachlan matrix, the Doolittle matrix, the Henikoff matrix, the Miyata matrix, the Fitch matrix, the Jones matrix, the Rao matrix, the Levin matrix, and the Risler matrix (summarized in, for example, Johnson & Overington (1993) J Mol Biol 233:716-738; see also the PROWL resource available at the website of The Rockefeller University, New York, New York, United States of America).

In determining amino acid substitutions, one may also consider the existence of intermolecular or intramolecular bonds, such as formation of ionic bonds (salt bridges) between positively charged residues (e.g., His, Arg, Lys) and negatively charged residues (e.g., Asp, Glu) or disulfide bonds between nearby cysteine residues.

Methods of substituting any amino acid for any other amino acid in an encoded peptide sequence are well known and a matter of routine experimentation for the skilled artisan, for example by the technique of site-directed mutagenesis or by synthesis and assembly of oligonucleotides encoding an amino acid substitution and splicing into an expression vector construct.

EXAMPLES

The following Examples provide illustrative embodiments. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the presently disclosed subject matter.

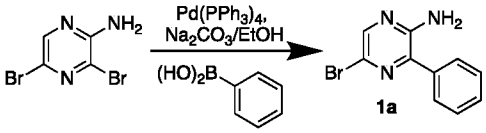
Materials and Methods for Examples 1-5

Synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases were purchased from Thermo Fisher Scientific. Q5 high-fidelity DNA polymerase and Taq DNA polymerase were purchased from NEB. Products of PCR and restriction digestion were purified by gel electrophoresis and Syd Laboratories Gel Extraction columns. Plasmid DNA was purified using Syd Laboratories Miniprep columns. DNA sequences were analyzed by Eurofins. Potassium D-luciferin was purchased from Thermo Fisher Scientific. Coelenterazine was purchased from Gold Biotechnology. Furimazine (Nano-Glo®) was purchased from Promega. AkaLumine-HCl was purchased from Aobious. CTZ and DTZ were obtained from GoldBio and Haoyuan Chemexpress, respectively. All other chemicals were purchased from Sigma-Aldrich, Fisher Scientific, or VWR and used without further purification. Bruker Avance DRX 600 and Varian NMRS 600 at the UVA Biomolecular Magnetic Resonance Facility was used to record all NMR spectra. Chemical shift (δ) is given in parts per million relative to ^{1}H (7.24 p.p.m.) and ^{13}C (77.23 p.p.m.) for $CDCl_3$; ^{1}H (2.50 p.p.m.) and ^{13}C (39.5 p.p.m.) for DMSO-d_6; ^{1}H (3.31 p.p.m.) and ^{13}C (49.15 p.p.m.) for methanol-d_4. Splitting patterns are reported as s (singlet), bs (broad singlet), d (doublet), t (triplet), dd (doublet of doublets), and m (multiplet). Coupling constant (J) is given in Hz. High resolution ESI-MS was run on an Agilent 6545 Q-TOF LC/MS system by direct infusion. A Waters Delta Prep ZQ 2000 LC-MS Purification System equipped with a XBridge BEH Amide OBD Prep Column (130Å,

5 μm, 30 mm X 150 mm) was used for preparative reverse-phase HPLC purifications. Nu/J mice were obtained from the Jackson Laboratory (Cat. # 002019) and maintained and treated in standard conditions that complied with all relevant ethical regulations. All animal procedures were approved by the UVA Institutional Animal Care and Use Committee. Images were analyzed using the Fiji image analysis software. Microsoft Excel and GraphPad Prism were used to analyze data and prepare figures.

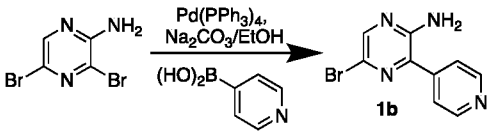
Chemical synthesis of compounds

*5-bromo-3-phenylpyrazin-2-amine (**1a**)*:



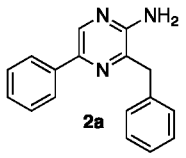
To a solution of $Pd(PPh_3)_4$ (460 mg, 0.4 mmol, 0.1 equiv.) in 200 mL EtOH was added 2-Amino-3,5-dibromopyrazine (1010 mg, 4 mmol, 1 equiv.), 1N Na_2CO_3 solution (8 mL, 8 mmol, 2 equiv.) and phenylboronic acid (490 mg, 4 mmol, 1 equiv.). The resultant mixture was stirred at 80 °C under argon for 12 h. The solvent was removed *in vacuo* and the residue was suspended in 200 mL ddH_2O, which was extracted twice with EtOAc (200 mL). The organic layers were combined and dried over anhydrous Na_2SO_4, filtered and removed in *vacuo*. The residue was purified by silica column chromatography with elution (DCM:MeOH = 100:1) to yield compound **1a** (above) as yellow solid (360 mg, 36%). ^1H NMR (600 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.71 (d, J = 7.4 Hz, 2H), 7.50 (t, J = 7.4 Hz, 2H), 7.45 (t, J = 7.4 Hz, 1H), 4.82 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 152.4, 142.4, 139.3, 135.9, 129.2, 128.8, 128.7, 128.1, 127.9, 123.9. HRMS (ESI-TOF) calcd for $C_{10}H_8BrN_3$ [M + H]$^+$: 249.9902, found: m/z 249.9916.

*5-bromo-3-(pyridin-4-yl)pyrazin-2-amine (**1b**)*:



The synthesis of **1b** (above) followed the same procedure as **1a**, whereas 4-pyridylboronic acid (492 mg, 4 mmol, 1 equiv.) was used. Crude **1b** was purified by column chromatography with elution (DCM:MeOH = 10:1) to yield **1b** as yellow solid (301 mg, 30%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.69 (d, J = 6.0 Hz, 2H), 8.17 (s, 1H), 7.67 (d, J = 6.0 Hz, 2H), 6.68 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 152.6, 150.1, 144.1, 143.3, 136.1, 124.0, 122.4. HRMS (ESI-TOF) calcd for $C_9H_7BrN_4$ [M + H]$^+$: 250.9854, found: m/z 250.9845.

*3-benzyl-5-phenylpyrazin-2-amine (**2a**):*



2a

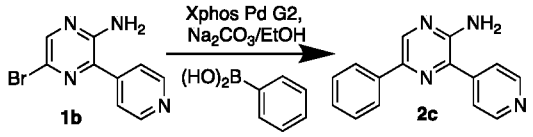
2a (above) was prepared following the published synthesis methods[35]. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.41 (s, 1H), 7.89 (d, J = 7.4 Hz, 2H), 7.39 (t, J = 7.7 Hz, 2H), 7.33 (d, J = 7.6 Hz, 2H), 7.27 (q, J = 7.7, 7.1 Hz, 3H), 7.18 (t, J = 7.3 Hz, 1H), 6.39 (s, 2H), 4.07 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 153.2, 140.5, 139.2, 138.6, 137.6, 137.4, 129.4, 129.1, 128.7, 127.8, 126.6, 125.2, 39.1. HRMS (ESI-TOF) calcd for $C_{17}H_{15}N_3$ [M + H]$^+$: 262.1266, found: m/z 262.1258.

*3,5-diphenylpyrazin-2-amine (**2b**):*

2b

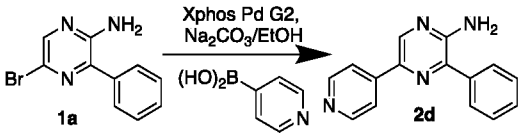
2b was reported previously[33].

*5-phenyl-3-(pyridin-4-yl)pyrazin-2-amine (**2c**):*



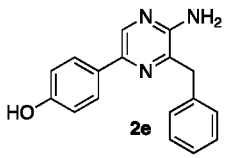
The synthesis and purification of **2c** (above) followed the same procedure as **2d**, whereas **1b** was used as the starting compound and phenylboronic acid (245 mg, 2 mmol, 2 equiv.) was used as boron reagent. Yellow solid (87 mg, 70%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.88 – 7.84 (m, 2H), 7.70 (s, 1H), 7.15 (dt, J = 7.8, 1.2 Hz, 2H), 7.12 – 7.08 (m, 2H), 6.65 – 6.59 (m, 2H), 6.57 – 6.51 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 152.3, 150.1, 144.9, 140.0, 139.4, 136.6, 134.7, 128.8, 127.9, 125.0, 122.7. HRMS (ESI-TOF) calcd for $C_{15}H_{12}N_4$ [M + H]$^+$: 249.1062, found: m/z 249.1059.

*3-phenyl-5-(pyridin-4-yl)pyrazin-2-amine (**2d**):*



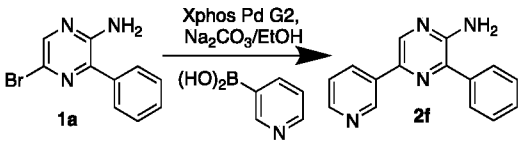
To a mixture of XPhos Pd G2 (79 mg, 0.1 mmol, 0.2 equiv.) and XPhos (24 mg, 0.05 mmol, 0.1 equiv.) in 5 mL EtOH was added **1a** (125 mg, 0.5 mmol, 1 euiqv.), 1N Na_2CO_3 (1 mL, 1 mmol, 2 equiv.) and 4-pyridylboronic acid (246 mg, 2 mmol, 4 equiv.). The resulting mixture was stirred at 80 °C under argon for 12 h. The solvent was then removed *in vacuo* and the residue was dissolved in 1N HCl (30 mL) and subsequently washed with EtOAc (30 mL). The aqueous layer was collected and the pH was then adjusted to 10 by the addition of 1N NaOH. Product **2d** precipitated as yellow solid, which was filtered, washed with EtOAc and dried under reduced pressure overnight. (93 mg, 75%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.71 (s, 1H), 8.58 (d, J = 6.2 Hz, 2H), 7.94 (d, J = 6.2 Hz, 2H), 7.78 (d, J = 7.2 Hz, 2H), 7.52 (t, J = 7.5 Hz, 2H), 7.46 (t, J = 7.3 Hz, 1H), 6.63 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 153.3, 150.1, 144.0, 139.2, 138.6, 137.1, 128.8, 128.2, 119.0. HRMS (ESI-TOF) calcd for $C_{15}H_{12}N_4$ [M + H]$^+$: 249.1062, found: m/z 249.1060.

*4-(5-amino-6-benzylpyrazin-2-yl)phenol (**2e**):*



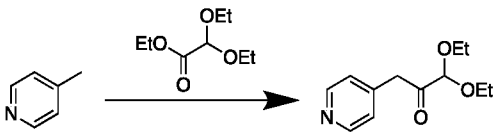
2e (above) was prepared following the published synthesis methods[36]. ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 9.49 (s, 1H), 8.29 (s, 1H), 7.72 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 7.3 Hz, 2H), 7.28 (t, *J* = 7.6 Hz, 2H), 7.18 (t, *J* = 7.3 Hz, 1H), 6.79 (d, *J* = 8.6 Hz, 2H), 6.19 (s, 2H), 4.06 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d$_6$) δ 157.1, 152.0, 139.7, 139.5, 138.3, 135.9, 135.9, 128.9,128.2,126.2, 126.1, 115.5, 115.4, 38.7. HRMS (ESI-TOF) calcd for $C_{17}H_{15}N_3O$ $[M + H]^+$: 278.1215, found: m/z 278.1208.

*3-phenyl-5-(pyridin-3-yl)pyrazin-2-amine (**2f**):*



The synthesis and purification of **2f** (above) followed the same procedure as **2d**, whereas 3-pyridylboronic acid (246 mg, 2 mmol, 4 equiv.) was used. Yellow solid (95 mg, 77%). ^{1}H NMR (600 MHz, DMSO-d$_6$) δ 9.19 (s, 1H), 8.64 (s, 1H), 8.54 (d, J = 3.9 Hz, 1H), 8.39 (d, J = 8.1 Hz, 1H), 7.79 (d, J = 7.3 Hz, 2H), 7.53 – 7.49 (m, 3H), 7.46 (t, J = 7.3 Hz, 1H), 6.47 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d$_6$) δ 152.6, 148.5, 146.3, 138.4, 138.4, 137.3, 137.2, 132.6, 132.3, 132.2, 128.8, 128.6, 128.3. HRMS (ESI-TOF) calcd for $C_{15}H_{12}N_4$ $[M + H]^+$: 249.1062, found: m/z 249.1060.

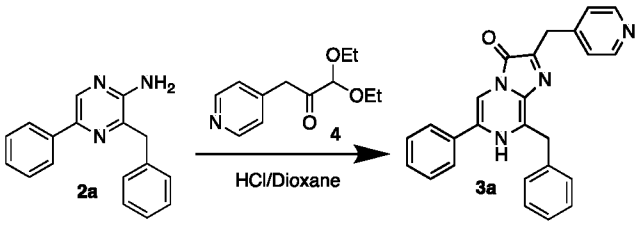
*3-pyridin-4-yl-1,1-diethoxyacetone (**4**):*



To a solution of 4-methylpyridine (931 mg, 10 mmol, 1 equiv.) in 50 mL anhydrous THF was added potassium tert-butoxide (5.6 g, 50 mmol, 5 equiv.), and the mixture was stirred at room temperature for 10 min. Ethyl diethoxyacetate (3.52 g, 20 mmol, 2 equiv.) in 20 mL THF was then added dropwise over 10 min. The resulting mixture was stirred overnight, and solvent was removed under *vacuo*. The residue was purified by silica column chromatography with elution (Hexane:EtOAc = 1:3 to 100% EtOAc) to yield product as light yellow solid (669 mg, 30%). ^{1}H NMR (600 MHz, DMSO-d$_6$) δ 8.47 (d, J = 5.8 Hz, 2H), 7.18 (d, J = 5.8 Hz, 2H), 4.81 (s, 1H), 3.91 (s, 2H), 3.63 (dq, J = 9.7, 7.1 Hz, 2H), 3.54

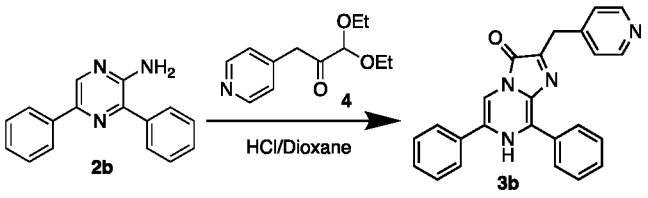
(dq, J = 9.7, 7.1 Hz, 2H), 1.15 (t, J = 7.1 Hz, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 202.0, 149.3, 143.2, 125.3, 101.6, 62.8, 42.6, 15.1. HRMS (ESI-TOF) calcd for $C_{12}H_{17}NO_3$ [M + H]$^+$: 224.1208, found: m/z 224.1195.

*8-benzyl-6-phenyl-2-(pyridin-4-ylmethyl)imidazo[1,2-a]pyrazin-3(7H)-one (**3a**):*



To a solution of **2a** (26 mg, 0.1 mmol, 1 equiv.) and **4** (89 mg, 0.4 mmol 4 equiv) in 5 mL degassed 1,4-dioxane was added 0.8 mL 6N HCl. The resulting mixture was stirred at 80°C in a seal tube for 12 h. The solvent was then removed *in vacuo* and the residue was dissolved in 1 mL (ACN:H_2O = 1:1) and next purified with preparative RP-HPLC. (acetonitrile/water = 1:99 to 90:10, 20 mL/min, UV 254 nm). Product fractions were combined and lyophilized to give **3a** (above) as yellow powder (15 mg, 38%), which has to be stored as solid at -80 °C for long-term stability. ^{1}H NMR (600 MHz, Methanol-d_4) δ 8.43 (d, J = 6.2 Hz, 2H), 7.74 (s, 1H), 7.65 (d, J = 6.8 Hz, 2H), 7.49 – 7.38 (m, 7H), 7.29 (t, J = 7.6 Hz, 2H), 7.23 (t, J = 7.4 Hz, 1H), 4.42 (s, 2H), 4.23 (s, 2H). ^{13}C NMR (150 MHz, Methanol-d_4) δ 142.6, 137.9, 131.1, 130.4, 130.0, 129.9, 129.1, 128.5, 128.3, 110.1, 49.7. HRMS (ESI-TOF) calcd for $C_{25}H_{20}N_4O$ [M + H]$^+$: 393.1637, found: m/z 393.1630.

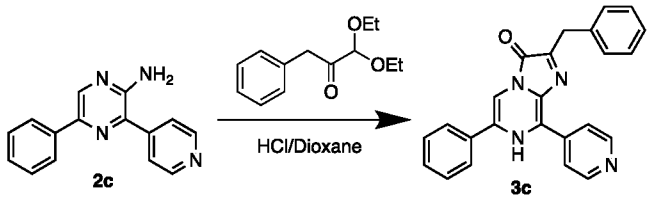
*6,8-diphenyl-2-(pyridin-4-ylmethyl)imidazo[1,2-a]pyrazin-3(7H)-one (**3b**):*



The synthesis and purification of **3b** (above) followed the same procedure as **3a**, whereas **2b** (25 mg, 0.1 mmol, 1 equiv.) was used as the starting compound. Orange powder (8 mg, 21%). 1H NMR (600 MHz, Methanol-d4) δ 8.79 (d, J = 6.5 Hz, 2H), 8.50 (s, 1H), 8.15 (d, J = 7.2 Hz, 2H), 8.06 (d, J = 6.5 Hz, 2H), 8.00 (d, J = 7.4 Hz, 2H), 7.69 (t, J = 7.4 Hz, 1H), 7.65 (t, J = 7.2 Hz, 2H), 7.58 (t, J = 7.2 Hz, 2H), 7.56 – 7.53 (m, 1H),

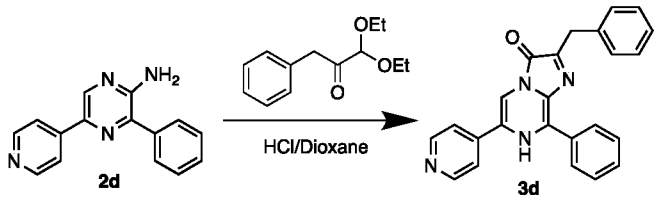
4.66 (s, 2H). ^{13}C NMR (150 MHz, Methanol-d$_4$) δ 161.0, 146.2, 142.7, 139.3, 134.7, 133.3, 133.0, 131.3, 131.0, 130.4, 130.3, 128.8, 128.5, 112.2. HRMS (ESI-TOF) calcd for $C_{24}H_{18}N_4O$ [M + H]$^+$: 379.1481, found: m/z 379.1480.

*2-benzyl-6-phenyl-8-(pyridin-4-yl)imidazo[1,2-a]pyrazin-3(7H)-one (**3c**):*



To a solution of **2c** (25 mg, 0.1 mmol, 1 equiv.) and 1,1-diethoxy-3-phenylpropan-2-one (89 mg, 0.4 mmol, 4 equiv.) in 5 mL degassed 1,4-dioxane was added 0.8 mL 6 N HCl, and the resulting mixture was stirred at 80°C in a sealed tube for 12 h. The solvent was then removed *in vacuo* and the residue was dissolved in 1 mL (ACN:H$_2$O = 1:1) and next purified with preparative RP-HPLC. (acetonitrile/water = 1:99 to 90:10, 20 mL/min, UV 254 nm). Product fractions were combined and lyophilized to give **3c** (above) as brown powder (9 mg, 23%). ^{1}H NMR (600 MHz, Acetonitrile-d$_3$ and D$_2$O, ratio = 9:1) δ 9.31 (d, J = 6.8 Hz, 2H), 8.84 (d, J = 6.8 Hz, 2H), 8.54 (s, 1H), 8.09 (d, J = 8.0 Hz, 2H), 7.52 (t, J = 7.6 Hz, 2H), 7.44 (t, J = 7.3 Hz, 1H), 7.35 (d, J = 7.7 Hz, 2H), 7.28 (t, J = 7.7 Hz, 2H), 7.24 – 7.21 (m, 1H), 4.19 (s, 2H). ^{13}C NMR (150 MHz, Methanol-*d*$_4$) δ 143.0, 140.7, 140.1, 139.5, 137.5, 132.0, 130.2, 129.9, 129.6, 129.4, 127.8, 127.5, 127.4, 126.5, 113.8, 33.6. HRMS (ESI-TOF) calcd for $C_{24}H_{18}N_4O$ [M + H]$^+$: 379.1481, found: m/z 379.1477.

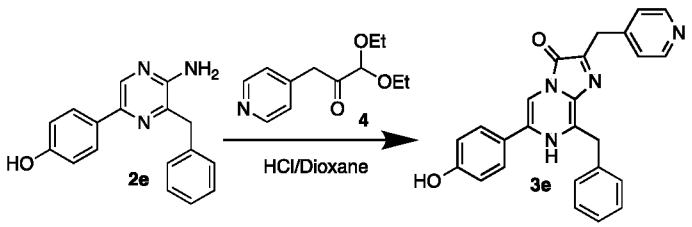
*2-benzyl-8-phenyl-6-(pyridin-4-yl)imidazo[1,2-a]pyrazin-3(7H)-one (**3d**):*



The synthesis and purification of **3d** (above) followed the same procedure as **3c**, whereas **2d** (25 mg, 0.1 mmol, 1 equiv.) was used as the starting compound. Yellow powder

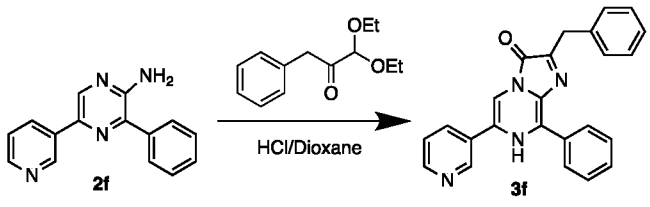
(6 mg, 16%). ^{1}H NMR (600 MHz, Methanol-d_4) δ 9.52 (s, 1H), 9.01 (d, J = 6.8 Hz, 2H), 8.97 (d, J = 6.8 Hz, 2H), 8.15 – 8.11 (m, 2H), 7.71-7.66 (m, 3H), 7.35-7.30 (m, 7.4 Hz, 4H), 7.25 (t, J = 7.4 Hz, 1H), 4.36 (s, 2H). ^{13}C NMR (150 MHz, Methanol-d_4) δ 154.5, 148.8, 143.5, 140.4, 138.4, 137.2, 135.0, 133.1, 130.6, 130.4, 130.4, 130.1, 129.8, 129.5, 128.7, 128.3, 125.3, 117.2, 30.5. HRMS (ESI-TOF) calcd for $C_{24}H_{18}N_4O$ $[M + H]^+$: 379.1481, found: m/z 379.1476.

8-benzyl-6-(4-hydroxyphenyl)-2-(pyridin-4-ylmethyl)imidazo[1,2-a]pyrazin-3(7H)-one (3e):



The synthesis and purification of **3e** (above) followed the same procedure as **3a**, whereas **2e** (28 mg, 0.1 mmol, 1 equiv.) was used as the starting compound. Yellow powder (14 mg, 34%). ^{1}H NMR ^{1}H NMR (600 MHz, Methanol-d_4) δ 8.78 (d, J = 5.2 Hz, 2H), 8.08 (d, J = 5.2 Hz, 2H), 8.01 (s, 1H), 7.72 (d, J = 7.7 Hz, 2H), 7.53 (d, J = 7.7 Hz, 3H), 7.42 (d, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 7.25 (t, J = 7.7 Hz, 1H), 4.58 (s, 2H), 4.52 (s, 2H). ^{13}C NMR (150 MHz, Methanol-d_4) δ 161.2, 142.6, 137.1, 130.3, 130.2, 130.1, 129.1, 128.7, 117.3, 111.3. HRMS (ESI-TOF) calcd for $C_{25}H_{20}N_4O_2$ $[M + H]^+$: 409.1586, found: m/z 409.1585.

2-benzyl-8-phenyl-6-(pyridin-3-yl)imidazo[1,2-a]pyrazin-3(7H)-one (3f):



The synthesis and purification of **3f** (above) followed the same procedure as **3c**, whereas **2f** (25 mg, 0.1 mmol, 1 equiv.) was used as the starting compound. Orange powder

(8 mg, 21%). ^{1}H NMR (600 MHz, Methanol-d_4) δ 9.73 (s, 1H), 9.47 (d, J = 8.3 Hz, 1H), 9.36 (s, 1H), 8.99 (d, J = 5.6 Hz, 1H), 8.32 – 8.27 (m, 1H), 8.09 (d, J = 6.6 Hz, 2H), 7.72 – 7.66 (m, 3H), 7.32 (7.67-7.30, 4H), 7.25 (t, J = 7.0 Hz, 1H), 4.36 (s, 2H). ^{13}C NMR (150 MHz, Methanol-d_4) δ 148.6, 145.3, 142.9, 141.5, 138.3, 137.4, 137.2, 134.8, 133.1, 130.5, 130.4, 130.1, 129.5, 129.1, 128.3, 128.2, 121.6, 114.8, 30.3. HRMS (ESI-TOF) calcd for $C_{24}H_{18}N_4O$ $[M + H]^+$: 379.1481, found: m/z 379.1478.

Plasmid and library construction

Polymerase chain reactions (PCRs) with various synthetic oligonucleotide pairs (see Table 5) were used to amplify genetic elements. To create a gene library with randomization at residues 18 and 19, oligo pairs pBAD-F and L18D19NNK-R, L18D19NNK-F and pBAD-R, were used to amplify two individual fragments from pBAD-teLuc; the corresponding products were used for assembly in a subsequent overlap PCR reaction by using oligos pBAD-F and pBAD-R. The assembled full-length fragment was digested with Xho I and Hind III restriction enzymes and ligated into a predigested, compatible pBAD/His B plasmid. Similarly, pBAD-F, 27VSSNNK-R, 27VSSNNK-F, and pBAD-R were used to create a library with randomization at residues 27, 28, and 29. To introduce random mutations across the gene, Taq DNA polymerase was used in all reactions with 0.2 mM $MnCl_2$ along with unbalanced dNTPs to promote amplification errors. To create mammalian expression plasmids, HindIII-pyr-F-Koz and pyr-R-XhoI were used to amplify the LumiLuc gene fragment, which was further treated with Hind III and Xho I restriction enzymes and ligated into a predigested, compatible pcDNA3 plasmid. The Akaluc gene was synthesized by Eurofins, and cloned into a pBAD plasmid for bacterial expression and a pcDNA3 plasmid for mammalian expression, by using Aka-F-XhoI and Aka-R-HindIII or Aka-F-HindIII-Kozak and Aka-R-XhoI oligonucleotides. To build mScarlet-LumiLuc fusion library, mScarlet-F-XhoI and mScar-NNK-pyr-R oligonucleotides were used to amplify mScarlet-I gene, while mScar-NNK-pyr-F and pyr-R-HindIII oligonucleotides were used for LumiLuc cloning, which were subsequently assembled by overlap PCR reaction. The product was digested with Xho I and Hind III restriction enzymes and ligated into a predigested, compatible pBAD/His B plasmid. The LumiScarlet gene was cloned into pcDNA3 for mammalian expression using HindIII-mScarlet-F-Koz and pyr-R-XhoI oligonucleotides. All ligation products were used to transform *Escherichia coli* DH10B

electrocompetent cells, which were next plated on LB agar plates supplemented with ampicillin (100 μg/mL).

Library screening

DH10B cells containing luciferase mutants were plated on LB agar plates supplemented with ampicillin (100 μg/mL) and L-arabinose (0.02%, w/v%) and incubated at 37°C overnight to form bacterial colonies. Agar plates were left at room temperature for another 6 h, and this was followed by bioluminescence imaging using a luminescence dark box (UVP Bio Spectrum) equipped with a QSI 628 cooled CCD camera (Quantum Scientific Imaging). Digital images were acquired after spraying about 200 μL of 10 μM substrates to each agar plate, and next, images were processed with the Fiji image analysis software to derive bioluminescence intensities of individual colonies. For each round of selection, the brightest 20 colonies from a total of about 10,000 colonies were chosen and inoculated in 5 mL liquid LB broth containing ampicillin (100 μg/mL) and L-arabinose (0.02%, w/v%). After overnight growth at 37°C and 250 r.p.m., the cultures were moved onto a shaker at room temperature for another 6 h. 500 μL cell cultures were centrifuged and next lysed with 100 μL B-PER (Thermo Fisher Scientific). Next, to 1 μL lysate from each sample was added 100 μL substrate at a final concentration of 20 μM in assay buffer. Bioluminescence activities of individual samples were measured on a Synergy Mx Microplate Reader (BioTek). Kinetics were followed for 0.1 s signal integration every 60 s for a total of 20 min. Top three Mutants showing exceptionally high bioluminescence activities or extended kinetics were chosen for next-round selection, sequencing, and other additional characterization. mScarlet-I and LumiLuc fusion libraries were screened for high BRET efficiency using a 600-700 nm bandpass filter. 20 colonies were picked from each library and inoculated in 5 mL liquid LB broth containing ampicillin (100 μg/mL) and L-arabinose (0.02%, w/v%). The cell lysates were prepared with B-PER and the bioluminescence emission spectra were measured by adding 20 μM 8pyDTZ. The construct showed highest BRET efficiency was designated LumiScarlet.

***In vitro* bioluminescence characterization**

Luciferases were expressed and purified as previously described.[33] A Synergy Mx Microplate Reader (BioTek) was used for all *in vitro* bioluminescence characterizations. 50 μL of luciferin substrates was injected into the wells of white 96-well plates containing 50

μL of pure enzymes in assay buffer (1 mM CDTA, 0.5% Tergitol NP-40, 0.05% Antifoam 204, 150 mM KCl, 100 mM MES pH 6.0, 1 mM DTT, and 35 mM thiourea). The final concentrations of all enzymes were 20 pM. Measurements were taken every 30 s post injection (0.1 s integration and 10 s shaking during intervals). Akaluc bioluminescence assays were performed at final concentration of 10 nM Akaluc and 100 μM AkaLumine in an assay buffer contained 30 mM MOPS (pH 7.0), 1.5 mM ATP, and 5 mM $MgSO_4$. To derive values for apparent Michaelis constants (Km), substrate concentrations varied from 0.78 to 50 μM, and peak bioluminescence intensities at individual substrate concentrations were used to fit the Michaelis–Menten equation. To record emission spectra, 50 μL of 20 μM substrates were injected into 50 μL of 2 nM pure enzymes, and the bioluminescence spectra were collected with 0.1 s integration and 1 nm increments from 350 to 750 nm.

Chemiluminescence measurement

0.63 g ammonium bicarbonate was dissolved in 12 mL water and 24 mL acetonitrile containing 30% aqueous hydrogen peroxide, resulting in an active peroxymonocarbonate solution. The solution was left at room temperature for 10 min. Each stock solution containing synthetic analogues (500 μM, 100 μL) was dispensed into wells of a 96-well plate, and chemiluminescence was triggered by addition of 100 μL of the peroxymonocarbonate solution. Light emission was recorded on a Synergy Mx Microplate Reader (BioTek) with 0.1 s integration and 1 nm increments from 350 to 750 nm.

Mammalian cell culture, transfection, and imaging

HEK 293T cells were cultured and transfected as previously described.[33] The number and density of cells in Dulbecco's phosphate-buffered saline (DPBS) were determined using a hemocytometer. Cells were next diluted in DPBS to gain the desired numbers in each 50 μL solution. To use the luminescence dark box to directly image cells, we added luciferase-expressing HEK 293T cells (5,000 cells per well with ~70% transfection efficiency) and the corresponding luciferin substrates into wells of a white-wall, 96-well plate. Bioluminescence was imaged using a luminescence dark box immediately after substrate addition. The camera exposure time was set at 2 s. A Chroma Red 600-700 nm filter was used to acquire far-red emission. All images were analyzed using the Fiji image analysis software.

Generation of luciferase-expressing stable cell lines

HeLa cells were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were transfected with pcDNA3-teLuc, pcDNA3-Antares2, pcDNA3-LumiLuc, pcDNA3-LumiScarlet, or pcDNA3-Akaluc as previously described.[33] 48 h after transfection, cells were passed into fresh DMEM containing 10% FBS and 1 mg/mL G418. The medium was removed and replaced every 3 days. Stable polyclonal cell lines were generated after ~ 2 weeks of G418 selection.

Xenograft mouse model

HeLa cells stably expressing luciferases were dissociated with trypsin and re-suspended in 10 mL DMEM. Cell numbers were determined using a hemocytometer, and cell viability was determined using a trypan blue exclusion test. 10^4 or 10^5 cells were re-suspended in 100 μL FBS-free DMEM containing 50% Matrigel matrix (Corning). 8-week-old female nude mice were first anesthetized using isoflurane. Cells were subcutaneously injected into the left and right dorsolateral trapezius regions or thoracolumbar regions. Mice were recovered on heat pads for 5 min while cells were allowed to settle. On day 1, 3, 5, 7, 14, and 28 post tumor implants, mice were subsequently imaged using a Caliper IVIS Spectrum (Perkin Elmer) approximately 5 min after intravenous (i.v.) administration of corresponding luciferins (100 μL solution for indicated doses). DTZ was dissolved in a 100 μL solution containing 8% glycerol, 10% ethanol, 10% hydroxypropyl-β-cyclodextrin, and 35% PEG 400 in water. 8pyDTZ and AkaLumine-HCl was dissolved in normal saline. All solutions were passed through 0.22 μm pore filters before administrations. The following conditions were used for image acquisition: open filter for total bioluminescence, exposure time = 60 s (Day 1, 3, and 5); 30 s (Day 7); 10 s (Day 14); 3 s (Day 28), binning = small, field of view = 21.6 × 21.6 cm, and f/stop = 1. Image analysis was performed using the Living Image 4.3.1 software.

Deep-tissue mouse model

10^6 HeLa cells stably expressing either LumiLuc, LumiScarlet or Akaluc were i.v. injected to female nude mice. After 4 h, images were acquired using a Caliper IVIS Spectrum immediately after i.v. delivery 0.2 μmol 8pyDTZ or 1.5 μmol AkaLumine-HCl in 100 μL normal saline. The following conditions were used for image acquisition: open filter

for total bioluminescence, exposure time = 10 s, binning = small, field of view = 21.6 × 21.6 cm, and f/stop = 1.

Fluorescence imaging of ATP in mammalian cells

HEK293T and HeLa cells were cultured and transfected as aforementioned. Images were acquired on a Leica DMi8 inverted microscope equipped with the SPE confocal module. Cells were cultured in DMEM (no phenol red) with 4.5 g/mL glucose. 405 and 488 nm laser was used to excite PercevalHR, and emission was collected from 510 nm to 600 nm. Intervals between each image were 5 second. 50 μM 8pyDTZ was added to cells. For the internal calibration purpose, iodoacetic acid (IAA) was added to a final concentration of 5 mM to completely deplete intracellular ATP. pHRFP was also used to monitor the cellular pH change before and after addition of 50 μM 8pyDTZ.

Statistical analysis.

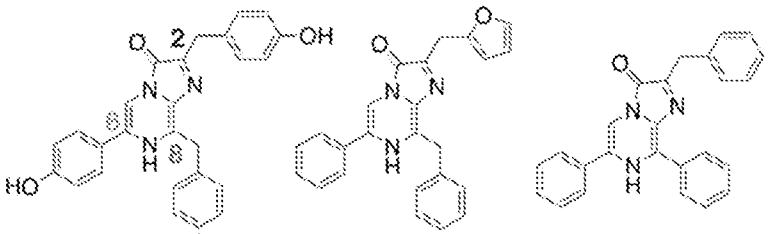
Unpaired two-tailed t-tests were used to determine all *p*-values. No statistical methods were used to predetermine the sample size. Animals were randomly assigned to receive various treatments. Unless otherwise indicated, data are shown as mean ± s.d., and error bars in figures represent s.d.

EXAMPLE 1

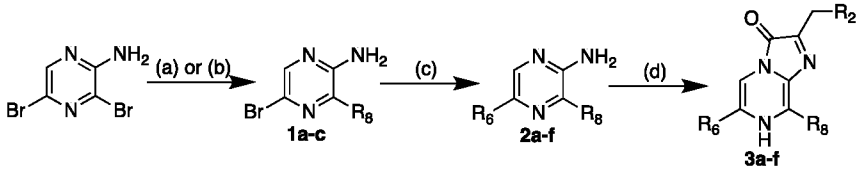
Design and synthesis of pyridyl CTZ and DTZ analogs with enhanced water solubility

Despite that recent studies have synthesized and tested a number of CTZ analogs with NanoLuc,[18, 19] the luciferase has not yet been optimized to pair with these new substrates and the water solubility issue of the substrates has not yet been tackled systematically. Thus, a need remained for CTZ and DTZ analogs with improved water solubility.

The chemical structures of coelenterazine (CTZ), furimazine (FRZ) and diphenylterazine (DTZ) are provided below.



The need was fulfilled, as disclosed herein, by using the concept of bioisostere replacements in medicinal chemistry. Pyridine is considered a biocompatible N-heterocycle substituent for benzene with enhanced water solubility, because pyridine-containing molecules can be readily turned into pyridinium salts. Therefore, a convergent synthetic route was designed to prepare a series of CTZ and DTZ analogs with pyridyl isomer substitutions at the C-2, C-6 and C-8 positions of the imidazopyrazinone core (Scheme 1, below). Briefly, Suzuki or Negishi cross-coupling reactions were first used to regioselectively functionalize 2-amino-3,5-dibromopyrazine with either pyridyl, phenyl, or benzyl functional groups to give monosubstituted products (1a-c), which were subsequently derivatized via Suzuki cross-coupling reactions to afford disubstituted intermediates (2a-f, see structures and synthetic methods above). In the second cross-coupling step, the XPhos-Pd-G2 catalyst was used to enhance reaction yields and minimize the protodeboronation of pyridyl boronic acids.[20] An acid-catalyzed ring closing reaction[21] in dioxane was also utilized to derive various pyridyl CTZ and DTZ analogs (3a-f, Table 2) from the disubstituted intermediates and corresponding α-ketoacetals.



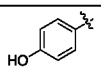
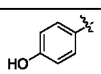
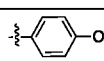
Scheme 1. Synthesis of pyridyl CTZ and DTZ analogs. (a) Suzuki coupling: $Pd(PPh_3)_4$, Na_2CO_3, R_8-$B(OH)_2$, and EtOH; (b) Negishi coupling: $PhCH_2MgCl$, $ZnCl_2$, $(PPh_3)_2PdCl_2$, and THF; (c) Suzuki coupling: XPhos-Pd-G2, Na_2CO_3, R_6-$B(OH)_2$, and EtOH; (d) Acid-catalyzed ring closing: corresponding α-ketoacetal, HCl, and dioxane.

Turbidimetric solubility assays[22] were used to evaluate water solubility of these CTZ and DTZ analogs (Table 2). Surprisingly, the newly synthesized pyridyl analogs enhanced the solubility by 4- to 14-fold as compared to CTZ and DTZ. The autoluminescence and stability of these new analogs was also evaluated, the results of which indicated that they are comparable or even better than CTZ and FRZ. Moreover, their

chemiluminescence was also evaluated, because the wavelength of bioluminescence is often related to the wavelength of substrate chemiluminescence, although the luciferase enzyme provides further electrostatic tuning which can reshape the emission (Figure 2). In addition, the bioluminescence of these new substrates in the presence of several representative, ATP-independent luciferases such as RLuc8, NanoLuc, teLuc, and aequorin was evaluated (Figures 3A-3D).[23]

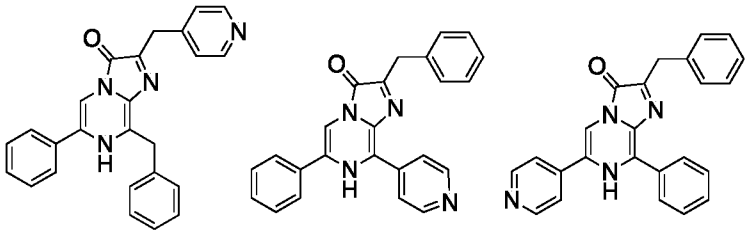
Although each luciferase has different substrate preferences, the compound 3a (pyCTZ) generated strong blue bioluminescence in the presence of each of these tested luciferases. When paired with aequorin, the bioluminescence intensity of pyCTZ was comparable to native CTZ, suggesting that pyCTZ may be directly used to replace CTZ for aequorin-based calcium sensing.[24] Furthermore, compared to DTZ, compounds 3c (8pyDTZ), and 3f were able to emit red-shifted chemiluminescence and/or bioluminescence, while 3b and 3d (6pyDTZ) caused hypsochromic shift (Table 2). Molecular mechanisms governing the spectral shift properties of these synthetic substrates remain to be investigated. Because 3c showed the most red-shifted emission and red-shifted photons can penetrate through tissue better,[25] 3c (8pyDTZ) was selected as the candidate substrate for further development of an optimized, red-shifted luciferase-luciferin pair.

Table 2. Chemical and photoluminescence properties of synthetic pyridyl CTZ and DTZ analogs.

Compound	R_6	R_8	R_2	Bioluminescence [a] λ_{max} (nm)	Chemiluminescence [b] λ_{max} (nm)	Water Solubility (μM)
3a				451	505	1416
3b				497	506	1813
3c				532	555	1711
3d				483	492	1736
3e				450	465	987
3f				518	503	1562
CTZ				455	461	256
DTZ				502	510	131

Note: [a] Determined with 1 nM teLuc in PBS; [b] Triggered by peroxymonocarbonate formed *in situ*.

The chemical structures of pyCTZ (3a in Table 2), 8pyDTZ (3c in Table 2) and 6pyDTZ (3d in Table 2) are provided below, as well as in Figure 1.



EXAMPLE 2

Directed evolution of the teLuc luciferase for improved brightness

teLuc was previously optimized for DTZ, a substrate with conjugated disubstitutions on the imidazopyrazinone core. 8pyDTZ exhibits about 30 nm red-shift but the emission of teLuc-8pyDTZ has been greatly attenuated compared to teLuc-DTZ. teLuc was then engineered for increased photon flux in the presence of 8pyDTZ. On the basis of a published apo-nanoKAZ structure[26] and our computational model,[7] random mutations were first introduced to residues 18 and 19 close to a putative substrate-binding pocket (Figures 4A and 4B). After screening for improved mutants, residues 27, 28, and 29 located deeper in the putative catalytic site were further randomized. From the first two rounds of protein engineering, a teLuc-L18Q/S19A/V27L/S28T mutant was identified, to which random mutations were further introduced using error-prone PCR. After eight additional rounds of mutagenesis and screening, a LumiLuc luciferase was derived with 12 total mutations and about 5-fold enhancement of 8pyDTZ bioluminescence from teLuc (Figures 4C and 5).

The resultant LumiLuc-8pyDTZ pair has an emission peak at 525 nm. Its *in vitro* maximal photon emission rate (Vmax) is about 60% and about 36% of NanoLuc-FRZ and teLuc-DTZ, respectively. The apparent Michaelis constant (KM) of LumiLuc-8pyDTZ was 4.6 μM, lower than that of teLuc-DTZ or NanoLuc-FRZ (Figure 6A). This reduced KM is practically beneficial, since LumiLuc-8pyDTZ would be relatively brighter when effective substrate concentrations are limited, such as in live cells (Figure 6B) and *in vivo*. Similar to NanoLuc and teLuc, the bioluminescence kinetics of LumiLuc is flash-type in phosphate buffer saline (PBS) and glow-type in a specially formulated assay buffer (Figure 6C).[12]

LumiLuc has broad substrate specificity. It improved the photon flux of 3a (pyCTZ) and 3d (6pyDTZ) from teLuc by about 120% and about 150%, respectively (Figure 4C). The directed evolution process to enhance photon flux of teLuc for 8pyDTZ did not preclude the luciferase from catalyzing other structurally relevant substrates. LumiLuc is capable of efficiently generating blue, teal, or yellow bioluminescence when paired with pyCTZ, 6pyDTZ or 8pyDTZ (λmax: 450, 476, and 525 nm, respectively; Figure 4D), thereby leading to a new family of ATP-independent bioluminescent reporters with water-soluble substrates.

EXAMPLE 3

LumiLuc-8pyDTZ in cultured mammalian cells

Next, LumiLuc-8pyDTZ was evaluated in human embryonic kidney (HEK) 293T cells transiently expressing the luciferase (Figure 7). The LumiLuc-8pyDTZ pair produced about 3- to 5-fold more bioluminescence than teLuc-8pyDTZ at all tested substrate concentrations. Moreover, despite that LumiLuc-8pyDTZ is less bright than teLuc-DTZ at saturated substrate concentrations, LumiLuc-8pyDTZ is notably brighter than teLuc-DTZ at low substrate concentrations (from 6.25 to 25 μM; Figure 8A). Far-red emission at wavelengths longer than 600 nm is more indicative of the *in vivo* performance of bioluminescent reporters, because mammalian tissue is more transparent in this spectral region.[25] To compare far-red emission intensities of bioluminescent reporters, HEK 293T cells were imaged in the presence of a 600-700 nm bandpass filter. At substrate concentrations from 6.25 to 100 μM, LumiLuc-8pyDTZ consistently produces 1.6- to 3.9-fold higher photon flux than teLuc-DTZ (Figure 8B).

ATP-dependent luciferases, such as FLuc and Akaluc consumes one ATP molecule in each catalytic cycle, leading to metabolic disruption.[8] Instead, ATP-independent LumiLuc does not use ATP for catalysis. ATP/ADP ratios were monitored in live HEK 293T cells using PercevalHR, a previously reported fluorescent ATP/ADP biosensor.[27] No ATP perturbation was observed from 8pyDTZ-treated, LumiLuc-expressing cells.

EXAMPLE 4

LumiLuc-8pyDTZ to track tumor growth in a mouse xenograft model

BLI has been a popular imaging modality for various animal models.[13, 25] The recently reported Akaluc-AkaLumine and Antares2-DTZ pairs are two benchmark reporters

for *in vivo* BLI.[6, 7] A biologically-relevant tumor xenograft mouse model[28] was adapted to compare these bioluminescent reporters. Cervical cancer HeLa cell lines stably expressing individual luciferases were generated, including teLuc, Antares2, LumiLuc, and Akaluc (Figures 9A and 9B). Next, 104 or 105 luciferase-expressing HeLa cells were injected into the left or right dorsolateral trapezius and thoracolumbar regions of immunodeficient NU/J mice (day 0) and monitored tumor growth over 4 weeks. Bioluminescence was quantified in days 1, 3, 5, 7, 14, and 28 after tail vein injection of corresponding substrates. AkaLumine-HCl was delivered at a dose of 1.5 μmol per mouse. This dosage (about 75 nmol/g), which is normalized against the body weights of mice, is identical to the previously reported dosage.[6] Moreover, when 3 μmol of AkaLumine-HCl per mouse (about 150 nmol/g) was used, death for 2 out of 3 mice was observed in the pilot experiment. 8pyDTZ was dissolved in normal saline to its saturation concentration and intravenously injected, resulting in a dose of 0.2 μmol per mouse (about 10 nmol/g). The LumiLuc-8pyDTZ pair showed detectable bioluminescence on day 1 at sites inoculated with 104 cells, and kept exhibiting about 3-fold higher photon flux over Akaluc-AkaLumine up to day 7 (Figures 10A and 10B). The signals for Akaluc-AkaLumine at sites inoculated 104 cells were not consistently higher than background until day 3. Furthermore, the *in vivo* brightness of LumiLuc-8pyDTZ is comparable to, if not higher than, the Antares2-DTZ pair (Figures 10A, 10B and 11A-11C), despite the fact that the majority of emitted photons from LumiLuc-8pyDTZ has not yet exceeded 600 nm. These data collectively support that LumiLuc-8pyDTZ is a superior bioluminescent reporter system for high-sensitivity *in vivo* BLI.

The bioluminescence of Akaluc-AkaLumine eventually surpassed LumiLuc-8pyDTZ from day 14 (Figures 11A-11C). In addition to differences in biodistribution and pharmacokinetic properties of AkaLumine and 8pyDTZ, it was interpret that 8pyDTZ may be a limiting reagent in large tumors because AkaLumine-HCl could be delivered into mice at a much higher dose than 8pyDTZ due to the higher solubility of AkaLumine-HCl. It may be possible further enhance the *in vivo* performance of marine luciferases and their derivatives by further increasing the water solubility and thus the administration dosage of CTZ and DTZ analogs.

This data provides for the monitoring of the disclosed luciferase-luciferin pairs for *in vivo* monitoring of tumor models. For example, bioluminescence can be monitored in a tumor model by establishing a luciferase expressing cell (e.g. a cell expressing LumiLuc,

teLuc, RLuc8 and OpyLuc) in the model, e.g. by transfecting cells in the model and/or by administering to that model already transfected cells expressing a luciferase, and administering to the model a luciferin (e.g. pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ or 8pyDTZ). Using these luciferase-luciferin pairs as reporter systems the bioluminescence can be used as a tool to monitor the tumor.

EXAMPLE 5

Engineering of BRET-based LumiScarlet and teScarlet for deep-tissue BLI

mScarlet-I is a recently reported red fluorescent protein with high quantum yield and excellent performance as a Förster resonance energy transfer (FRET) acceptor.[29] It was thus hypothesized that LumiLuc could be genetically fused to mScarlet-I for BRET, thereby red-shifting the emission of LumiLuc. Several fusion strategies between LumiLuc and mScarlet-I were explored, libraries were constructed by randomizing the linkers, and mutants with high BRET efficiency were screened (Figures 12A and 12B). A mutant was identified, namely LumiScarlet (Figure 13A), which is a fusion protein of mScarlet-I (residues 1-225; Bindels et al, 2016, Nature Methods, 14(1) , 53-56; incorporated herein by reference) linked to the N-terminus of LumiLuc (residues 2-169; SEQ ID NO. 3) through a single-residue "Lys" linker (nucleotide and polypeptide sequences provided herein as SEQ ID NOs. 4 and 5, respectively).

High BRET efficiency was achieved with LumiScarlet in the presence of either pyCTZ, or 6pyDTZ, or 8pyDTZ (Figure 13B). In particular, because the emission spectrum of LumiLuc-8pyDTZ overlaps well with the excitation spectrum of mScarlet-I (Figure 12C), about 51% of the total emission of LumiScarlet, when paired with 8pyDTZ, was longer than 600 nm (Table 3).

Table 3. BRET-based bioluminescent reporters that are based on NanoLuc and its derivatives.

BRET Construct	BRET Donor	BRET Acceptor	Size (kDa)	λ_{max} (nm)	Luciferin	Photon > 600 nm (%)	Ref.
LumiScarlet	LumiLuc	mScarlet-I	44	527, 600	8pyDTZ	51	This work
	LumiLuc	mScarlet-I	44	476, 600	6pyDTZ	38	
	LumiLuc	mScarlet-I	44	450, 600	pyCTZ	26	

Antares	NanoLuc	CyOFP	70	456, 583	FRZ	23	([1])
Antares2	teLuc	CyOFP	70	501, 583	DTZ	33	([2])
ReNL	NanoLuc	tdTomato	72	459, 583	FRZ	24	([3])

Next, the newly engineered LumiLuc-8pyDTZ and LumiScarlet-8pyDTZ were compared with Akaluc-AkaLumine for deep-tissue BLI. A million HeLa cells stably expressing corresponding luciferases were injected into each of NU/J mice via tail vein and performed BL imaging 4 h later. Immunodeficient mice were used here to minimize immune responses to HeLa cells, so that signals will be mostly from live cells trapped in the lungs. LumiScarlet gave about 3-fold higher detectable signals than LumiLuc under this condition (Figure 13C and 13D), even though the *in vitro* brightness of LumiScarlet is only about 70% of LumiLuc (Figure 12D). Moreover, the signals from LumiScarlet-8pyDTZ were comparable to the signals from Akaluc-AkaLumine.

Of note, some diffuse signals were observed from areas other than the lungs. These signals were not caused by substrate background, as injection of 8pyDTZ into blank mice resulted in only weak background much lower than what was observed in Figure 13C. Luciferase activities were detectable in blood after tail vein injection of luciferase-expressing HeLa cells, suggesting partial lysis of luciferase-labeled cells during cell injection. Also, in contrast to ATP-independent LumiLuc and LumiScarlet, ATP-dependent Akaluc is enzymatically inactive in serum because of relatively low ATP levels and further deactivation by serum components.[8] Thus, diffuse signals from LumiScarlet in Figure 13C were likely from the LumiScarlet luciferase released into blood.

Collectively, the deep-tissue BLI results confirm that red-shifted BRET-based LumiScarlet has better mammalian tissue penetration than LumiLuc. Moreover, LumiScarlet-8pyDTZ is a novel, ATP-independent bioluminescent reporter with exceptional deep-tissue BLI performance comparable to ATP-dependent Akaluc-AkaLumine.

Similar to the BRET-based strategy of creating LumiScarlet, a BRET-based fusion of teLuc and mScarlet-I was engineered. Libraries were constructed to randomize the linker between teLuc and mScarlet-I. A mutant was identified, namely teScarlet (Figure 12E), which is a fusion protein of mScarlet-I (residues 1-221) linked to the N-terminus of teLuc (residues 2-169) through a three-residue "His-Leu-Asp" linker (nucleotide and polypeptide

sequences provided herein as SEQ ID NOs. 7 and 8, respectively). High BRET efficiency was also achieved with teScarlet in the presence of DTZ (Figure 12F).

Discussion of Examples 1-5

Conventionally, ATP-dependent bioluminescent reporters, such as FLuc and Akaluc, are considered to be more useful for *in vivo* BLI than ATP-independent marine luciferases, because the emission of ATP-dependent insect luciferases is often at the red end of the visible spectrum where the mammalian tissue is relatively transparent. However, these insect luciferases require ATP and Mg2+ for bioluminescence. The ATP- and Mg2+-dependency is sometimes problematic because ATP and Mg2+ levels may vary under different biological circumstances.[30] In particular, ATP-dependent luciferases are inactive in extracellular space and common biological fluids such as blood and urine, where ATP accessibility is limited.[8] Moreover, ATP-dependent luciferases consume ATP in bioluminescence reactions and may cause concerns such as metabolic disruption.[8] In contrast, most ATP-independent marine luciferases are enzymatically active in extracellular space and common biological fluids; they do not consume ATP for bioluminescence. Furthermore, some marine luciferase derivatives have fast catalytic turnover and thus give high photon flux. It is therefore not surprising that marine luciferase and their derivatives, such as NanoLuc and Gaussia luciferase, have been widely used for *in vitro* bioluminescence assays. However, currently, the *in vivo* applications of marine luciferases are hindered by their blue emission and poor substrate water solubility. As disclosed herein, combined chemical synthesis and protein engineering approaches yielded enhanced ATP-independent marine luciferases for *in vivo* BLI by developing red-shifted colors and water-soluble substrates.

First, a series of pyridyl CTZ and DTZ analogs with diverse emission profiles were prepared. The water solubility of these synthetic analogs generally increased by about 10-fold from their ancestors. These substrate analogs can not only be paired with the new luciferases engineered here, but also existing ATP-independent reporters, such as RLuc and aequorin.

Further, a luciferase was engineered for the 8pyDTZ substrate via directed protein evolution. The resultant LumiLuc-8pyDTZ bioluminescent reporter system exhibited reduced KM and red-shifted emission. These factors favored *in vivo* BLI. As a result, LumiLuc-8pyDTZ showed high sensitivity in a mouse xenograft model. In addition,

LumiLuc-8pyDTZ did not perturb the intracellular ATP/ADP level, and 8pyDTZ could be dissolved up to about 2 mM in low-viscosity saline without using irritative and toxic organic cosolvent. Therefore, the efforts disclosed herein enhanced not only the biocompatibility of bioluminescent reporters, but also reproducibility for intravenous injections.

Furthermore, a BRET-based LumiScarlet reporter was developed for further red-shifted emission. The emission of LumiLuc-8pyDTZ overlaps well with the excitation of mScarlet-I, an excellent red-emitting resonance energy transfer acceptor. LumiScarlet-8pyDTZ exhibited high brightness, significant emission longer than 600 nm, and excellent tissue penetration. LumiScarlet-8pyDTZ was comparable to NIR-emitting Akaluc-AkaLumine in a mouse model for deep-tissue BLI. Moreover, because LumiScarlet is enzymatically active in blood, it will be an excellent reporter for monitoring targets of interest in the blood of *in vivo* models.

LumiLuc is a luciferase with broad substrate specificity. When it was paired with different substrates, intense blue, teal, and yellow bioluminescence was generated. Subsequently, different emission profiles from LumiScarlet were gained in the presence of different substrates. The use of LumiScarlet-8pyDTZ for deep-tissue imaging was also demonstrated. In addition, because the two emission peaks of LumiScarlet-pyCTZ or LumiScarlet-6pyDTZ are more separated than LumiScarlet-8pyDTZ, LumiScarlet-pyCTZ and LumiScarlet-6pyDTZ will be useful for studying protein-protein interactions or constructing BRET-based biosensors.

In summary, disclosed herein are several engineered luciferase-luciferin pairs that emit photons spanning an appreciable range in the visible spectrum. The discoveries disclosed herein greatly enhance the biocompatibility and sensitivity of ATP-independent bioluminescent reporters for *in vivo* BLI. Future studies are likely to continuously increase the water-solubility of CTZ and DTZ analogs and red-shift the emission of marine luciferases. Subsequently, it is expected that a large array of bioluminescent biosensors will be developed on the basis of these bright, ATP-independent bioluminescent reporters.[31] The new reporters and biosensors will further ease non-invasive imaging of freely moving animals, leading to new biological insights.

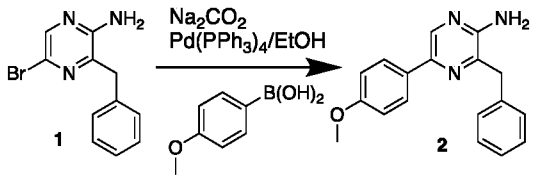
Materials and Methods for Examples 6-9

Materials and general methods

Synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases were purchased from Thermo Fisher Scientific. Q5 high-fidelity DNA polymerase and Taq DNA polymerase were purchased from NEB. Products of PCR and restriction digestion were purified by gel electrophoresis and Syd Laboratories Gel Extraction columns. Plasmid DNA was purified using Syd Laboratories Miniprep columns. DNA sequences were analyzed by Eurofins. AkaLumine-HCl was purchased from Aobious. All other chemicals were purchased from Sigma-Aldrich, Fisher Scientific, or VWR and used without further purification. Bruker Avance DRX 600 and Varian NMRS 600 at the UVA Biomolecular Magnetic Resonance Facility was used to record all NMR spectra. Chemical shift (δ) is given in parts per million relative to ^{1}H (7.24 p.p.m.) and ^{13}C (77.23 p.p.m.) for $CDCl_3$; ^{1}H (2.50 p.p.m.) and ^{13}C (39.5 p.p.m.) for DMSO-d_6; ^{1}H (3.31 p.p.m.) and ^{13}C (49.15 p.p.m.) for methanol-d_4. Splitting patterns are reported as s (singlet), bs (broad singlet), d (doublet), t (triplet), dd (doublet of doublets), and m (multiplet). Coupling constant (J) is given in Hz. High resolution ESI-MS was run on an Agilent 6545 Q-TOF LC/MS system by direct infusion. A Waters Delta Prep ZQ 2000 LC-MS Purification System equipped with a XBridge BEH Amide OBD Prep Column (130Å, 5 µm, 30 mm X 150 mm) was used for preparative reverse-phase HPLC purifications. Images were analyzed using the Fiji image analysis software. Microsoft Excel and GraphPad Prism were used to analyze data and prepare figures.

Chemical synthesis

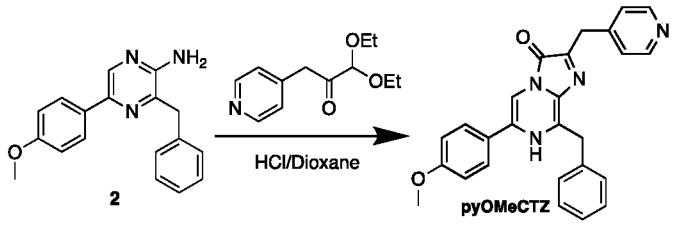
3-benzyl-5-(4-methoxyphenyl)pyrazin-2-amine (2):



1 was prepared following the published synthesis methods[64]. To a solution of $Pd(PPh_3)_4$ (230 mg, 0.2 mmol, 0.1 equiv.) in 50 mL EtOH was added **1** (528 mg, 2 mmol, 1 equiv.), 1N Na_2CO_3 solution (4 mL, 4 mmol, 2 equiv.) and 4-Methoxylphenylboronic acid (304 mg, 2 mmol, 1 equiv.). The resultant mixture was stirred at 80 °C under argon for 12

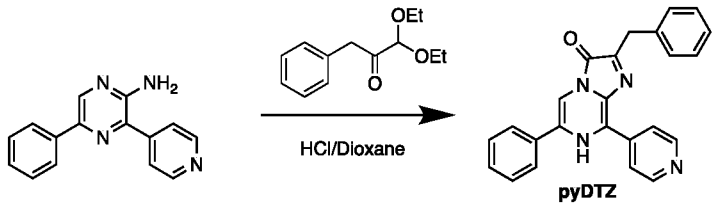
h. The solvent was removed *in vacuo* and the residue was suspended in 100 mL ddH_2O, which was extracted twice with EtOAc (100 mL). The organic layers were combined and dried over anhydrous Na_2SO_4, filtered and removed in *vacuo*. The residue was purified by silica column chromatography with elution (Ethyl acatate:Hexane = 1:1) to yield compound **2** as yellow solid (413 mg, 71%). ^1H NMR (600 MHz, DMSO-d_6) δ 8.33 (s, 1H), 7.82 (d, J = 8.8 Hz, 2H), 7.32 (d, J = 6.7 Hz, 2H), 7.26 (t, J = 7.6 Hz, 2H), 7.17 (t, J = 7.4 Hz, 1H), 6.95 (d, J = 8.8 Hz, 2H), 6.26 (s, 2H), 4.05 (s, 2H), 3.76 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) 158.9, 152.2, 139.8, 139.0, 138.2, 136.2, 128.9, 128.2, 126.1, 126.1, 114.1, 55.1, 38.6. HRMS (ESI-TOF) calcd for $C_{18}H_{17}N_3O$ $[M + H]^+$: 292.1372, found: m/z 292.1369.

*8-benzyl-6-(4-methoxyphenyl)-2-(pyridin-4-ylmethyl)imidazo[1,2-a]pyrazin-3(7H)-one (**pyOMeCTZ**):*



To a solution of **2** (29 mg, 0.1 mmol, 1 equiv.) and 3-pyridin-4-yl-1,1-diethoxyacetone (45 mg, 0.2 mmol, 2 equiv) in 2 mL degassed 1,4-dioxane was added 1 mL 6N HCl. The resulting mixture was stirred at 80°C in a seal tube for 12 h. The solvent was then removed *in vacuo* and the residue was dissolved in 1 mL (ACN:H_2O = 1:1) and next purified with preparative RP-HPLC. (acetonitrile/water = 1:99 to 90:10, 20 mL/min, UV 254 nm). Product fractions were combined and lyophilized to give **pyOMeCTZ** as yellow powder (10 mg, 24%), which has to be stored as solid at -80 °C for long-term stability. ^1H NMR (600 MHz, Methanol-d_4) δ 8.82 (d, J = 6.4 Hz, 2H), 8.27 (s, 1H), 8.09 (d, J = 6.2 Hz, 2H), 7.73 (d, J = 8.7 Hz, 2H), 7.43 (d, J = 7.5 Hz, 2H), 7.32 (t, J = 7.6 Hz, 2H), 7.26 (t, J = 7.2 Hz, 1H), 7.10 (d, J = 8.7 Hz, 2H), 4.66 (s, 2H), 4.61 (s, 2H), 3.87 (s, 3H). ^{13}C NMR (150 MHz, Methanol-d_4) δ 142.7, 137.0, 130.2, 130.1, 129.1, 128.7, 126.5, 115.9, 56.2. HRMS (ESI-TOF) calcd for $C_{26}H_{22}N_4O_2$ $[M + H]^+$: 423.1743, found: m/z 423.1740.

*2-benzyl-6-phenyl-8-(pyridin-4-yl)imidazo[1,2-a]pyrazin-3(7H)-one (**pyDTZ**):*



To a solution of 5-phenyl-3-(pyridin-4-yl)pyrazin-2-amine (25 mg, 0.1 mmol, 1 equiv.) and 1,1-diethoxy-3-phenylpropan-2-one (89 mg, 0.4 mmol, 4 equiv.) in 5 mL degassed 1,4-dioxane was added 0.8 mL 6 N HCl, and the resulting mixture was stirred at 80°C in a sealed tube for 12 h. The solvent was then removed *in vacuo* and the residue was dissolved in 1 mL (ACN:H_2O = 1:1) and next purified with preparative RP-HPLC. (acetonitrile/water = 1:99 to 90:10, 20 mL/min, UV 254 nm). Product fractions were combined and lyophilized to give **pyDTZ** as brown powder (8 mg, 22%). ^{1}H NMR (600 MHz, Acetonitrile-d_3 and D_2O, ratio = 9:1) δ 9.31 (d, J = 6.8 Hz, 2H), 8.84 (d, J = 6.8 Hz, 2H), 8.54 (s, 1H), 8.09 (d, J = 8.0 Hz, 2H), 7.52 (t, J = 7.6 Hz, 2H), 7.44 (t, J = 7.3 Hz, 1H), 7.35 (d, J = 7.7 Hz, 2H), 7.28 (t, J = 7.7 Hz, 2H), 7.24 – 7.21 (m, 1H), 4.19 (s, 2H). ^{13}C NMR (150 MHz, Methanol-d_4) δ 143.0, 140.7, 140.1, 139.5, 137.5, 132.0, 130.2, 129.9, 129.6, 129.4, 127.8, 127.5, 127.4, 126.5, 113.8, 33.6. HRMS (ESI-TOF) calcd for $C_{24}H_{18}N_4O$ $[M + H]^+$: 379.1481, found: m/z 379.1477.

Plasmid construction

Polymerase chain reactions with various synthetic oligonucleotide pairs (see Table 4) were used to amplify genetic elements. Generating gene libraries with randomizations were previously described. Abovementioned screening approach was applied to the selection process of random mutagenesis by Error prone-PCR. Oligonucleotides pBAD-F and pBAD-R were used to create a library with randomization by using Taq DNA polymerase, 0.2 mM $MnCl_2$, and unbalanced dNTPs to promote amplification errors. The PCR product was digested with Xho I and Hind III restriction enzymes and ligated into a predigested, compatible pBAD/His B plasmid. To create mammalian expression plasmids containing NFκB response element, NFkB_SacI_F and NFkB_BgIII_R were used to amplify the fragment from pHAGE NFkB-TA-LUC-UBC-GFP-W plasmid (Addgene:49343), which was further treated with Sac I and BgI II restriction enzymes and ligated into a predigested, compatible SRE reporter vector_559 plasmid (Addgene:82686).

For Antioxidant response element, the DNA fragment was synthesized by IDT and ligated into Sac I and BgI II predigested SRE reporter vector_559 plasmid. The OpyLuc, RLuc8, and Akaluc gene were cloned into corresponding plasmids containing desired response element by using opyluc_AscI_Kozak_F/opyluc_FseI_R, Rluc_ AscI_Kozak_F/Rluc_FseI_R, or Akaluc_AscI_Kozak_F/Akaluc_FseI_R oligonucleotide pairs with Asc I and Fse I double digestion. All ligation products were used to transform *Escherichia coli* DH10B electrocompetent cells, which were next plated on LB agar plates supplemented with ampicillin (100 μg/mL).

Table 4. Oligonucleotides used in this study.

Oligo name	**SEQ ID NO.**	**Nucleotide sequence (5′–> 3′)**
pBAD-F	19	ATGCCATAGCATTTTTATCC
pBAD-R	20	GATTTAATCTGTATCAGG
NFkB_SacI_F	21	TACCGAGCTCATCCAGTTTGGACTAGTGG
NFkB_BgIII_R	22	AGCCCAGATCTCCTCTAGAGTCTAGATCTGG
opyluc_AscI_Kozak_F	23	AAAGCCACCGGCGCGCCGCCGCCACCATGGTCTTCACTCTCGAAGATTTTGT
opyluc_FseI_R	24	TCGAAGCGGCCGGCCTTACGCCAGAATGCGTTCATGCA
Akaluc_AscI_Kozak_F	25	AAAGCCACCGGCGCGCCGCCGCCACCATGGAAGATGCCAAAAACATTAAGA
Akaluc_FseI_R	26	TCGAAGCGGCCGGCCTTACACGGCGATCTTGCCGTCCTTCTT
Rluc_ AscI_Kozak_F	27	AAAGCCACCGGCGCGCCGCCGCCACCATGGCTTCCAAGGTGTACGACC
Rluc_FseI_R	28	TCGAAGCGGCCGGCCTTACTGCTCGTTCTTCAGCACGCGCT

Preparation of mammalian cell culture and cell lysate

HEK 293T cells were cultured and transfected as previously described.[63] The number and density of cells in Dulbecco's phosphate-buffered saline (DPBS) were determined using a hemocytometer. Cells were next diluted in DPBS to gain the desired numbers in each 50 μL solution. Cell lysates were obtained by incubating desired number of cell in a CelLytic M solution for 15 minutes and centrifuged.

Library screening

DH10B cells containing luciferase mutants were plated on LB agar plates supplemented with ampicillin (100 μg/mL) and l-arabinose (0.02%, w/v%) and incubated at 37°C overnight to form bacterial colonies. Agar plates were left at room temperature for another 6 h, and this was followed by bioluminescence imaging using a luminescence dark box (UVP Bio Spectrum) equipped with a QSI 628 cooled CCD camera (Quantum Scientific Imaging). Digital images were acquired after spraying about 200 μL of 50 μM pyDTZ to each agar plate, and next, images were processed with the Fiji image analysis software to derive bioluminescence intensities of individual colonies. For each round of selection, colonies showed bright bioluminescence were chosen and inoculated in 1 mL liquid LB broth containing ampicillin (100 μg/mL) and L-arabinose (0.02%, w/v%) in 96-well deep plates. After overnight growth at 37°C and 250 r.p.m., the cultures were moved onto a shaker at room temperature for another 6 h. The 96-well plates were centrifuged and the pellet in each well was lysed with 200 μL B-PER. After 30-minute incubation, the 96-well plates were centrifuged again. Next, 2 μL lysate from each sample was transferred to the wells of new white 96-well plates where 100 μL of 20 μM pyDTZ in assay buffer was added to each well. Bioluminescence activities of individual samples were measured on a microplate reader. Kinetics were followed for 0.1 s signal integration every 30 s for a total of 10 min. Meanwhile, 2 μL lysate from each sample was added 100 μL of 20 μM pyOMeCTZ in assay buffer. The selectivity was determined by the specific activity toward pyDTZ/activity toward pyOMeCTZ. Top three mutants showing exceptionally high bioluminescence selectivity of pyDTZ over pyOMeCTZ were chosen for next-round selection, sequencing, and other additional characterization.

***In vitro* bioluminescence characterization**

Luciferases were expressed and purified as previously described.[53] A microplate reader was used for all *in vitro* bioluminescence characterizations. To record bioluminescence emission spectra, 50 μL of luciferin substrates was injected into the wells of white 96-well plates containing 50 μL of pure enzymes in PBS (1.5 mM ATP and 5 mM $MgSO_4$ were supplemented for Akaluc). Kinetic measurements were taken every 30 s post injection with 0.1 s integration and 10 s shaking during intervals. To derive values for apparent Michaelis constants (Km), substrate concentrations varied from 0.78 to 50 μM, and

10-min integrated bioluminescence at individual substrate concentrations were used to fit the Michaelis–Menten equation.

Bioluminescence imaging with luminescence dark box

UVP Bio Spectrum luminescence dark box was used for all bioluminescence imaging. To record bioluminescence imaging with pure enzymes, 50 μL of 60 μM substrates were injected into corresponding 50 μL pure enzymes (final concentration: 10 nM for RLuc8 and OpyLuc; 100 nM for Akaluc), and the bioluminescence imaging was collected with 10 s exposure time. A filter wheel equipped with a Chroma Blue 360-500 nm, a Chroma Green 495-580 nm, and a Chroma Red 600-700 nm filter was used to acquire emission in each channel. To use the luminescence dark box to directly image cells, we added luciferase-expressing HEK 293T cells (5,000 cells per well for RLuc8 and OpyLuc; 30,000 cells per well for Akaluc) and the indicated luciferin substrates solution were injected into wells of a white 96-well plate. Final concentration of each substrate were 25 μM for pyOMeCTZ, 10 μM for pyDTZ, and 100 μM for AkaLumine-HCl. Bioluminescence was imaged in the luminescence dark box immediately after substrate addition. The camera exposure time was set at 30 s. All images were analyzed using the Fiji image analysis software.

Transfection and activation of signaling pathways in HEK293T cell line

HEK293T cells were transfected at about 70% confluency by using plasmid DNA:PEI = 3:9 mixture. Plasmids used in this study included SRE-RLuc8, ARE-OpyLuc, and NF-κB-Akaluc, NF-κB-RLuc8, SRE-OpyLuc, ARE-Akaluc and CMV-Akaluc. 3 h after transfection, the medium was removed and replaced by fresh medium. The cells were allowed to recover for another 3 h. 20% fetal bovine serum (FBS), 50 μM *tert*-butylhydroquinone (tBHQ), or 10 ng/mL tumor necrosis factor alpha (TNFα) were used to activate serum response element (SRE), antioxidant response element (ARE), or nuclear factor kappa B (NF-κB) responsive element. Bioluminescence signals were acquired 16 h post induction. An un-transfected sample was used for background subtraction and an un-induced sample was used as a negative control.

EXAMPLE 6

Design of triple luciferase system and the directed evolution of luciferase to improve substrate selectivity for synthetic pyDTZ

To choose bioluminescent reporters that can generate different colors of emission, available luciferase-luciferin pairs that have been reported previously in literature were first screened.[54] RLuc8 (nucleotide and polypeptide sequences provided herein as SEQ ID NOs.13 and 14, respectively) is able to produce intense bioluminescence in a violet wavelength range (λ_{max}: ~405 nm) when methoxy-eCoelenterazine (me-eCTZ) was used as the substrate.[55] *Renilla* luciferase (RLuc) is also known to be not tolerant to C-8 chemical modifications.[56] It was reasoned that the blue-shifted emission might be due to dihedral angle twist caused by the C-6 methoxyl substitution. Therefore, a me-eCTZ analog, pyOMeCTZ (Figure 14A), with a pyridyl substitution on C-2 was synthesized to improve the water solubility by taking advantage of the fact that pyridine-containing molecules can be readily turned into pyridinium salts. As a result, RLuc8-pyOMeCTZ pair is able to generate violet emission with λ_{max} at about 416 nm, or yield a bioluminescence of about 380-470 (nm).

According to our result, teLuc is tolerant to a variety of C-8 chemical modifications, including both electronic and steric derivatives.[53] Herein, pyDTZ (Figure 14B) that can emit green to yellow photons (λ_{max}: about 530 nm, or yield a bioluminescence of about 480-600 (nm)) when paired with teLuc was synthesized. Since the emission wavelength between RLuc8-pyOMeCTZ and teLuc-pyDTZ pairs are well resolved, they are readily available to pair with Akaluc (nucleotide and polypeptide sequences provided herein as SEQ ID NOs. 15 and 16, respectively)-AkaLumine pair that produces near infrared (NIR) photons[57] (λ_{max}: about650 nm, or yield a bioluminescence of about 600-750 (nm); Figure 14C) to yield a triple-color luciferase reporter system. It has been known that CTZ-utilizing murine luciferases do not have substrate cross-talk with D-luciferin-utilizing insect luciferases, so the remaining issue is the substrate selectivity between RLuc8 and teLuc to engineer a fully orthogonal triple-color luciferase system.

To address this issue, it was noticed that teLuc exhibited a substrate preference to pyDTZ over pyOMeCTZ by about 50-fold activity, suggesting that it might be feasible to engineer a mutant via directed evolution to more selectively access pyDTZ rather than pyOMeCTZ. Instead of screening the library for only enhanced bioluminescence output, a

method was designed where the BL activity of the mutants to both of pyDTZ (positive screening) and pyOMeCTZ (negative screening) were screened in parallel. The "hit" mutants showing not only high specific activity in positive screening but also low activity in negative screening were selected for the next round selection (Figure 15A). After 8 rounds of selection, a mutant (designated OpyLuc; SEQ ID NO. 6) carried 11 mutations was obtained (Figure 15B, and Figure 16) and showed about 250-fold selectivity to pyDTZ over pyOMeCTZ (Figure 15C). Notably, Q20K and V21A mutations were acquired during random mutagenesis, suggesting residues nearby the catalytic site contribute to the substrate selectivity.

Collectively, provided herein are three luciferase-luciferin pairs (RLuc8-pyOMeCTZ; λ_{max}: 416 nm, OpyLuc-pyDTZ; λ_{max}: 520 nm, and Akaluc-AkaLumine; λ_{max}: 650 nm) that can access its specific luciferin and produce distinct colors of emission across the visible spectrum (Figure 17A, 18A and 18B). Their emission spectra are well separated and with only minimal spectra cross-talk. These features allow researcher to either initiate a specific luciferase activity by adding its corresponding luciferin substrate or scan the full spectra or use commercial filters to determine individual luciferase signals, thereby providing flexible data acquisition methods for any chosen purpose (Figure 17B).

EXAMPLE 7

Triple luciferase system produces orthogonal BL signals in purified enzyme and in transfected HEK293T cells

To validate that the emission of these three luciferase-luciferin pairs are indeed spectrally separated, and can be resolved by filters, recombinant luciferases were first purified from *E. coli* and the respective BL signals were imaged with/without 360-500 nm, 495-580 nm, or 600-700 nm bandpass filters (Figure 19A and 19B). The result indicated RLuc8-pyOMeCTZ, OpyLuc-pyDTZ, and Akaluc-AkaLumine pairs all give the highest signal under the filter set-up that matches its respective emission color, suggesting their emission spectra can be well separated simultaneously by a set of commercial filters. Since these signals can be recorded in the same time, it solved the need of sequential sampling by Promega Dual-Luciferase Reporter assay.

To demonstrate that the disclosed triple luciferase system is a practical tool to monitor gene expression levels in live mammalian cells, the photon flux of each luciferase

was first evaluated in the presence of a series of substrate concentrations. It would have been ideal to explore an optimal concentration for each luciferin (pyOMeCTZ, pyDTZ, and AkaLumine) that can provide a similar level of photon flux from individual luciferase-luciferin pair. Unfortunately, the photon flux of Akaluc-AkaLumine at saturated concentration is about 10-fold lower than that of RLuc8-pyOMeCTZ and OpyLuc-pyDTZ pairs, possibly due to its nature BL mechanism of ATP-dependency and two-step reaction. In order to at least keep the photon flux of RLuc8-pyOMeCTZ and OpyLuc-pyDTZ pairs at the same level, a condition containing 25 μM pyOMeCTZ, 10 μM pyDTZ, and 100 μM AkaLumine-HCl was selected as the "Optimal Mix" for live cell imaging. By comparing Optimal Mix and only its respective substrate, only OpyLuc exhibited slightly unspecific inhibition by Optimal Mix while RLuc8 and Akaluc remained unaffected.

Next, the performance of each luciferase was examined for *in cellulo* imaging in the presence of chosen luciferin concentration. The indicated luciferin substrate solution was injected into luciferase-expressing HEK 293T cells in a 96-well plate. As expected, pyDTZ initiated the BL emission only in the presence of OpyLuc. The excellent substrate selectivity was also observed in both Akaluc for AkaLumine and RLuc8 for pyOMeCTZ (Figure 19C and 19D). Taken together, the results indicated again that each of luciferase in the disclosed triple luciferase system can process its distinct substrate and generate distinguishable emission wavelength. To briefly sum up, each luciferase can be selectively activated by its specific luciferin and the emission photons can also be distinguished by wavelength, which provided the flexibility to monitor multiple transcriptional activities specifically, stepwise, or simultaneously as a versatile experimental design. Moreover, the equations to calculate the activities of the individual luciferases by splitting emissions is not necessarily required since the spectra cross-talks are minimized.

While reporter assays are typically performed in lysates from cultured cells, the disclosed triple luciferase system was also evaluated in lysates. The results indicated that RLuc8-pyOMeCTZ and OpyLuc-pyDTZ pairs showed about 2 to 3-fold higher BL signals in lysates while Akaluc-AkaLumine exhibited decreased signal even after supplementing with additional ATP. Therefore, using lysates in not required in the disclosed triple luciferase system because all three luciferins described here are cell-permeable and work well with intact mammalian cells. This feature is beneficial to expand the real-time measurement of BL assays without lysing cultured cells.

EXAMPLE 8

Monitor serum response, antioxidant, and NF-κB promoter activities in HEK293T cells by triple luciferase system

Subsequently, the disclosed triple luciferase system was used to monitor three signaling pathway activations in HEK293T cells where each of the luciferase expression was under control by a growth factor-regulated promoter element (serum response element, SRE),[58] a Nrf2-antioxidant response element (ARE),[59] or a transcription factor–nuclear factor kappa B (NF-κB) responsive promoter element (Table 5).[60] A reporter system was designed based on SRE promoter driving the expression of RLuc8, ARE promoter driving the expression of OpyLuc, and NF-κB promoter driving the expression of Akaluc. The response element promoters can be specifically activated by its respective stimuli – fetal bovine serum (FBS), tert-butylhydroquinone (tBHQ), and tumor necrosis factor alpha (TNFα) (Figure 20A).

Table 5. Oligonucleotides used in this study.

Oligo name	SEQ ID NO.	Nucleotide sequence (5′–> 3′)
L18D19NNK-F	29	CAGACAGCCGGCTACAACNNKNNKCAAGTC CTTGAACAGGGAGGTGTG
L18D19NNK-R	30	CACACCTCCCTGTTCAAG GACTTGMNNMNNGTTGTAGCCGGCTGTCTG
27VSSNNK-F	31	CAAGTCCTTGAACAGGGAGGTNNKNNKNNKTTG TTTCAGAATCTCGGGGTG
27VSSNNK-R	32	CACCCCGAGATTCTGAAACAAMNNMNNMNNAC CTCCCTGTTCAAGGACTTG
pBAD-F	33	ATGCCATAGCATTTTTATCC
pBAD-R	34	GATTTAATCTGTATCAGG
HindIII-pyr-F-Koz	35	AATAAAGCTTGCCGCCACCATGGTCTTCACTCTC GGGGATTTT
pyr-R-XhoI	36	TAATTCTCGAGTTACGCCAGAATGCGTTCATGCA G
Aka-F-HindIII-Kozak	37	ATTATAAAGCTTGCCGCCACCATGGAAGATG CCAAAAACATTAAGA
Aka-R-XhoI	38	TTATTCTCGAGTTACACGGCGATCTTGCCGTCCTT CTT
Aka-F-XhoI	39	ATAACTCGAGCATGGAAGATGCCAAAAACATTA AGA

Aka-R-HindIII	40	TTGCCAAGCTTACACGGCGATCTTGCCGTCCTTCT T
HindIII-mScarlet-F-Koz	41	ATTATAAAGCTTGCCGCCACCATGGTGAGCAAGG GCGAGGCAGT
mScarlet-F-XhoI	42	ATAACTCGAGCATGGTGAGCAAGGGCGAGGCAG TG
pyr-R-HindIII	43	TTGCCAAGCTTACGCCAGAATGCGTTCATGCA
mScar-NNK-pyr-F	44	GAGGGCCGCCACTCCACCGGANNKACTCTCGGG GATTTTGTTGGG
mScar-NNK-pyr-R	45	CCCAACAAAATCCCCGAGAGTMNNTCCGGTGGA GTGGCGGCCCTC

The basal promoter activities of all SRE, ARE, and NF-κB response elements were low (Figure 6A). The individual luciferase activity was measured (RLuc8, OpyLuc, and Akaluc) by adding its corresponding luciferin (pyOMeCTZ, pyDTZ, and AkaLumine). As expected, treating with single stimuli enabled the activation of its specific pathway and drove the downstream expression of genetically encoded luciferase. Stimulating the cells with either two or three stimuli resulted in activation of multi-pathway and were reported correctly by our triple luciferase system (Figure 20B). Next, the emission spectra were recorded after injection of Optimal Mix solution to the transfected HEK293T cells (Figure 20B and Figure 21C, D, and E). Due to the fact that all three emission spectra are well-separated, this system demonstrated a proof-of-concept of simultaneous recording of three pathway activations. Again, the results suggested that there is no cross-reactivity between each luciferase-luciferin pair, providing an advantage in future studies that require either sequential or simultaneous multicomponent monitoring.

Herein, the ability of the disclose triple luciferase system to monitor the simultaneous activation of two or all three labeled pathways was demonstrated. The ability to qualitatively detect three signaling activation states after treating with stimuli mixtures was demonstrated (Figure 20B and 21F, G, H, and I).

Next, the promoters were switched over to drive the downstream luciferase expression by using an alternative combination (NF-κB-RLuc8, SRE-OpyLuc, and ARE-Akaluc). After inducing with all stimuli (TNFα, FBS, and tBHQ), the signals from RLuc8 and OpyLuc were obvious as expected, while the signal from Akaluc was overwritten by the broad emission tailing of OpyLuc (Figure 21). This result suggested that Akaluc is only suitable to monitor strong promoter activity when paired with the other two luciferases,

because the photon flux of Akaluc is relatively lower. This is a factor that needs to be taken into account when researchers conducting the initial experimental design.

These data support the use of the new luciferase-luciferin pairs for assays of cell signaling pathways. Particularly, the data summarized in Figure 21 suggest that the use of the disclosed luciferase-luciferin pairs can allow for the monitoring of particular cell signaling pathways, which can be implemented to for candidate compound and drug screening applications.

EXAMPLE 9

Akaluc-AkaLumine pair is more suitable as an internal control

As mentioned above, it is recommended to normalize the BL assay results by an internal control for cell number, and transfection efficiency normalizations. Another set of plasmids containing NF-κB-RLuc8, SRE-OpyLuc, and a control of the constitutively active cytomegalovirus (CMV) promoter (CMV-Akaluc) was prepared. The cells were transfected with all three plasmids and incubated with two stimuli (TNFα and FBS) for 16 h. In this case, the BL signal was selectively triggered from individual luciferase by adding its respective luciferin to intact cells (Figure 6A). The BL kinetics were monitored for each luciferase after the addition of its respective luciferin (Figure 6B). All three luciferase generated BL signal, and the BL signal attenuated by the function of time. The advantage of incorporating an internal control here is not only limited to the normalization of cell number and transfection efficiency of each sample, but also the kinetic of each BL signal can be normalized to give a more constant ratio readout, which is more amenable to high-throughput screening (Figure 6C). Thus, this system enabled the monitoring of two signaling activations and improved the accuracy of assay by including another orthogonal control to exclude factors such as cell number, transfection efficiency, and BL emission kinetic.

EXAMPLE 10

Analysis of bioluminescent Ca^{2+} biosensors

Ca^{2+} is one of the most important signaling cations in biological systems. Bioluminescent Ca^{2+} biosensors are expected to have broad applications in non-invasive imaging and drug screening. Recent studies have reported a few bioluminescent Ca^{2+} biosensors based on NanoLuc.[65-68] To address the limitations of these existing sensors, such

as small dynamic range, low brightness, and/or relatively blue emission, Ca^{2+} biosensors based on the disclosed brighter and redder luciferase-luciferin pairs were developed. Moreover, in contrast to existing approaches, multiple Ca^{2+} binding elements were introduced to modulate bioluminescence through two different mechanisms. In particular, a calcium sensory element (e.g., a modified Troponin C) was sandwiched between a LumiLuc and Scarlet-I BRET pair. After testing several linker lengths, a prototypic biosensor was derived, whose bioluminescence at wavelengths longer than 600 nm increases by about 4-fold in response to Ca^{2+}. Further, calmodulin and M13 were inserted between the residue 133 and 134 of Lumiluc to modulate its intensity, resulting in LumiCameleon1 (Figures 22A and 22B; SEQ ID NOs. 9 and 10) showing a total of greater than about 20-fold increase of greater than about 600 nm emission in response to Ca^{2+}. The brightness of LumiCameleon1 in the Ca^{2+} saturated state is about 52% of LumiScarlet. This sensor is thus well suited for *in vivo* and *in vitro* applications, because of its high brightness, large dynamic range, and red-shifted emission compared to any previously reported NanoLuc-based Ca^{2+} sensor. Along the same line, a similar biosensor based on teLuc was created. The resultant LumiCameleon2 (Figures 22A and 22C; SEQ ID NOs. 11 and 12) also showed a large bioluminescence increase in response to Ca^{2+}.

DISCUSSION OF EXAMPLES 6-10

Herein, substrate selectivity was utilized to engineer a mutually orthogonal luciferase-luciferin pair for multiplexed cell-based BL assay. In combination with RLuc8 and Akaluc, this triple-color BL system features the selectivity of synthetic substrates and production of well separated emission spectra from 400 nm to 650 nm. Several advantages of previous bioluminescence technology (Table 6) were combined to develop a spectral-resolved triple-color BL system, which provides flexible and convenient approaches to monitor multiple biological events in either qualitative or quantitative manners.

New bioluminescent Ca^{2+} biosensors were also developed based on the modified luciferase compounds disclosed herein. These bioluminescent Ca^{2+} biosensors showed large bioluminescence increase in response to Ca^{2+}, making them well suited for *in vivo* and *in vitro* applications.

Table 6. Qualitative comparison of this study and commercial luciferase reporter systems

	Promega Dual-Luciferase Assay	Promega Chroma-Glo	Pierce Cypridina-Firefly Luciferase Dual Assay	Orthogonal Triple Luciferase Assay **(this work)**
Number of Substrates	2	1	2	3
Types of Enzymes	2 (Rluc, FLuc)	1 (2 CBLucs)	2 (VLuc, Red FLuc)	3
Gene Identity	low	>99%	low	low
Orthogonal Signals	Yes	No	Yes	Yes
Simultaneous Detection of 2 Signals	No	Yes	Yes	Yes
Simultaneous Detection of 3 Signals	No	No	No	Yes
Data Calculations Required	No	Yes	No	Yes/No
Luminometer Filters Required	No	Yes	Yes	Yes/No
Single Reagent Solution	No (CTZ, D-luciferin)	Yes (D-luciferin)	Yes (Vargulin, D-luciferin)	Yes (pyDTZ, pyOMeCTZ, Akalumine-HCl)
Emission Signals Well-Separated	Yes	No	Yes	Yes
Cell Lysis Required	Yes	Yes	Yes	Yes/No

By using this triple luciferase system, it was demonstrated that the activations of cell signaling can be detected simultaneously or separately from live cells in a single experiment where each individual BL signal can be distinguished from the other two luciferase-luciferin pairs. It is expected that there is also an ability to combine newly discovered luciferase-luciferin pairs[61] to independently activate even more innate processes in the same sample to study the cross-talks of cellular signaling pathways. Moreover, multiplexed BL assay is

compatible with modern genetically encoded fluorescent biosensors to further investigate complexed biological events via functional imaging.[62] The development of a such versatile tool ensures an accurate and precise analysis of signaling pathways which will extend to study other physiologically transcriptional activation and is critical to improve the design and screening of new drugs, as well as the diagnosis and treatment of disease.

REFERENCES

All references listed in the instant disclosure, including but not limited to all patents, patent applications and publications thereof, scientific journal articles, and database entries (including but not limited to UniProt, EMBL, and GENBANK® biosequence database entries and including all annotations available therein) are incorporated herein by reference in their entireties to the extent that they supplement, explain, provide a background for, and/or teach methodology, techniques, and/or compositions employed herein. The discussion of the references is intended merely to summarize the assertions made by their authors. No admission is made that any reference (or a portion of any reference) is relevant prior art. Applicants reserve the right to challenge the accuracy and pertinence of any cited reference.

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It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the presently disclosed subject matter. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. A bioluminescent protein, comprising a substituted luciferase polypeptide comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 2 with amino acid substitutions at one or more of positions corresponding to positions 4, 18, 19, 27, 28, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2.

2. The bioluminescent protein of claim 1, comprising amino acid substitutions at positions corresponding to positions 18, 19, 27 and 28, and further comprising one or more amino acid substitutions at one or more of positions corresponding to positions 4, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2.

3. The bioluminescent protein of claim 1, comprising an amino acid sequence having at least 95% homology to SEQ ID NO: 2 with amino acid substitutions at least eight positions selected from positions corresponding to positions 4, 18, 19, 27, 28, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2.

4. The bioluminescent protein of claim 1, wherein the luciferase polypeptide is substituted at positions corresponding to positions 4, 18, 19, 27, 28, 67, 71, 85, 90, 112, 119 and 136 of SEQ ID NO: 2.

5. The bioluminescent protein of claim 1, wherein the luciferase polypeptide comprises SEQ ID NO: 3.

6. The bioluminescent protein of any of claims 1 to 5, further comprising a fluorescent protein connected to the substituted luciferase polypeptide.

7. The bioluminescent protein of claim 6, wherein the fluorescent protein is connected to the substituted luciferase polypeptide so as to allow bioluminescence resonant energy transfer (BRET) between the substituted luciferase polypeptide and the fluorescent protein.

8. The bioluminescent protein of any of claims 6 or 7, wherein the substituted luciferase polypeptide comprises an amino acid sequence having at least 90% homology to SEQ ID NO: 5.

9. The bioluminescent protein of any of claims 6 to 8, wherein the substituted luciferase polypeptide comprises the amino acid sequence of SEQ ID NO: 5.

10. A bioluminescent protein comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 6.

11. A bioluminescent protein comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 8.

12. A bioluminescent protein comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 10.

13. A bioluminescent protein comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 12.

14. A nucleic acid encoding the bioluminescent protein of any of claims 1 to 13.

15. A vector encoding the nucleic acid of claim 14.

16. An expression vector encoding the nucleic acid of claim 14 functionally connected to a promoter.

17. A cell line containing the expression vector of claim 16 and expressing the bioluminescent protein.

18. A non-human cell transfected with the expression vector of claim 16 and expressing the bioluminescent protein.

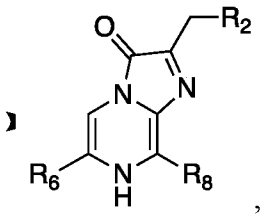
19. A combination comprising a bioluminescent protein of any of claims 1 to 13 and a luciferin.

20. The combination of claim 19, wherein the luciferin comprises a luciferin selected from pyCTZ, 6pyDTZ, and 8pyDTZ.

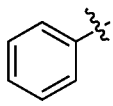
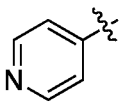
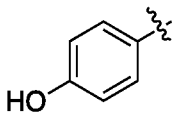
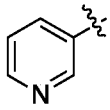
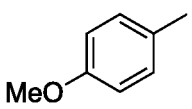
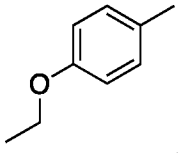
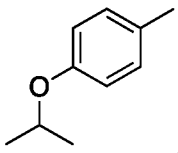
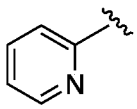
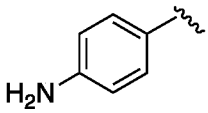
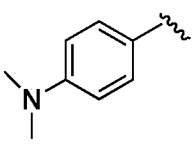
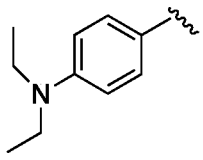
21. A method of producing luminescence, comprising reacting a luciferin with the bioluminescent protein of any of claims 1 to 13.

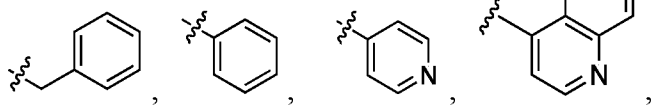
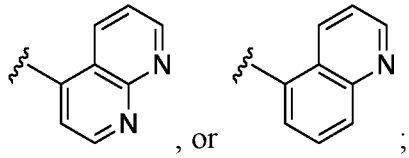
22. The method of claim 21, wherein the bioluminescent protein further comprises a fluorescent protein connected to the substituted luciferase polypeptide so as to allow BRET activity between the substituted luciferase polypeptide and the fluorescent protein.

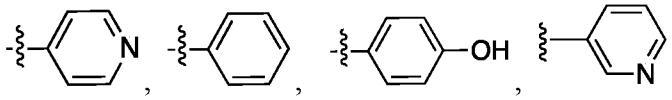
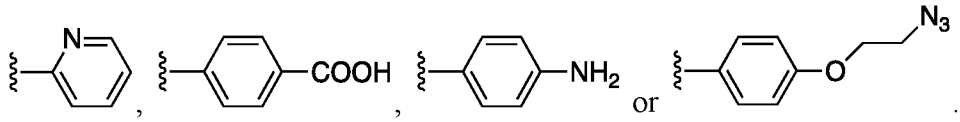
23. A luciferin compound comprising the following structure:



,

wherein R6 is selected from  ,  ,  ,  ,  ,  , r  ,  ,  ,  , or  ;

wherein R8 is selected from  ,  , or ; and

wherein R2 is selected from  ,  , or .

24. The luciferin compound of claim 23, wherein the luciferin compound comprises an analog of pyridyl coelenterazine (CTZ), pyridyl furimazine (FRZ) and/or a pyridyl diphenylterazine (DTZ).

25. The luciferin compound of any of claims 23 to 24, wherein the compound comprises a water solubility increased by at least about 4-fold as compared to CTZ and/or DTZ, optionally by at least about 10-fold as compared to CTZ and/or DTZ.

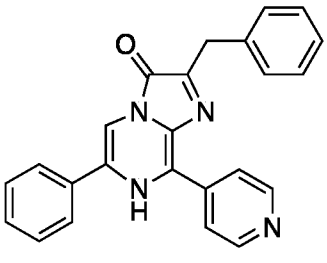
26. The luciferin compound of any of claims 23 to 25, wherein the compound comprises a water solubility ranging from about 1,000 μM to about 1,800 μM.

27. The luciferin compound of any of claims 23 to 26, wherein the compound comprises a bioluminescence ranging from about 450 λ_{max} (nm) to about 550 λ_{max} (nm).

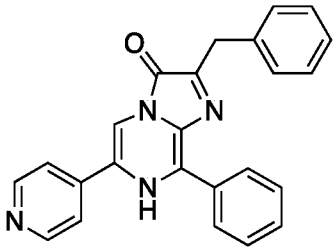
28. The luciferin compound of any of claims 23 to 27, wherein the compound is compatible with any luciferase, optionally any ATP-independent luciferase.

29. The luciferin compound of claim 28, wherein the luciferase comprises a polypeptide comprising SEQ ID NO: 3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

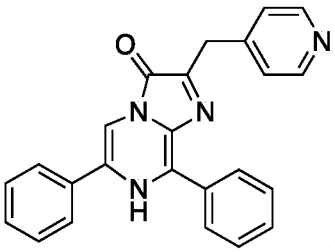
30. The luciferin compound of any of claims 23 to 28, wherein the compound is selected from:



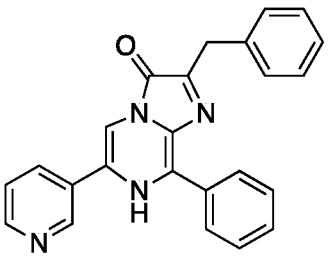
8pyDTZ;



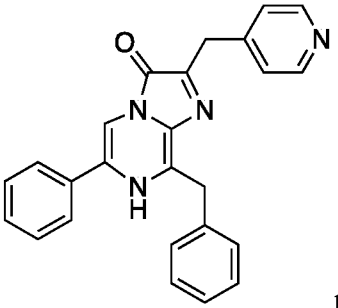
6pyDTZ;



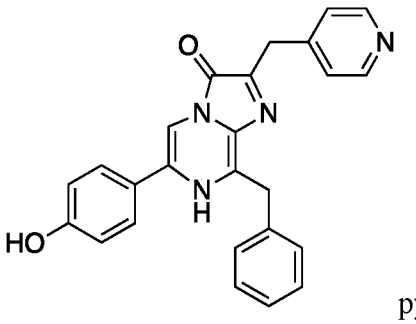
2pyDTZ;



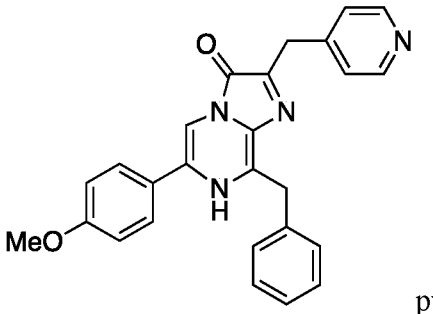
6opyDTZ;



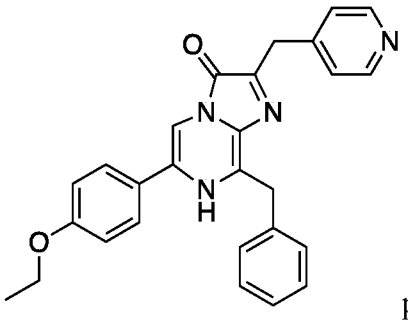
pyCTZ;



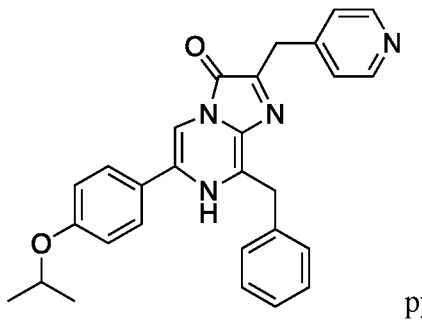
pyOHCTZ;



pyOMeCTZ;

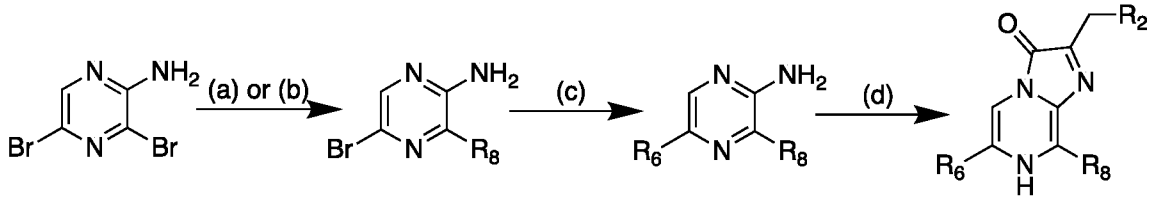


pyOEtCTZ; and



pyiPrCTZ.

31. A method of making a luciferin compound of any of claims 23 to 30, the method comprising making one or more pyridyl isomer substitutions at a C-2, C-6 and C-8 position of an imidazopyrazinone core according to the following chemical synthetic route:



,

wherein step (a) comprises Suzuki coupling, comprising $Pd(PPh_3)_4$, Na_2CO_3, R_8-$B(OH)_2$, and/or EtOH; step (b) comprises Negishi coupling, comprising $PhCH_2MgCl$, $ZnCl_2$, $(PPh_3)_2PdCl_2$, and/or THF; step (c) comprises Suzuki coupling, comprising XPhos-Pd-G2, Na_2CO_3, R_6-$B(OH)_2$, and/or EtOH; and step (d) comprises acid-catalyzed ring closing, comprising corresponding α-ketoacetal, HCl, and/or dioxane.

32. A luciferin compound made according to the method of claim 31.

33. A bioluminescent reporter system, the system comprising:
at least two bioluminescent proteins selected from RLuc8, OpyLuc and Akaluc; and
at least two luciferin compounds select from pyOMeCTZ, pyDTZ and AkaLumine.

34. The bioluminescent reporter system of claim 33, the system comprising:
bioluminescent proteins consisting of RLuc8, OpyLuc and Akaluc; and
luciferin compounds consisting of pyOMeCTZ, pyDTZ and AkaLumine.

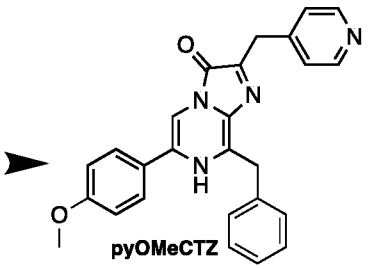
35. The bioluminescent reporter system of any of claims 33 or 34, wherein the bioluminescent proteins and luciferin compounds are provided in pairs as follows:
RLuc8 with pyOMeCTZ;
OpyLuc with pyDTZ; and
Akaluc with AkaLumine.

36. The bioluminescent reporter system of claim 35, wherein the pairs of bioluminescent proteins and luciferins produce distinct colors of emission across the visible spectrum.

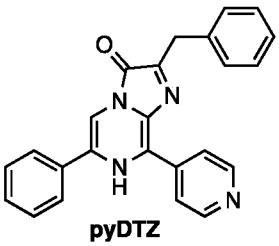
37. The bioluminescent reporter system of any of claims 35 to 36, wherein RLuc8 paired with pyOMeCTZ yields a bioluminescence of about 380-470 (nm), OpyLuc paired with

pyDTZ yields a bioluminescence of about 480-600 (nm), and Akaluc paired with AkaLumine yields a bioluminescence of about 600-750 (nm).

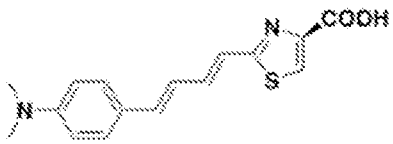
38. The bioluminescent reporter system of any of claims 33 to 37, wherein the luciferin compounds consisting of pyOMeCTZ, pyDTZ and AkaLumine comprise the following chemical structures:



;



; and



AkaLumine .

39. The bioluminescent reporter system of any of claims 33 to 38, wherein RLuc8 comprises a polypeptide sequence comprising SEQ ID NO: 14, OpyLuc comprises a polypeptide sequence comprising SEQ ID NO: 6, and Akaluc comprises a polypeptide sequence comprising SEQ ID NO: 16.

40. A series of nucleic acids encoding the bioluminescent proteins of any of claims 33 to 39.

41. A series of vectors encoding the nucleic acids of claim 40.

42. A series of expression vectors encoding the nucleic acids of claim 40, each functionally connected to a promoter.

43. A cell line containing the series of expression vector of claim 42 and expressing the bioluminescent proteins.

44. A non-human cell transfected with the series of expression vectors of claim 42 and expressing the bioluminescent proteins.

45. A method of producing luminescence, comprising reacting the luciferins with the bioluminescent proteins of any of claims 33 to 39.

46. A method of monitoring bioluminescence in a subject, the method comprising:

providing a subject;

establishing in the subject a luciferase expressing cell, wherein the luciferase is selected from the group consisting of LumiLuc, teLuc, RLuc8 and OpyLuc;

administering to the subject a luciferin selected from the group consisting of pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ and 8pyDTZ; and

measuring bioluminescence in the subject.

47. The method of claim 46, wherein the subject comprises a tumor or a cancer cell.

48. The method of any of claims 46 to 47, wherein the luciferase expressing cell comprises a tumor or a cancer cell.

49. The method of any of claims 46 to 48, wherein the subject comprises an *in vivo* tumor or cancer model.

50. The method of any of claims 46 to 49, wherein the subject is a non-human animal.

51. The method of any of claims 46 to 50, wherein the bioluminescence is measured in a tumor or cancer cell in the subject.

52. A cell containing an expression vector for expressing a bioluminescent protein, wherein the bioluminescent protein is selected from the group consisting of LumiLuc, teLuc, RLuc8 and OpyLuc, wherein the expression vector comprises a responsive promoter element, wherein the responsive promoter element is responsive to a stimuli of a cell signaling pathway.

53. The cell of claim 52, wherein the cell is cultured *in vitro*.

54. A cell signaling assay system, the system comprising:

a cell of any of claims 52 to 53; and

a luciferin selected from the group consisting of pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ and 8pyDTZ,

wherein the assay is configured to indicate activation of a cell signaling pathway by bioluminescence.

55. A method for monitoring a cell signaling pathway, the method comprising:

culturing a cell of any of claims 52 to 53;

exposing the cell to a luciferin selected from the group consisting of pyCTZ, pyOHCTZ, pyOMeCTZ, pyOEtCTZ, pyiPrCTZ, 2pyDTZ, 6pyDTZ, 6opyDTZ and 8pyDTZ;

exposing the cell to a compound of interest; and

measuring bioluminescence in the cell,

wherein an increase in bioluminescence in the cell is indicative of an activation of the cell signaling pathway in the cell.

56. The method of claim 55, wherein the compound of interest comprises a drug candidate compound.

57. A stable cell line integrated with the series of nucleic acids of claim 40 and expressing the bioluminescent proteins.

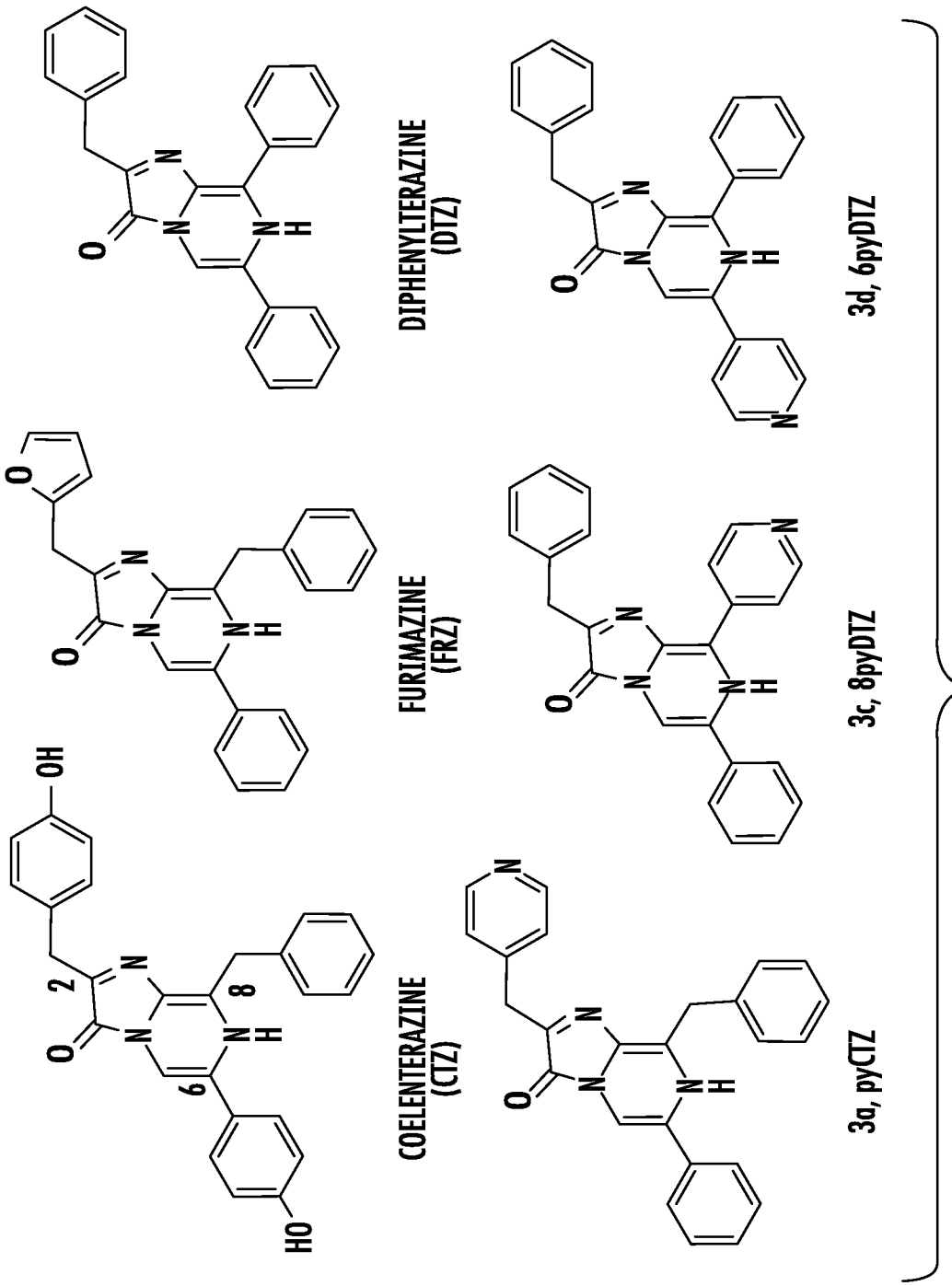

DIPHENYLTERAZINE
(DTZ)
FURIMAZINE
(FRZ)
COELENTERAZINE
(CTZ)
3d, 6pyDTZ
3c, 8pyDTZ
3a, pyCTZ

FIG. 1

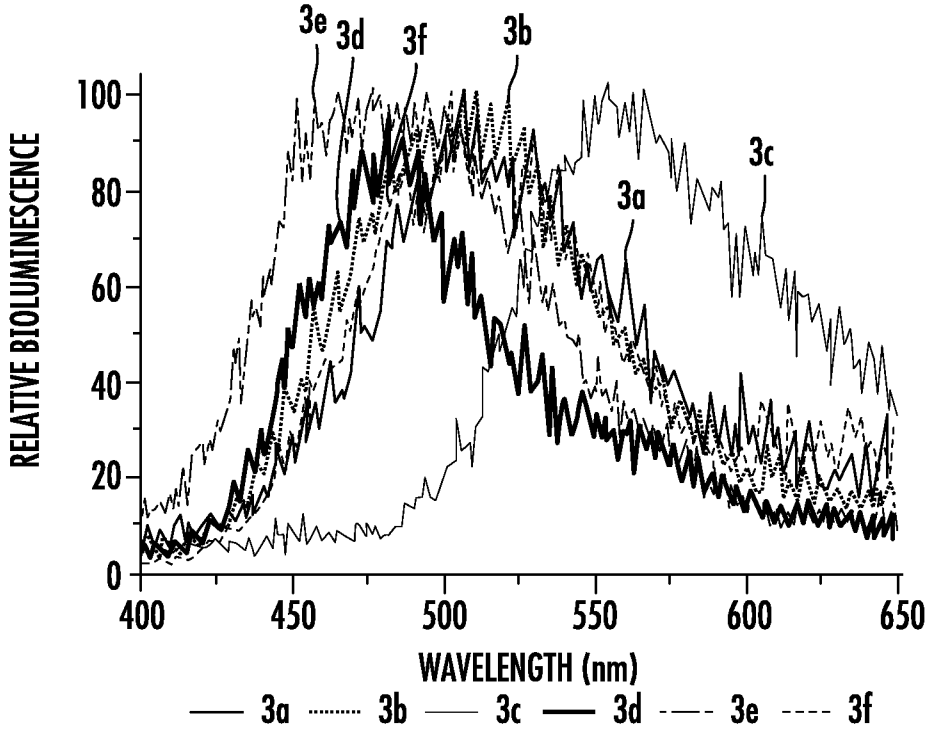

3e
3d
3f
3b
3c
3a
100
80
60
40
20
0
RELATIVE BIOLUMINESCENCE
400
450
500
550
600
650
WAVELENGTH (nm)
3a
3b
3c
3d
3e
3f

FIG. 2A

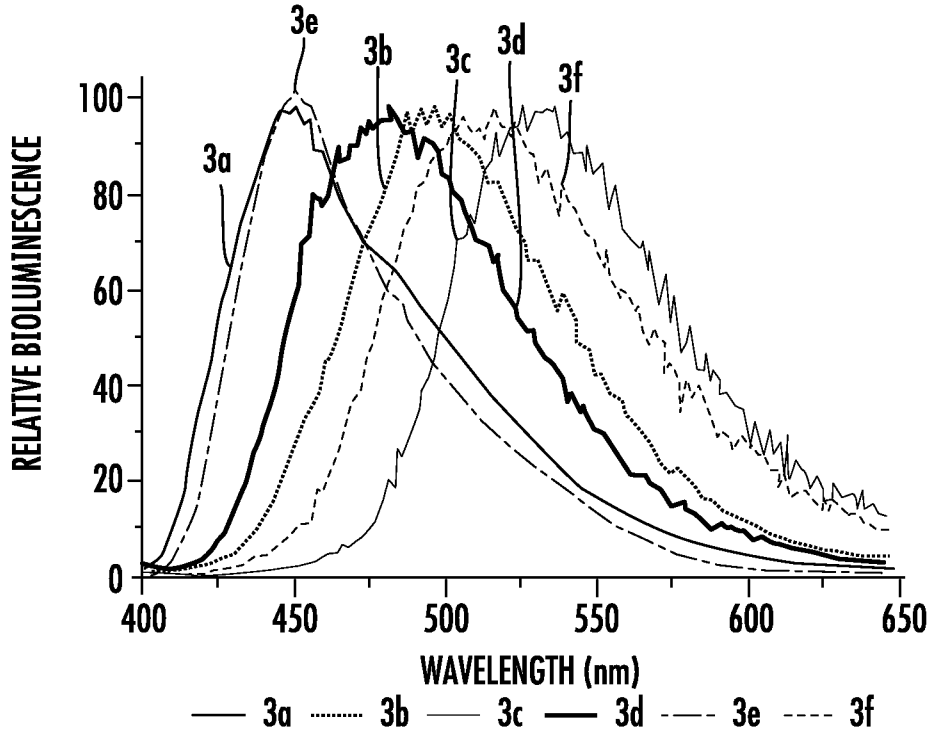

3e
3b
3c
3d
3f
3a
100
80
60
40
20
0
RELATIVE BIOLUMINESCENCE
400
450
500
550
600
650
WAVELENGTH (nm)
3a
3b
3c
3d
3e
3f

FIG. 2B

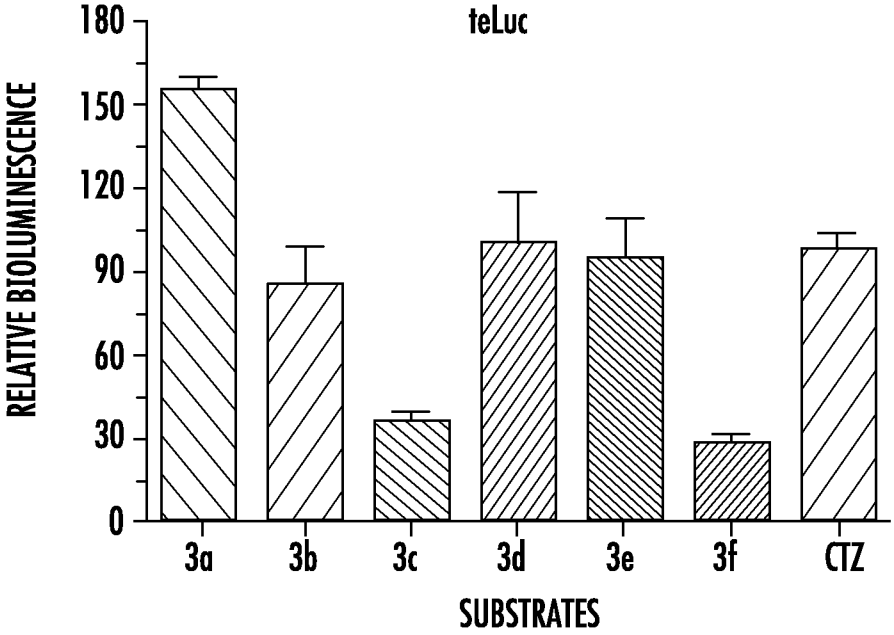

teLuc
RELATIVE BIOLUMINESCENCE
180
150
120
90
60
30
0
3a
3b
3c
3d
3e
3f
CTZ
SUBSTRATES

FIG. 3A

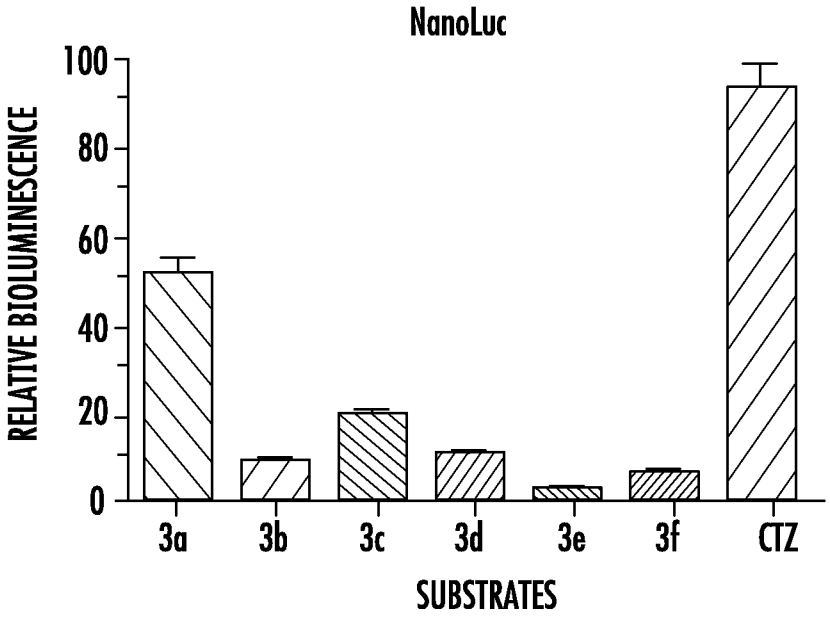

NanoLuc
RELATIVE BIOLUMINESCENCE
100
80
60
40
20
0
3a
3b
3c
3d
3e
3f
CTZ
SUBSTRATES

FIG. 3B

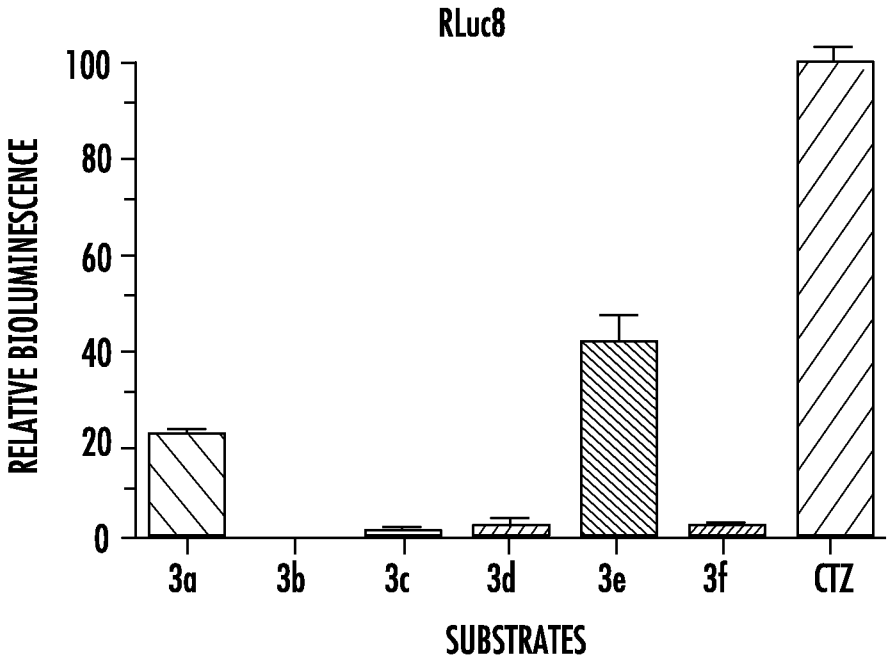

RLuc8
RELATIVE BIOLUMINESCENCE
100
80
60
40
20
0
3a
3b
3c
3d
3e
3f
CTZ
SUBSTRATES

FIG. 3C

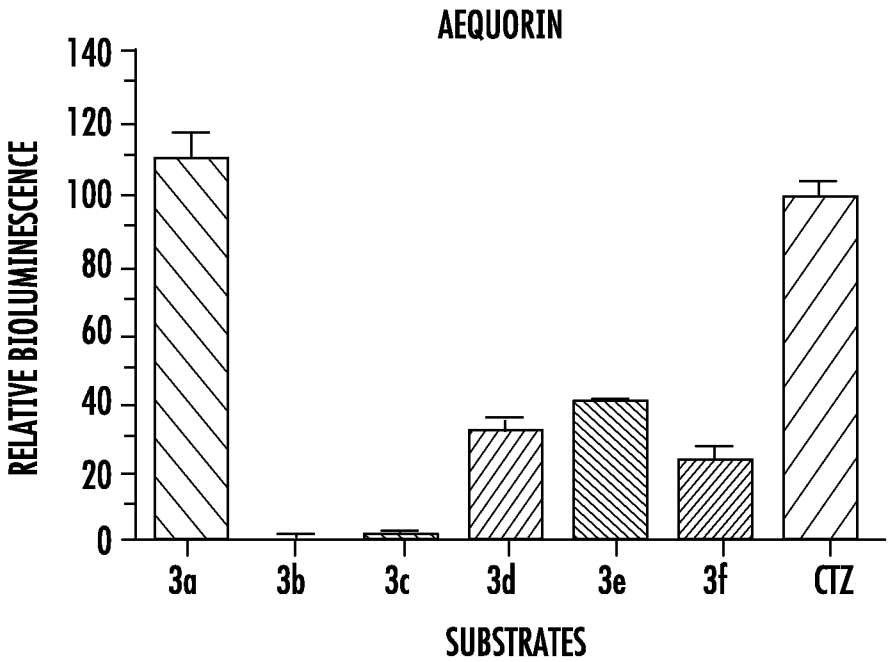

AEQUORIN
RELATIVE BIOLUMINESCENCE
140
120
100
80
60
40
20
0
3a
3b
3c
3d
3e
3f
CTZ
SUBSTRATES

FIG. 3D

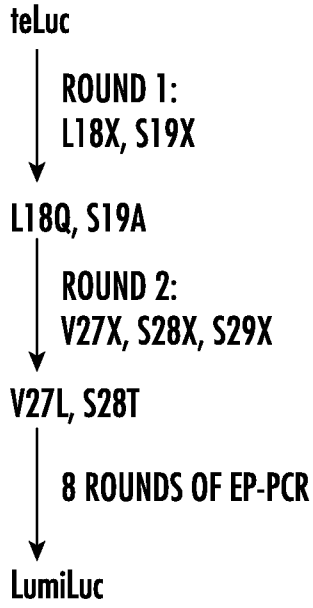

teLuc
ROUND 1:
L18X, S19X
L18Q, S19A
ROUND 2:
V27X, S28X, S29X
V27L, S28T
8 ROUNDS OF EP-PCR
LumiLuc

FIG. 4A

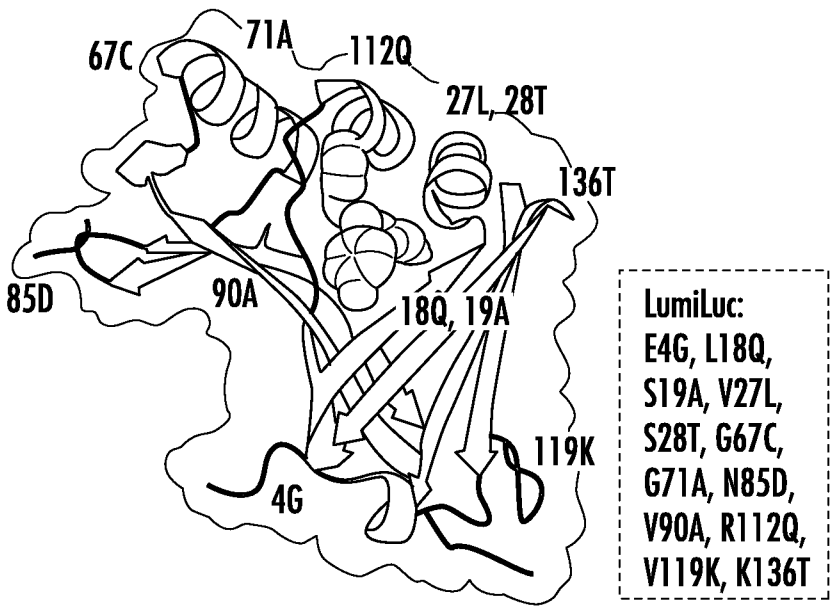

67C
71A
112Q
27L, 28T
136T
85D
90A
18Q, 19A
119K
4G
LumiLuc:
E4G, L18Q,
S19A, V27L,
S28T, G67C,
G71A, N85D,
V90A, R112Q,
V119K, K136T

FIG. 4B

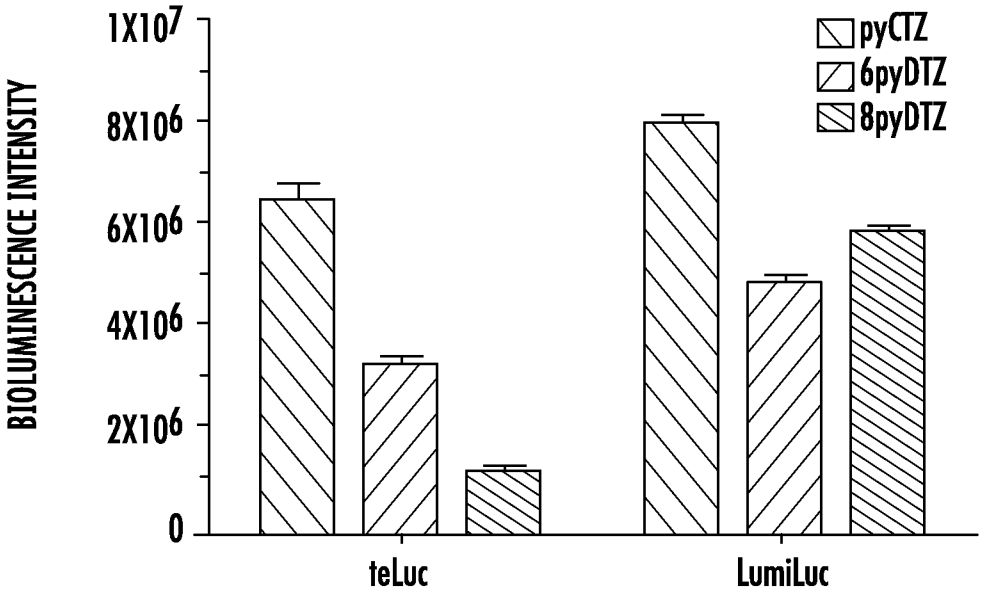

BIOLUMINESCENCE INTENSITY
1X10^7
8X10^6
6X10^6
4X10^6
2X10^6
0
pyCTZ
6pyDTZ
8pyDTZ
teLuc
LumiLuc

FIG. 4C

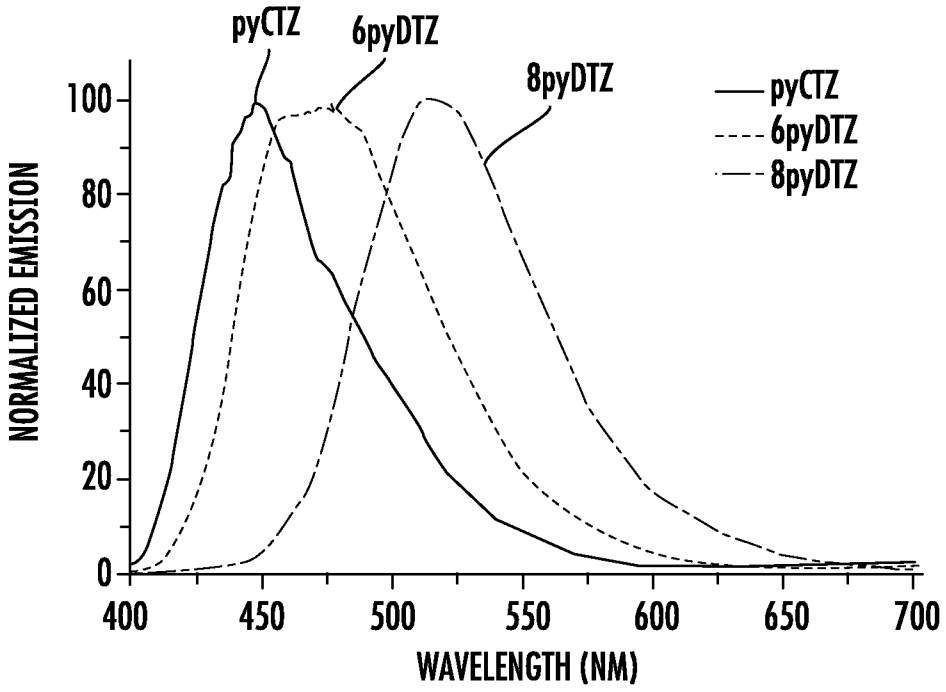

NORMALIZED EMISSION
100
80
60
40
20
0
400
450
500
550
600
650
700
WAVELENGTH (NM)
pyCTZ
6pyDTZ
8pyDTZ
pyCTZ
6pyDTZ
8pyDTZ

FIG. 4D

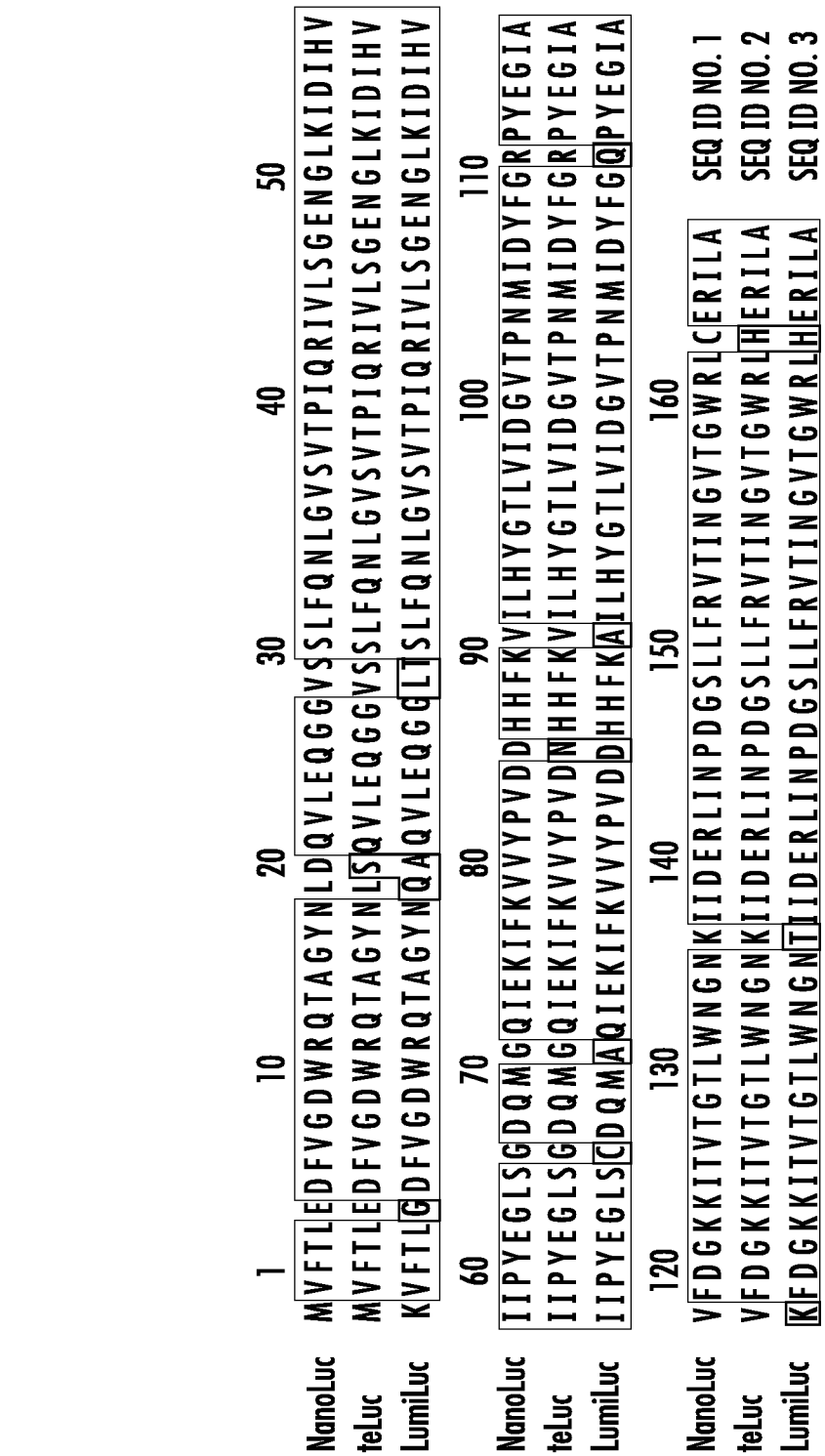

1 10 20 30 40 50
NanoLuc MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHV
teLuc MVFTLEDFVGDWRQTAGYNLSQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHV
LumiLuc KVFTLGDFVGDWRQTAGYNQAQVLEQGGLTSLFQNLGVSVTPIQRIVLSGENGLKIDIHV
60 70 80 90 100 110
NanoLuc IIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIA
teLuc IIPYEGLSGDQMGQIEKIFKVVYPVDDNHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIA
LumiLuc IIPYEGLSCDQMAQIEKIFKVVYPVDDHHFKAILHYGTLVIDGVTPNMIDYFGQPYEGIA
120 130 140 150 160
NanoLuc VFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILA SEQ ID NO. 1
teLuc VFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLHERILA SEQ ID NO. 2
LumiLuc KFDGKKITVTGTLWNGNTIIDERLINPDGSLLFRVTINGVTGWRLHERILA SEQ ID NO. 3

FIG. 5

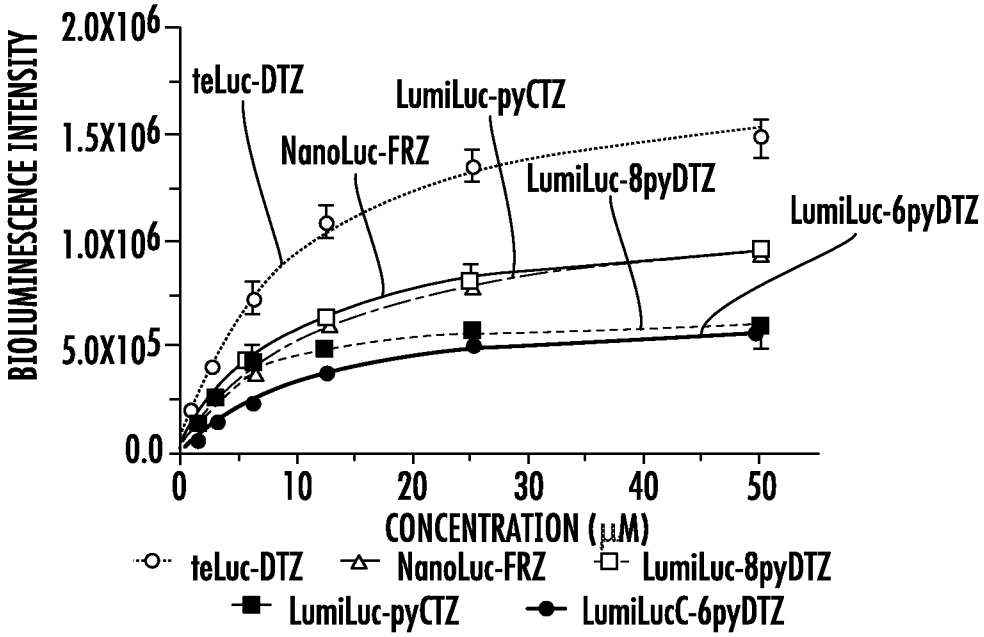

BIOLUMINESCENCE INTENSITY
2.0X10^6
1.5X10^6
1.0X10^6
5.0X10^5
0.0
0
10
20
30
40
50
CONCENTRATION (μM)
teLuc-DTZ
LumiLuc-pyCTZ
NanoLuc-FRZ
LumiLuc-8pyDTZ
LumiLuc-6pyDTZ
teLuc-DTZ
NanoLuc-FRZ
LumiLuc-8pyDTZ
LumiLuc-pyCTZ
LumiLucC-6pyDTZ

FIG. 6A

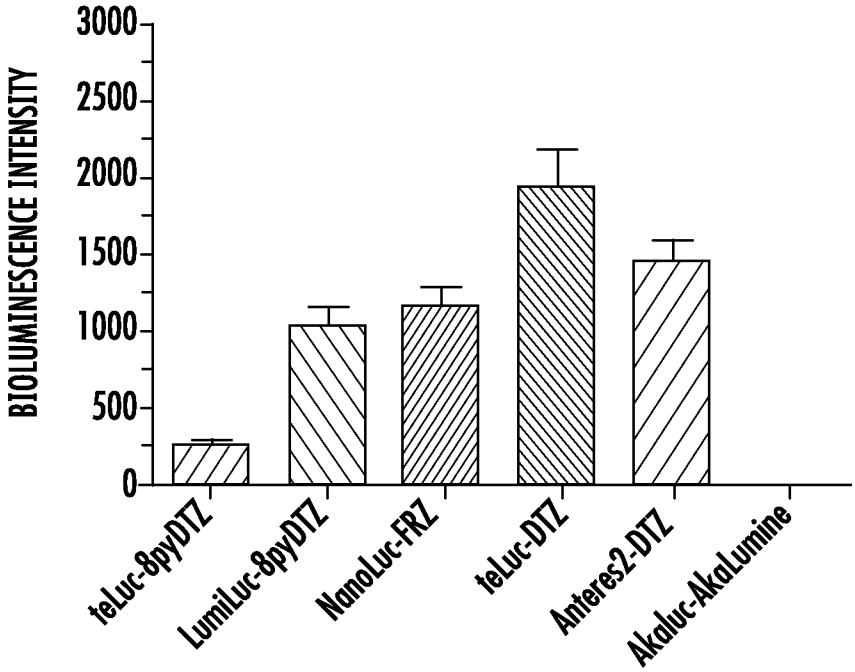

BIOLUMINESCENCE INTENSITY
3000
2500
2000
1500
1000
500
0
teLuc-8pyDTZ
LumiLuc-8pyDTZ
NanoLuc-FRZ
teLuc-DTZ
Antares2-DTZ
Akaluc-AkaLumine

FIG. 6B

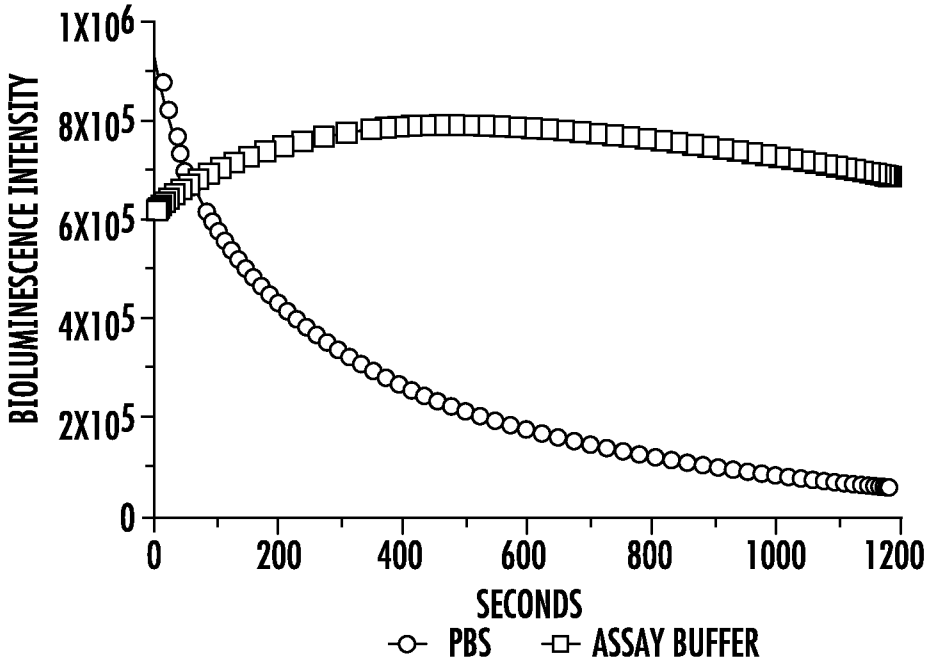

1X10^6
8X10^5
6X10^5
4X10^5
2X10^5
0
BIOLUMINESCENCE INTENSITY
0
200
400
600
800
1000
1200
SECONDS
PBS
ASSAY BUFFER

FIG. 6C

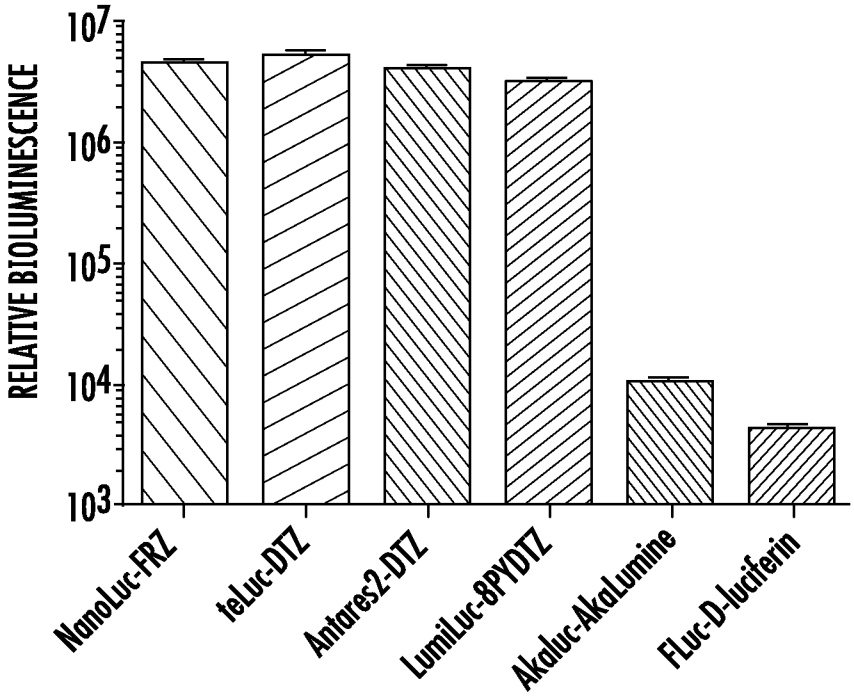

10^7
10^6
10^5
10^4
10^3
RELATIVE BIOLUMINESCENCE
NanoLuc-FRZ
teLuc-DTZ
Antares2-DTZ
LumiLuc-8PYDTZ
Akaluc-AkaLumine
FLuc-D-luciferin

FIG. 7

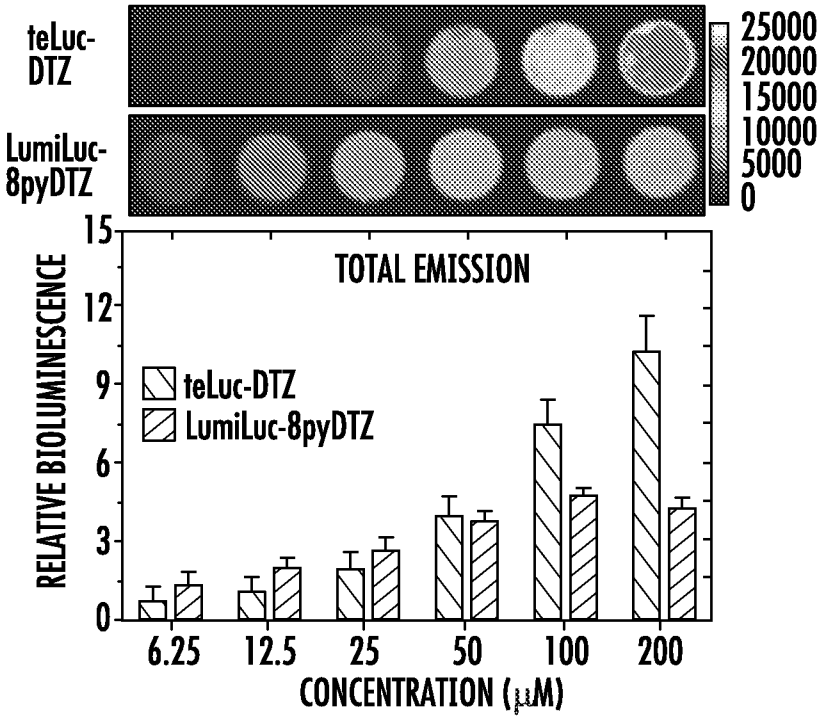

teLuc-DTZ
LumiLuc-8pyDTZ
25000
20000
15000
10000
5000
0
TOTAL EMISSION
RELATIVE BIOLUMINESCENCE
15
12
9
6
3
0
teLuc-DTZ
LumiLuc-8pyDTZ
6.25
12.5
25
50
100
200
CONCENTRATION (μM)

FIG. 8A

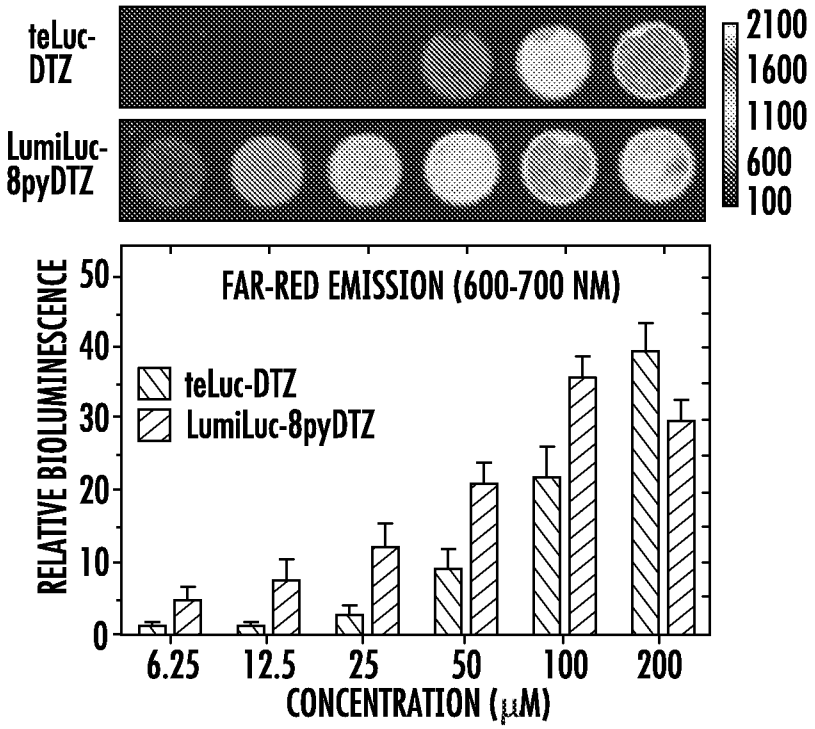

teLuc-DTZ
LumiLuc-8pyDTZ
2100
1600
1100
600
100
FAR-RED EMISSION (600-700 NM)
RELATIVE BIOLUMINESCENCE
50
40
30
20
10
0
teLuc-DTZ
LumiLuc-8pyDTZ
6.25
12.5
25
50
100
200
CONCENTRATION (μM)

FIG. 8B

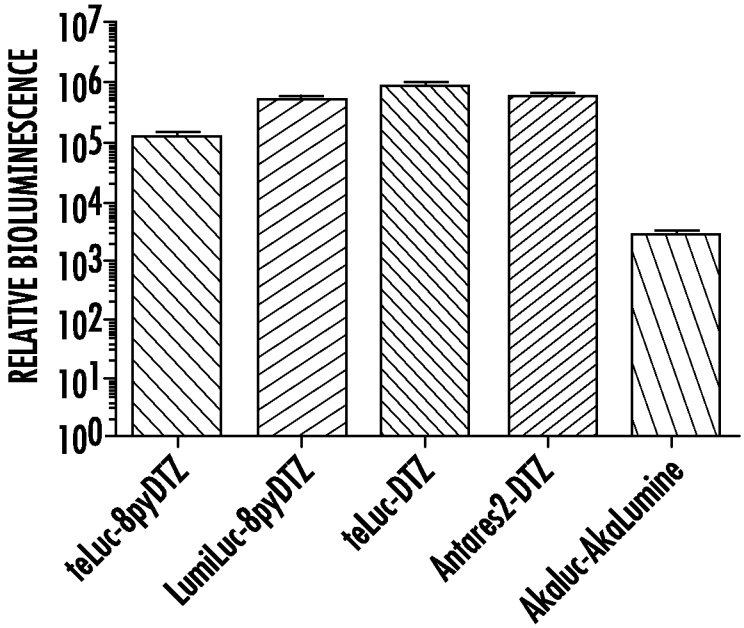

RELATIVE BIOLUMINESCENCE
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
teLuc-8pyDTZ
LumiLuc-8pyDTZ
teLuc-DTZ
Antares2-DTZ
Akaluc-AkaLumine

FIG. 9A

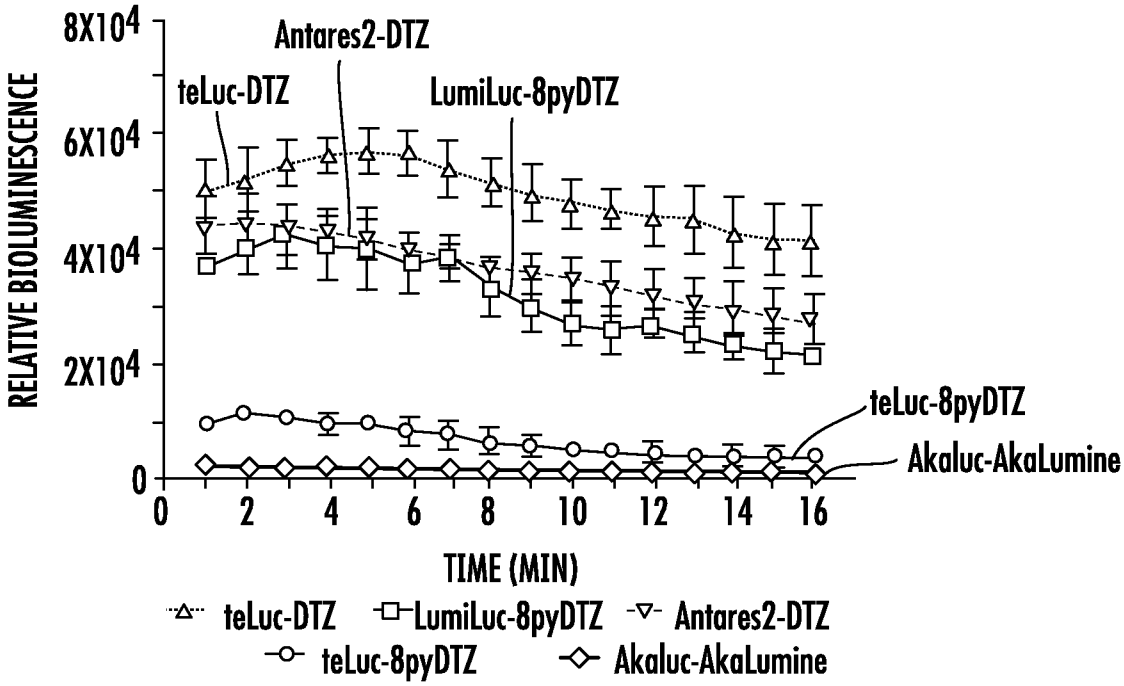

RELATIVE BIOLUMINESCENCE
8X10^4
6X10^4
4X10^4
2X10^4
0
Antares2-DTZ
teLuc-DTZ
LumiLuc-8pyDTZ
teLuc-8pyDTZ
Akaluc-AkaLumine
0 2 4 6 8 10 12 14 16
TIME (MIN)
teLuc-DTZ
LumiLuc-8pyDTZ
Antares2-DTZ
teLuc-8pyDTZ
Akaluc-AkaLumine

FIG. 9B

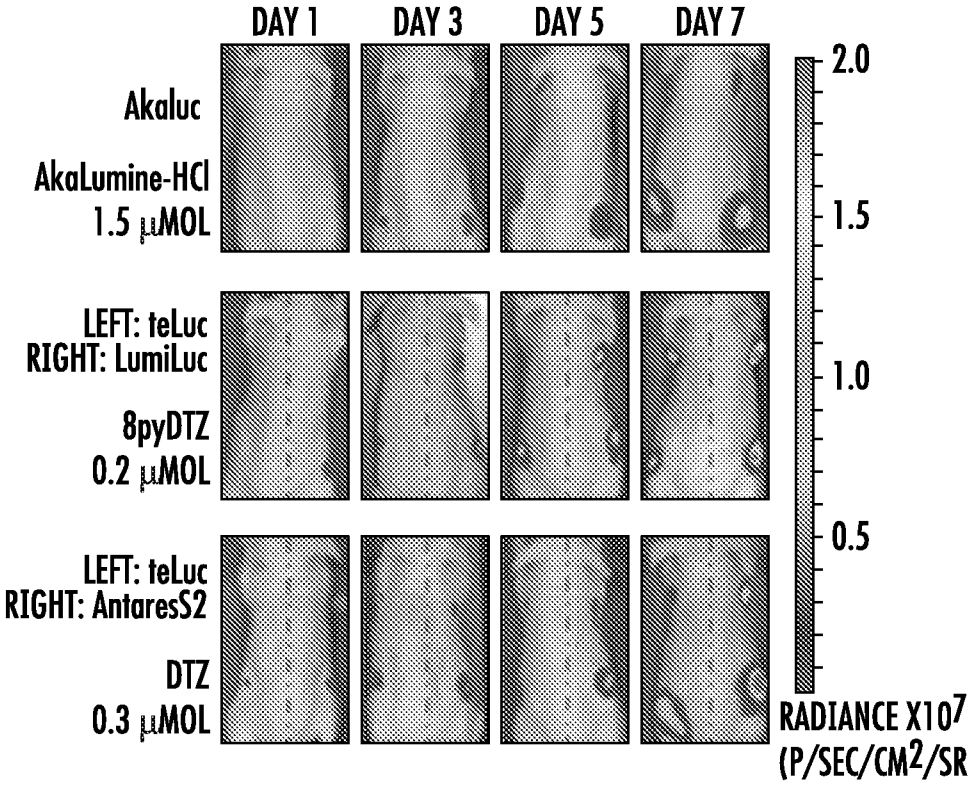


FIG. 10A

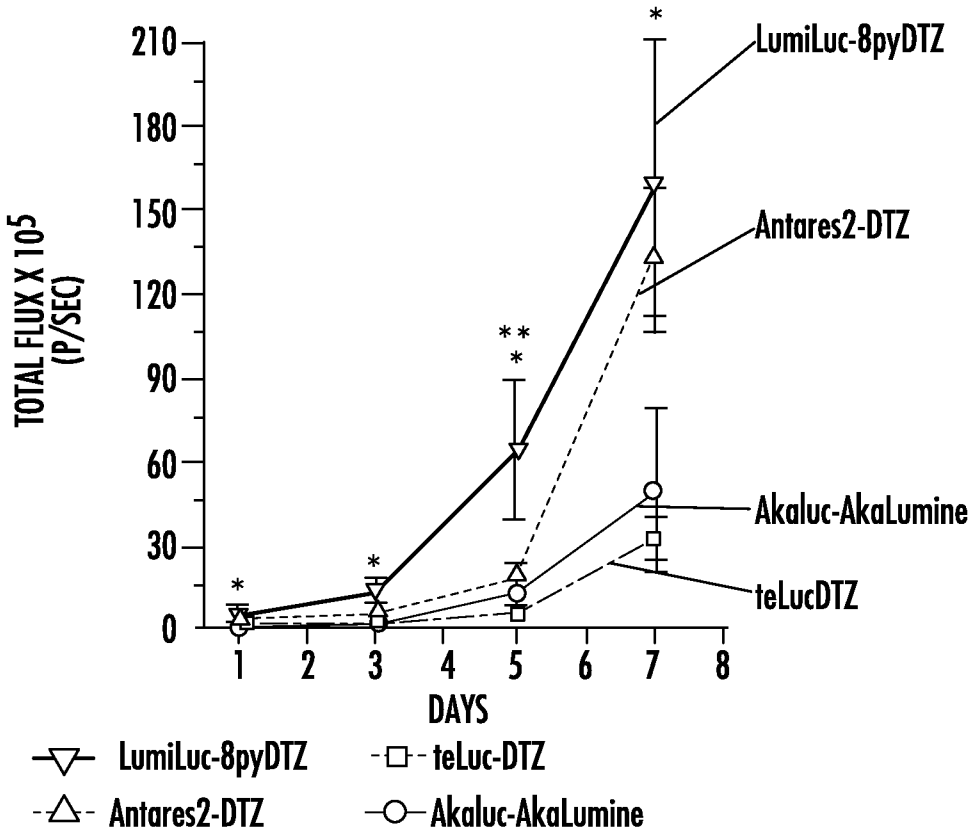


FIG. 10B

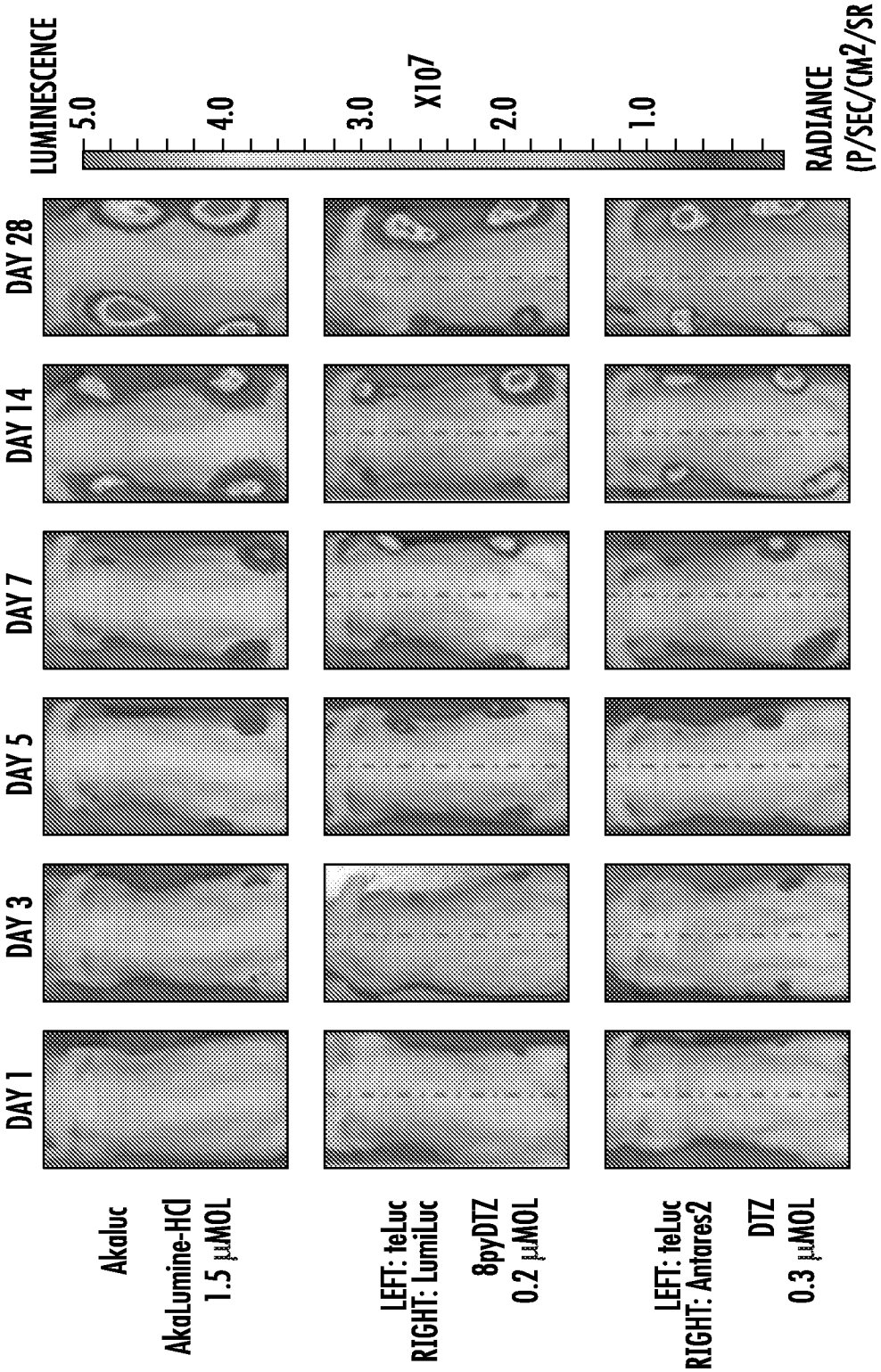

LUMINESCENCE
5.0
4.0
3.0
X10^7
2.0
1.0
RADIANCE (P/SEC/CM^2/SR
DAY 28
DAY 14
DAY 7
DAY 5
DAY 3
DAY 1
Akaluc
AkaLumine-HCl
1.5 μMOL
LEFT: teLuc
RIGHT: LumiLuc
8pyDTZ
0.2 μMOL
LEFT: teLuc
RIGHT: Antares2
DTZ
0.3 μMOL

FIG. 11A

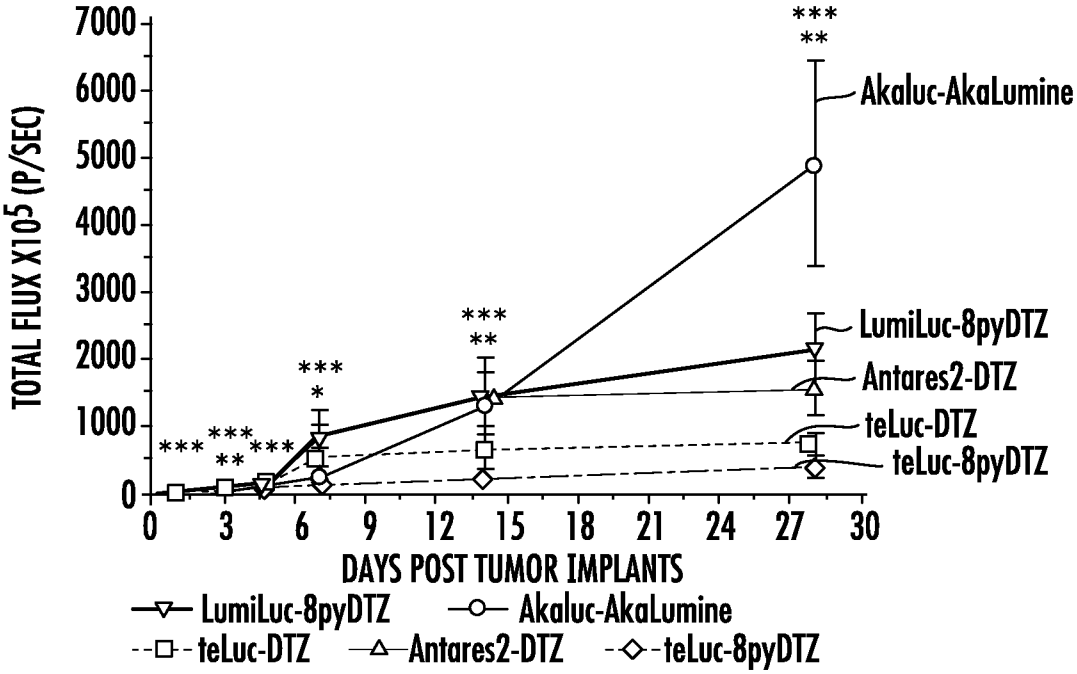


FIG. 11B

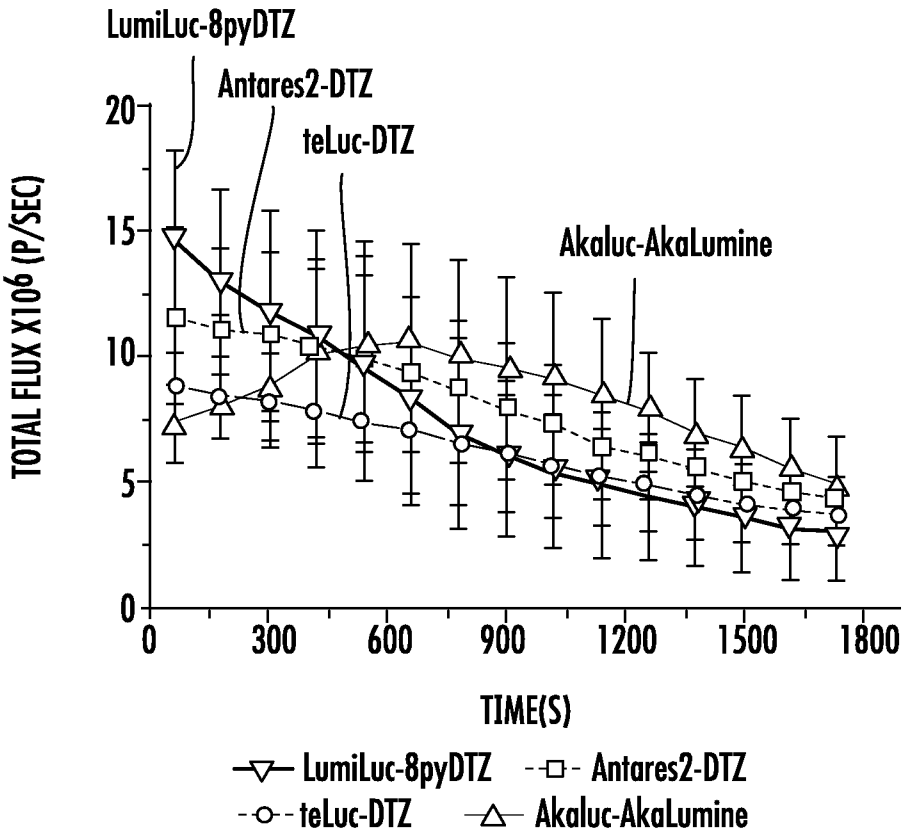


FIG. 11C

1	LumiLuc (-2-169)	XX	mScarlet-I (1-232)
2	mScarlet-I (1-232)	XX	LumiLuc (1-169)
3	mScarlet-I (1-225)	X	LumiLuc (2-169)

FIG. 12A

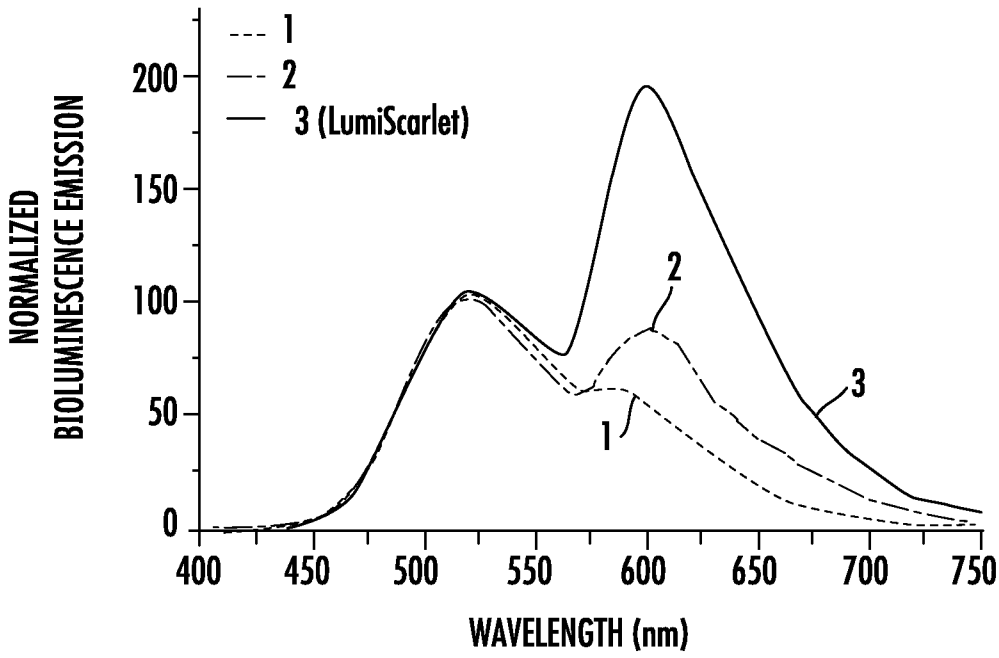


FIG. 12B

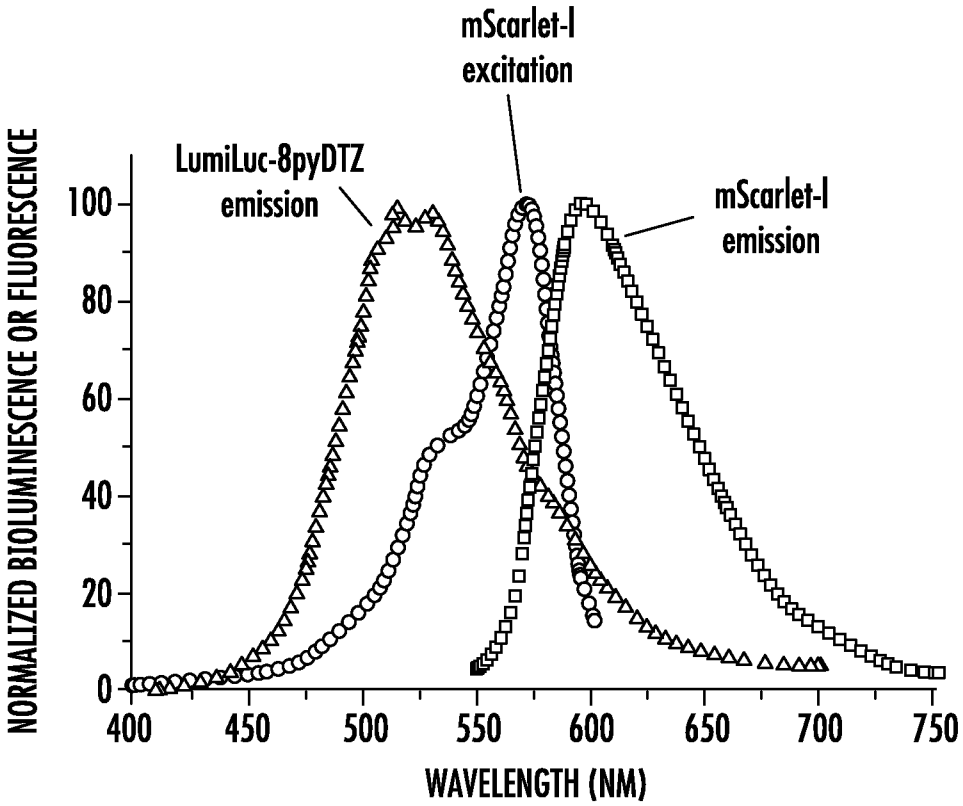


FIG. 12C

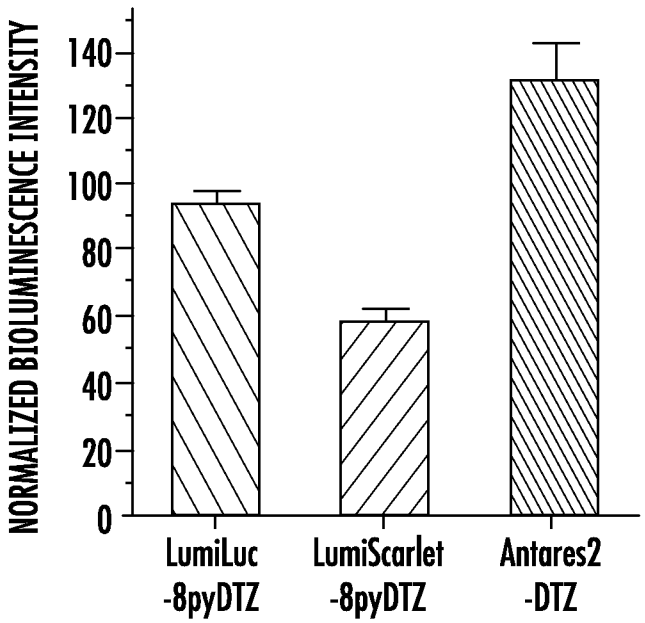


FIG. 12D

FIG. 12E

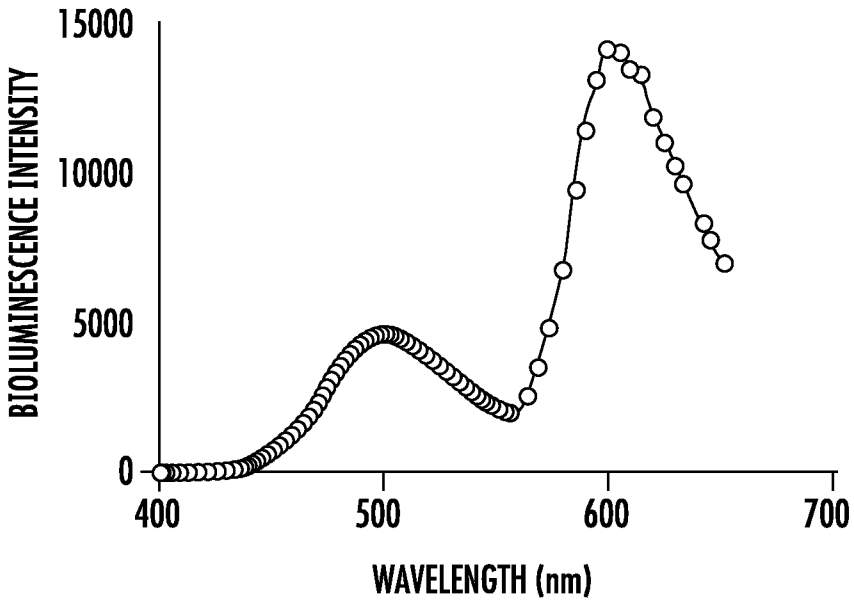


FIG. 12F

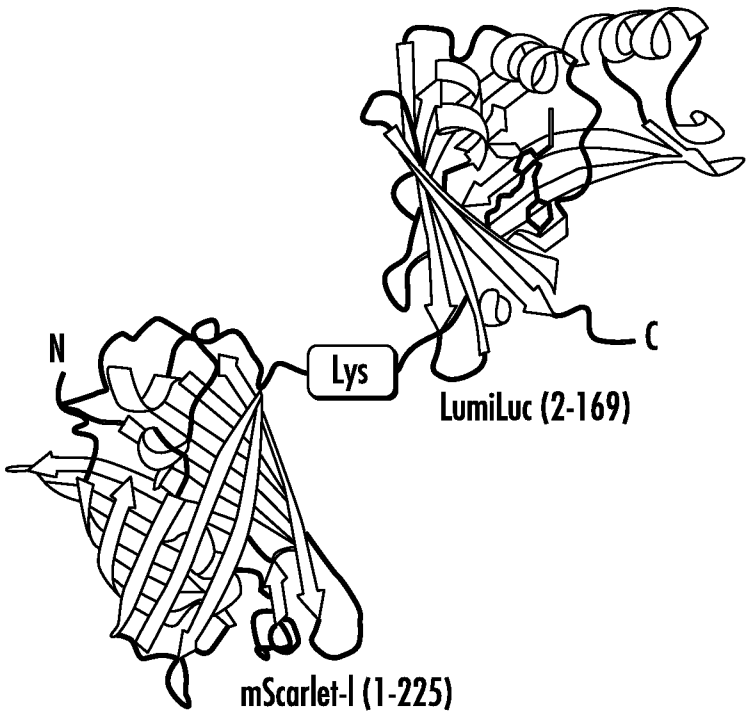


FIG. 13A

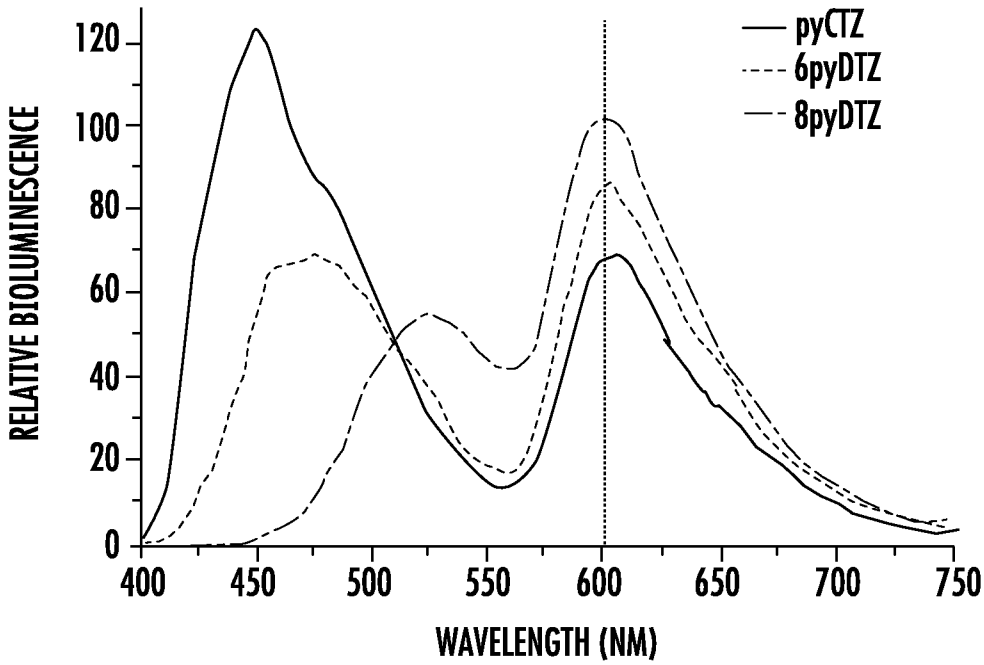


FIG. 13B

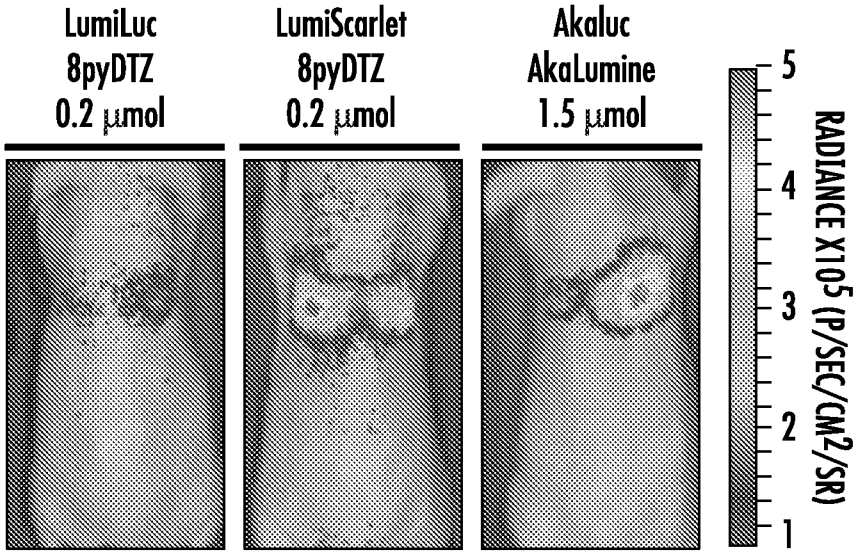


FIG. 13C

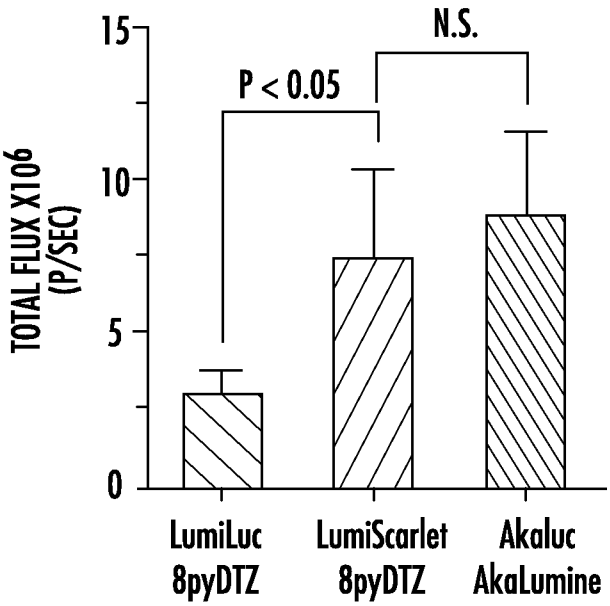
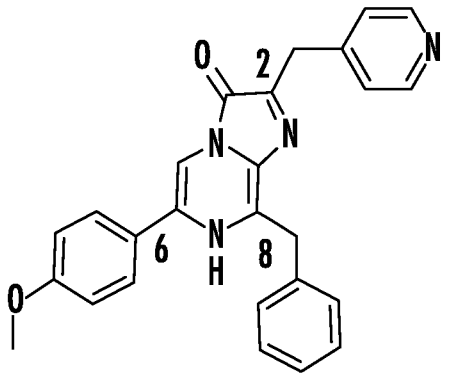
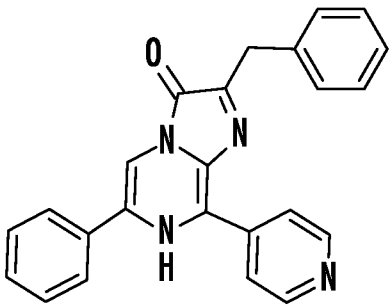


FIG. 13D



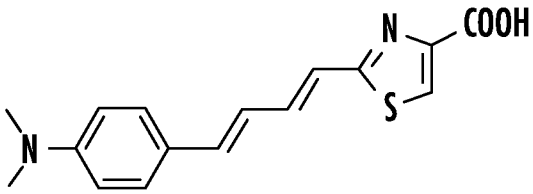
pyOMeCTZ, 416 NM

FIG. 14A



pyDTZ, 520 NM

FIG. 14B



AkaLumine, 650 NM

FIG. 14C

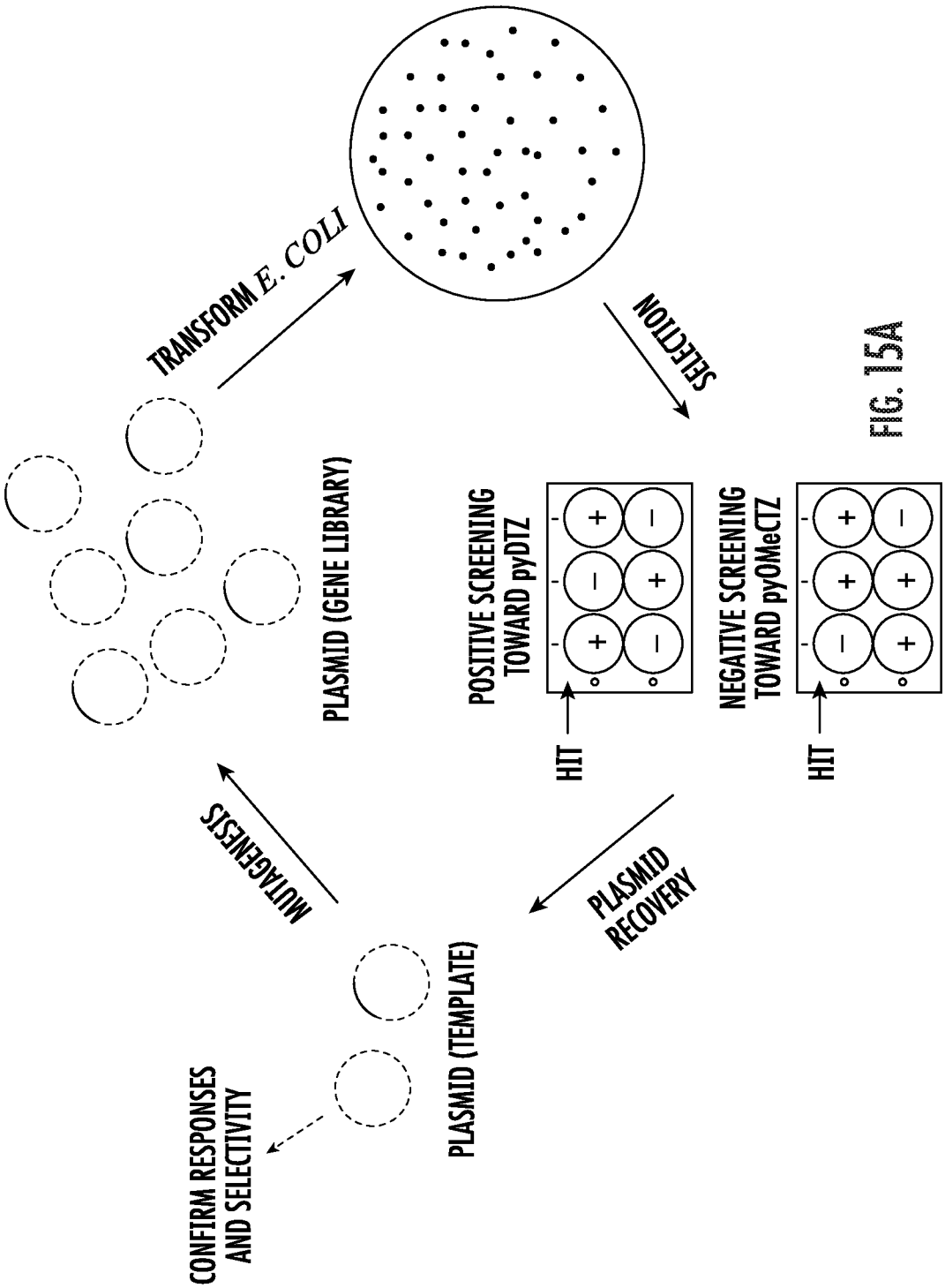
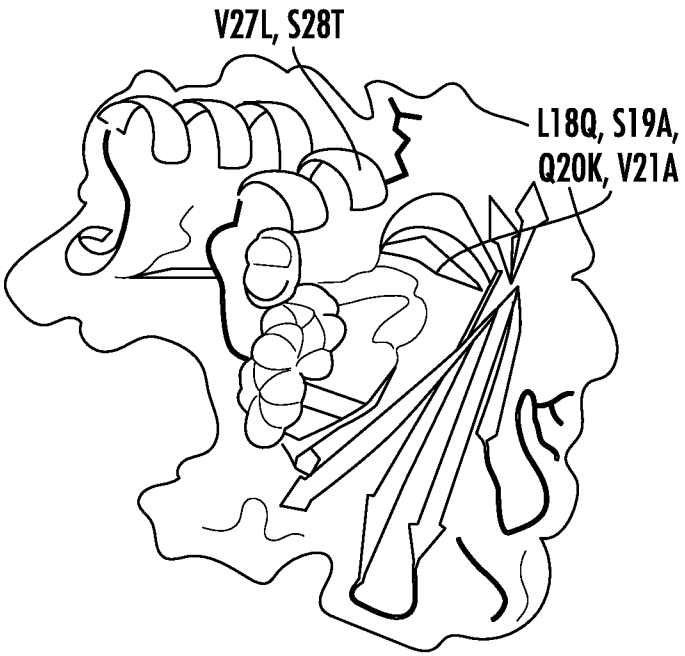

TRANSFORM E. COLI
SELECTION
PLASMID (GENE LIBRARY)
POSITIVE SCREENING TOWARD pyDTZ
NEGATIVE SCREENING TOWARD pyOMeCTZ
HIT
HIT
MUTAGENESIS
PLASMID RECOVERY
PLASMID (TEMPLATE)
CONFIRM RESPONSES AND SELECTIVITY

FIG. 15A



OpyLuc: L18Q, S19A, Q20K, V21A,
V27L, S28T, G67C, G71A, N85D,
R112Q, V119K

FIG. 15B

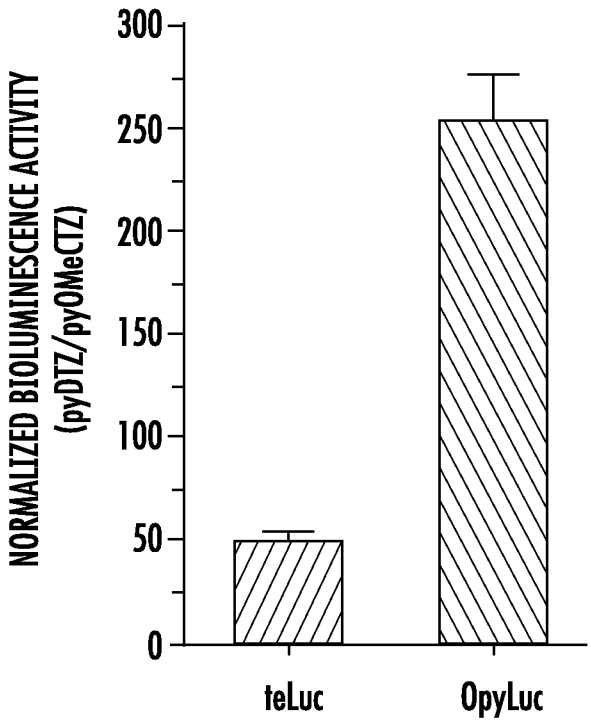


FIG. 15C

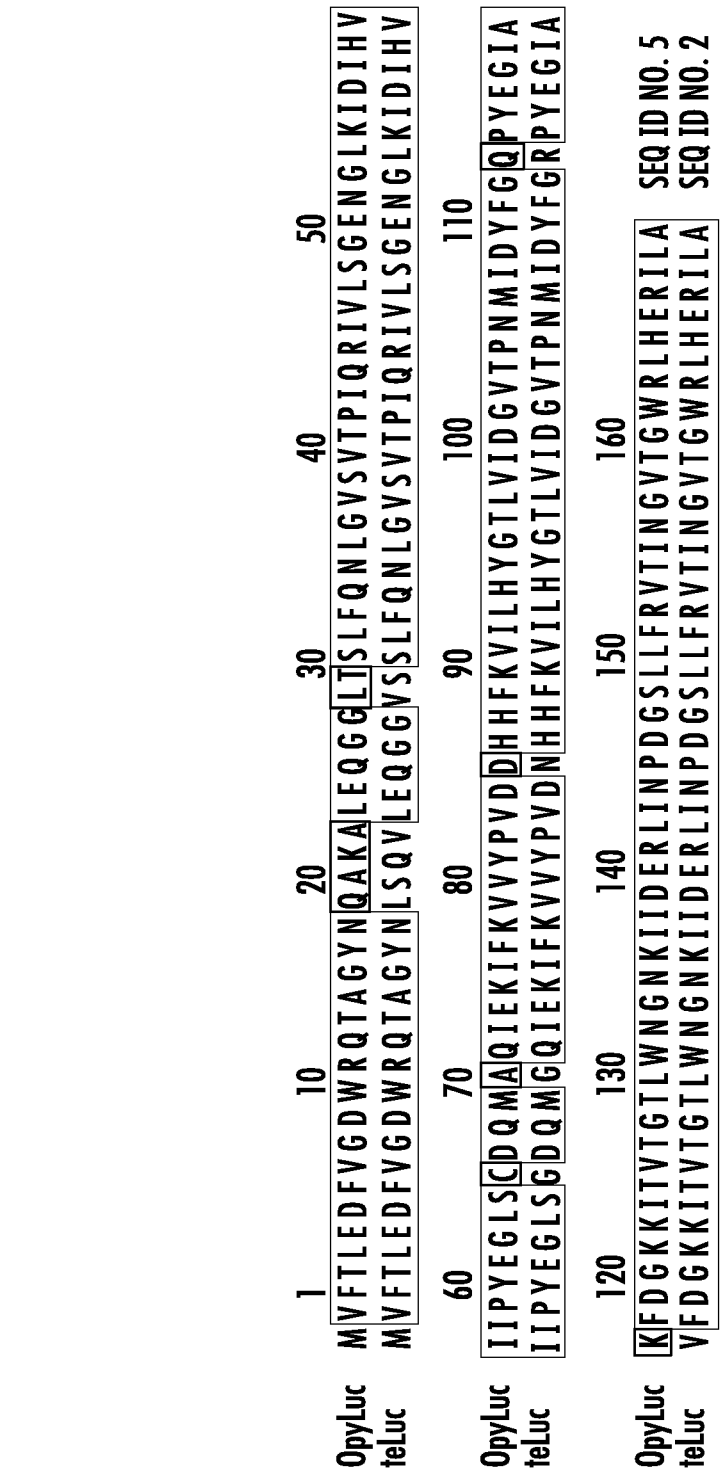

1 10 20 30 40 50
OpyLuc MVFTLEDFVGDWRQTAGYNQAKALEQGGLTSLFQNLGVSVTPIQRIVLSGENGLKIDIHV
teLuc MVFTLEDFVGDWRQTAGYNLSQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHV
60 70 80 90 100 110
OpyLuc IIPYEGLSCDQMAQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGQPYEGIA
teLuc IIPYEGLSGDQMGQIEKIFKVVYPVDNHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIA
120 130 140 150 160
OpyLuc KFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLHERILA SEQ ID NO. 5
teLuc VFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLHERILA SEQ ID NO. 2

FIG. 16

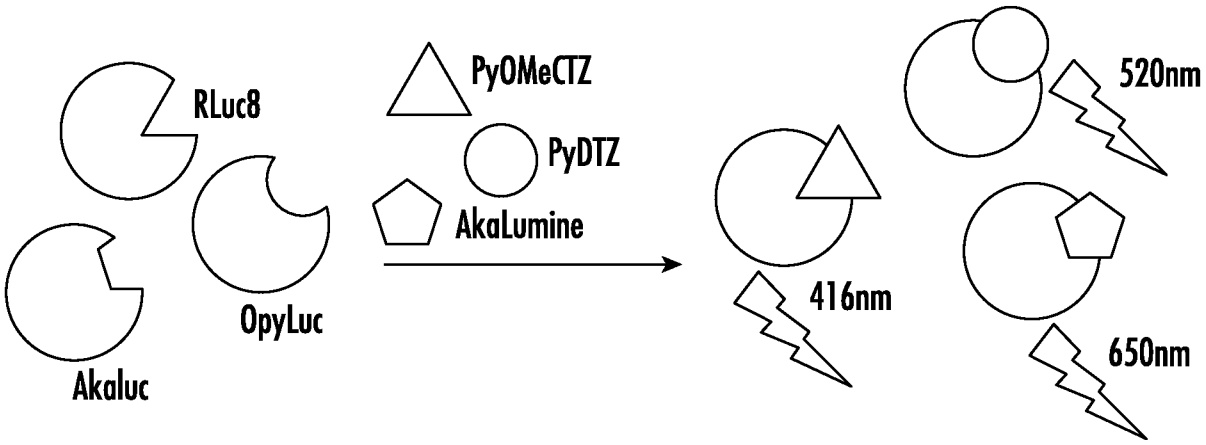


FIG. 17A

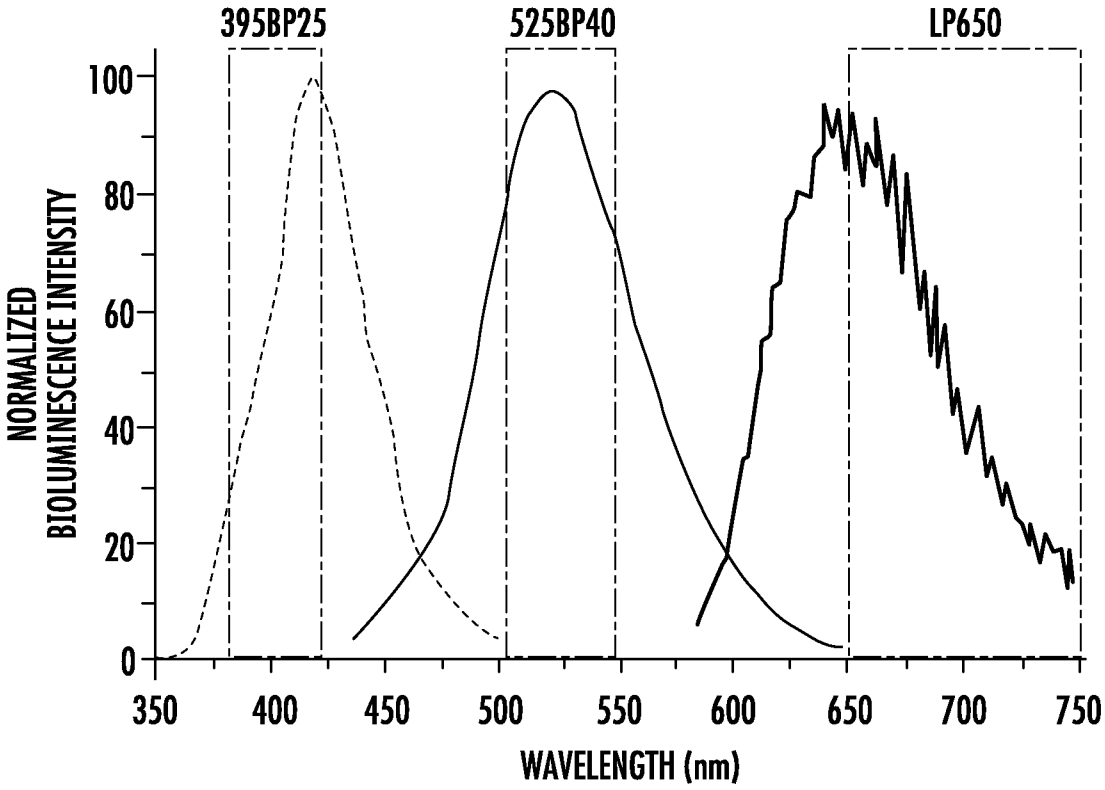


FIG. 17B

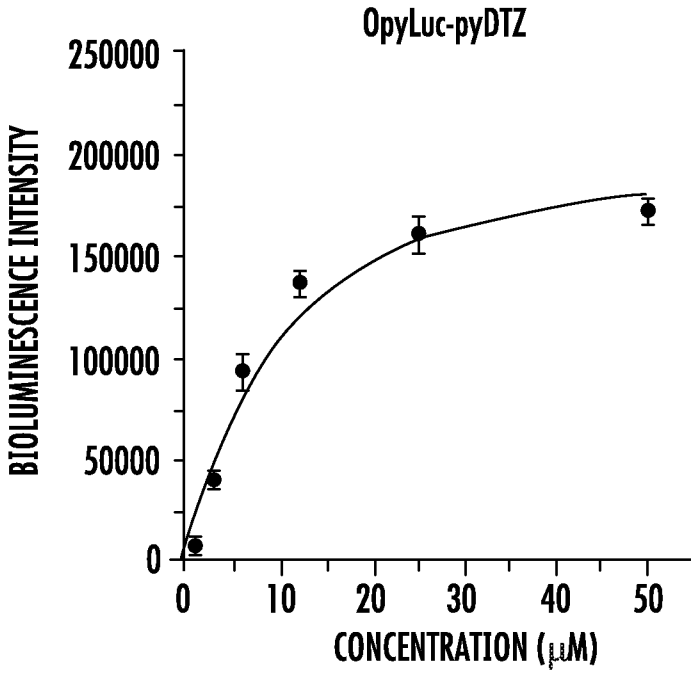


FIG. 18A

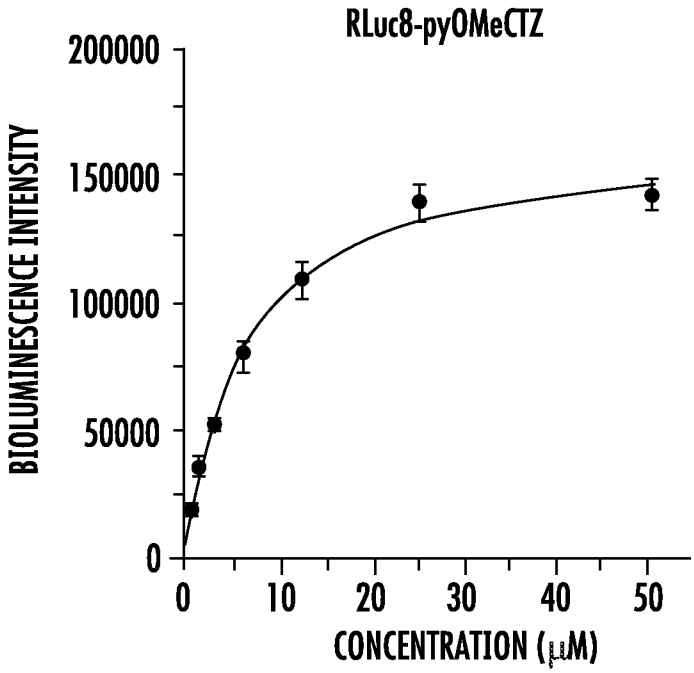


FIG. 18B

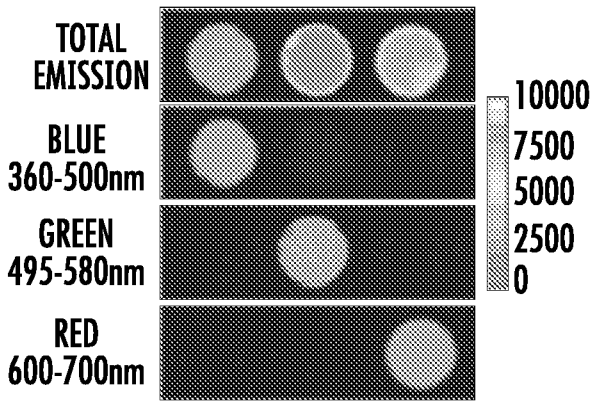


FIG. 19A

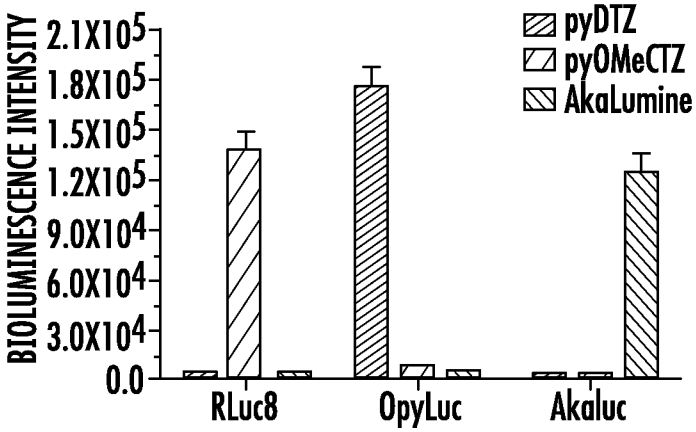


FIG. 19B

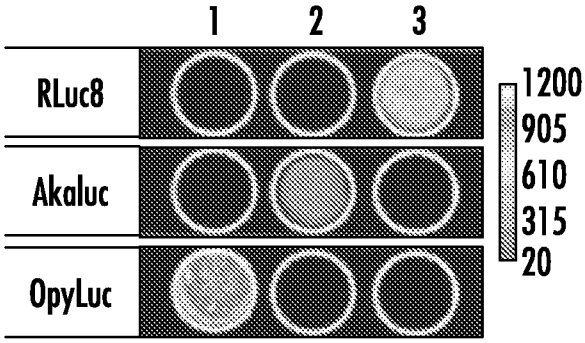


FIG. 19C

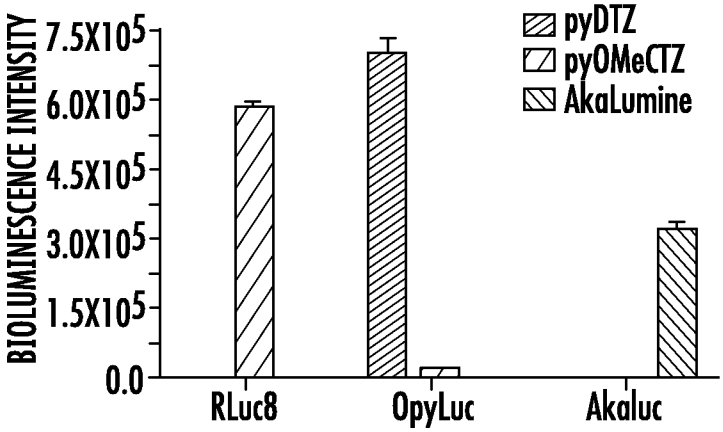


FIG. 19D

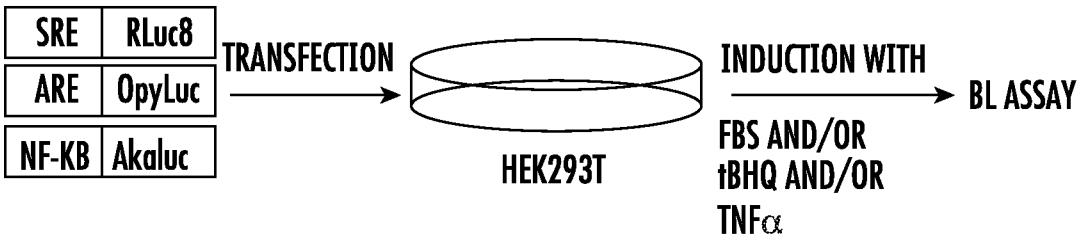


FIG. 20A

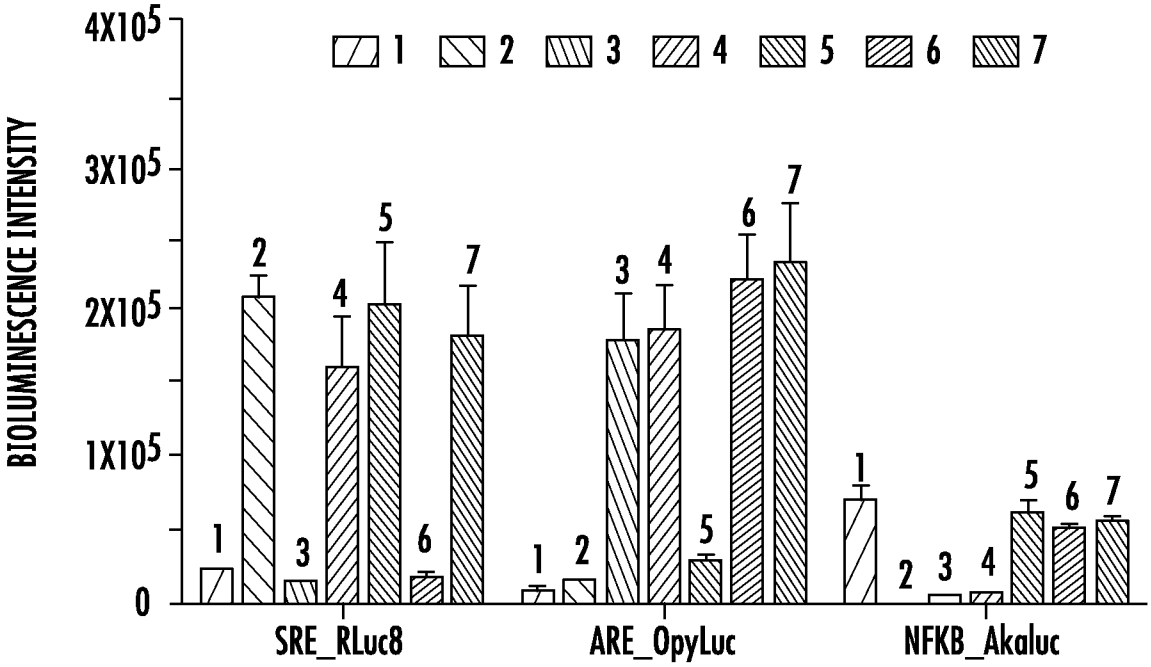


FIG. 20B

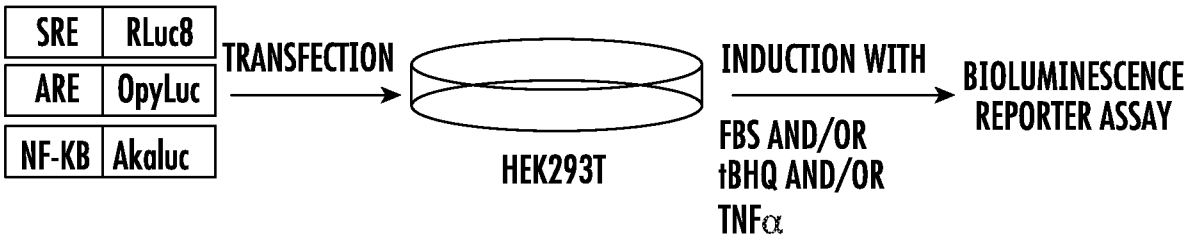


FIG. 21A

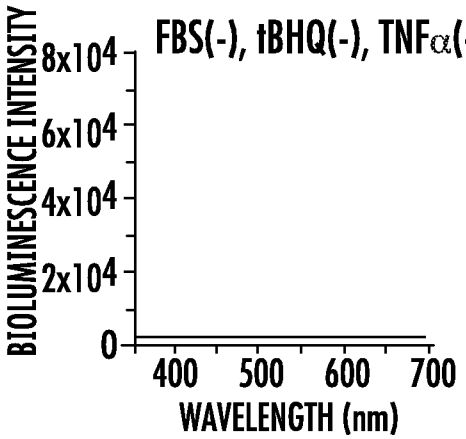


FIG. 21B

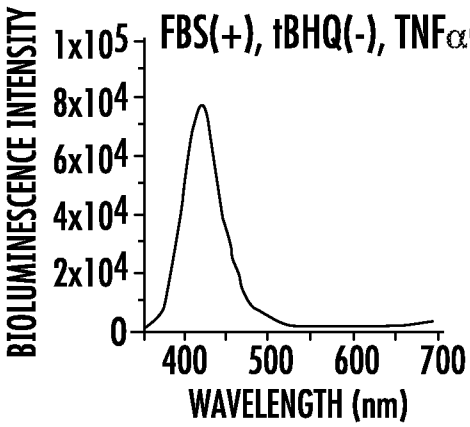


FIG. 21C

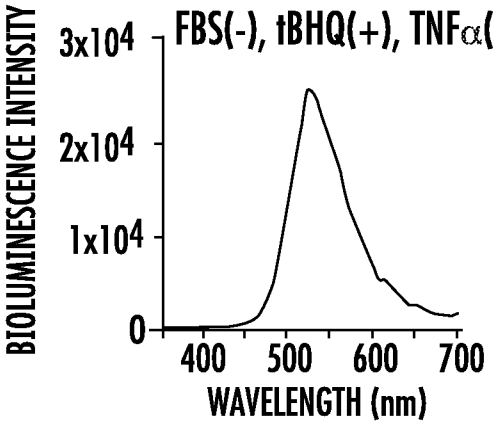


FIG. 21D

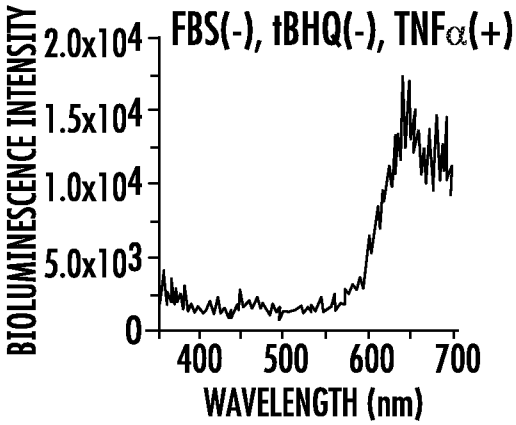


FIG. 21E

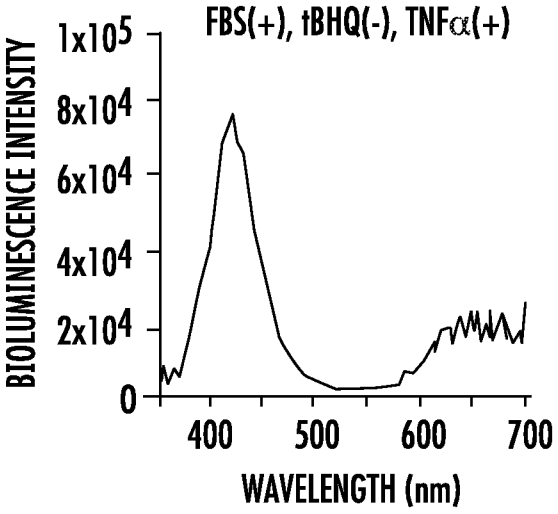


FIG. 21F

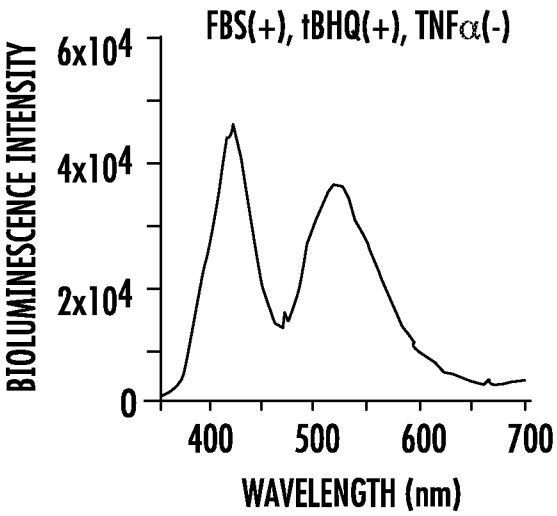


FIG. 21G

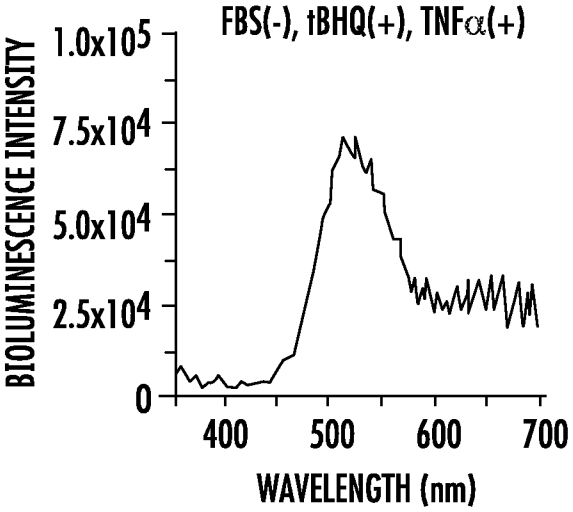


FIG. 21H

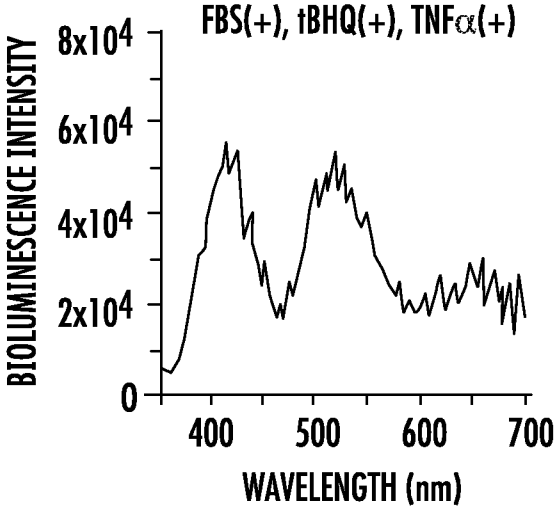


FIG. 21I

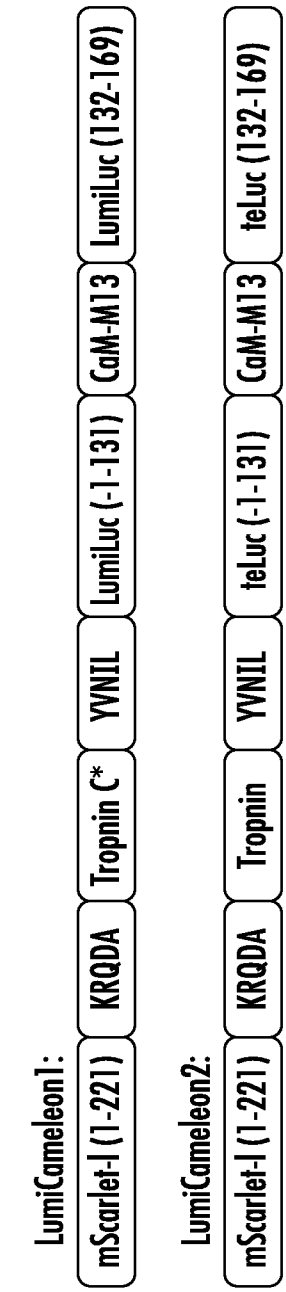


FIG. 22A

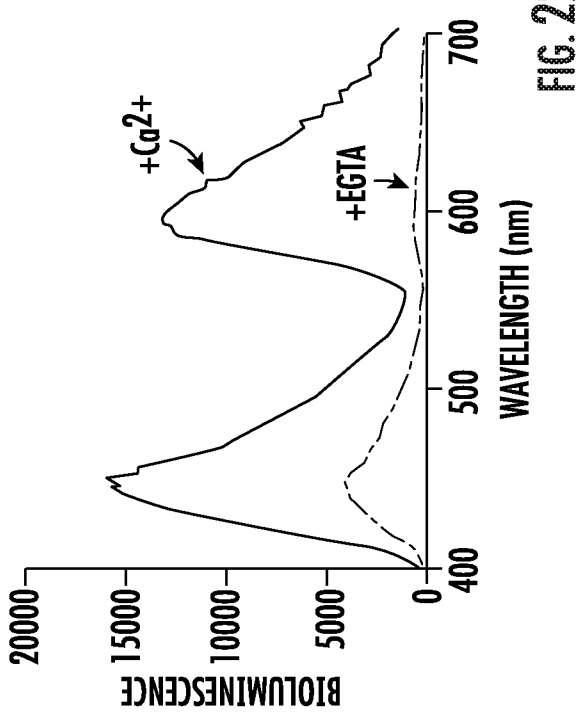


FIG. 22B

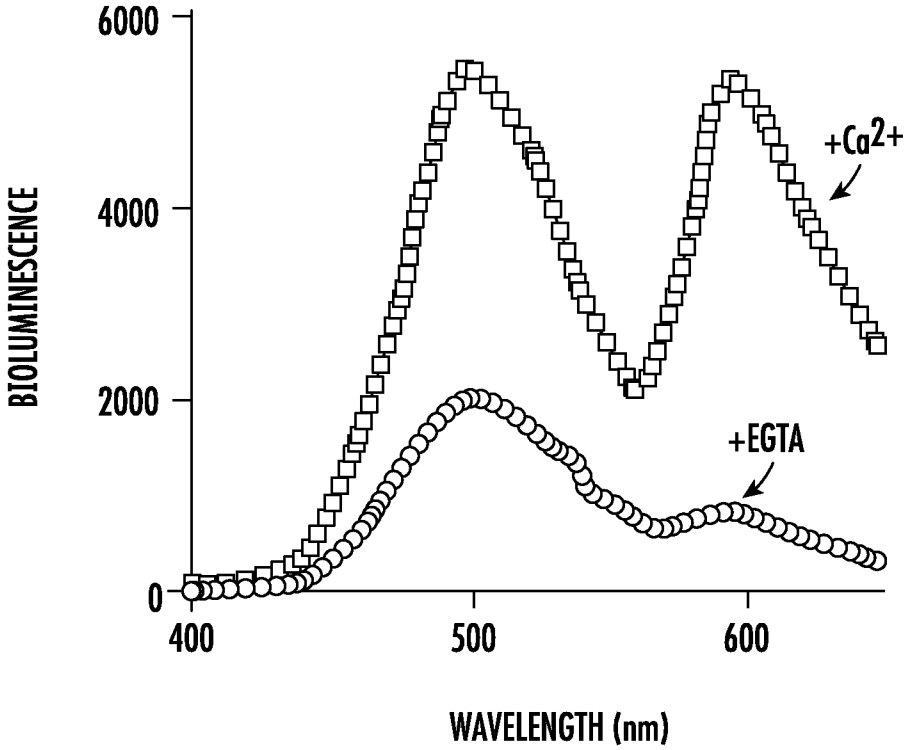

6000
4000
2000
0
BIOLUMINESCENCE
400
500
600
WAVELENGTH (nm)
+Ca2+
+EGTA

FIG. 22C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/20144

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.: 8-9, 14-22, 26-32, 37-45, 49-51, 57
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 extra sheet for Box No. III Observations where unity of invention is lacking -

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 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, 6(in part), 11 limited to amino acid substitution at position 4 of SEQ ID NO: 2 and SEQ ID NO: 8

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/20144

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 9/02, C07D 487/04, C12Q 1/66, A61K 49/00 (2020.01)

CPC - C12N 9/0069, A61K 49/0013, A61K 49/0045, C12Q 1/66, G01N 2333/90241

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2018/0057801 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 01 March 2018 (01.03.2018) Claim 1, SEQ ID NO: 1	1, 6/1, 11
A	YEH et al. "Red-shifted luciferase-luciferin pairs for enhanced bioluminescence imaging".Nat Methods. October 2017, Vol 14, No 10, pp 971-974; abstract, pg 2, para 4	1, 6/1, 11
A	US 2018/0327810 A1 (PROMEGA CORPORATION) 15 November 2018 (15.11.2018) Claim 132, SEQ ID NO: 1	1, 6/1, 11
A	US 2016/0376332 A1 (LIN et al.) 29 December 2016 (29.12.2016) abstract, SEQ ID NO:2	1, 6/1, 11

☐ 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
"D" document cited by the applicant in the international application
"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	Date of mailing of the international search report
02 July 2020	24 JUL 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 20/20144

Continuation of:
Box No. III. Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Groups I+: Claims 1-7, 10-13, drawn to a bioluminescent protein, comprising a substituted luciferase polypeptide. The composition will be searched to the extent that the substituted luciferase encompasses amino acid substitution at position 4 of SEQ ID NO: 2. It is believed that claims 1, 6(in part), 11 (Note, SEQ ID NO: 8 is the first amino acid sequence comprises amino acid substitution T4K) encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass amino acid substitution at position 4 of SEQ ID NO: 2. Additional amino acid substitution(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected amino acid substitution(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a substituted luciferase encompasses amino acid substitution at position 18 of SEQ ID NO: 2 (Claims 1, 6(in part), 12 (Note, SEQ ID NO: 10 comprises amino acid substitution Y18H).

Group II: claims 23-25, drawn to a luciferin compound.

Group III: claims 33-36, 46-48, 52-56, drawn to a bioluminescent reporter system and a method of monitoring bioluminescence in a subject.

The inventions listed as Groups I+, II and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Groups I+ include the special technical feature of a substituted luciferase polypeptide comprising amino acid substitution(s), not required by Groups II and III.

Group II includes the special technical feature of a luciferin compound of specific structure (see illustration in claim 23), not required by Groups I+ and III.

Group III includes the special technical feature of a bioluminescent reporter system comprising at least two luciferase proteins and at least two luciferin compounds and a method of use, not required by Groups I+ and II.

No technical features are shared between the amino acid sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Common Technical Features

The inventions of Groups I+ and III share the technical feature of a luciferase polypeptide.
The inventions of Groups II and III share the technical feature of a luciferin compound.

However, these shared technical features do not represent a contribution over prior art in view of US 2018/0057801 A1 to The Regents of the University of California (hereinafter "Univ California").

Univ California teaches (instant claim 1) a bioluminescent protein, comprising a substituted luciferase polypeptide comprising an amino acid sequence having at least 90% homology to SEQ ID NO: 2 with amino acid substitutions at one or more of positions corresponding to positions of SEQ ID NO: 2 (Claim 1, A bioluminescent protein, comprising a substituted luciferase polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1 with amino acid substitutions at positions corresponding to positions 21 and 166 of SEQ ID NO: 1, and comprising one or more amino acid substitutions at one or more positions.; Note, SEQ ID NO: 1 displays 98% homology to SEQ ID NO: 2 of instant claim 1.).

Univ California teaches (instant claim 23) a luciferin compound comprising the following structure wherein R6 is selected from -phenyl; R8 is selected from -phenyl; R2 is selected from -phenyl (para [0016], diphenyltetrazine.).

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I+, II and III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.