(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 October 2003 (09.10.2003)

PCT

(10) International Publication Number
WO 03/082904 A2

(51) International Patent Classification[7]: C07K 1/00

(21) International Application Number: PCT/US03/07979

(22) International Filing Date: 14 March 2003 (14.03.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/107,545 25 March 2002 (25.03.2002) US

(71) Applicant: CHEMICON INTERNATIONAL INC. [US/US]; 28820 Single Oak Drive, Temecula, CA 92590 (US).

(74) Agent: CHAN, Raymond, Y.; David and Raymond Patent Group, 108 N. Ynez Ave., Suite 128, Monterey Park, CA 91754 (US).

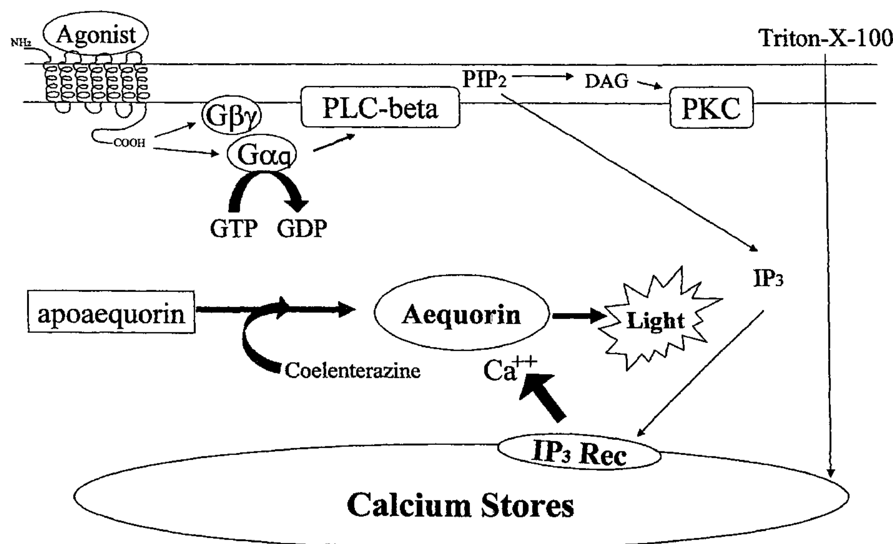
(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** MODIFIED AEQUORINS HAVING ENHANCED GLOWING KINETICS AND METHODS OF USING SAME



(57) **Abstract:** Modified apoaequorin polypeptides that exhibit enhanced glowing are provided, as are functional fragments of the polypeptides, and polynucleotides encoding the polypeptides and functional fragments. A modified glowing aequorin photoprotein also is provided. In addition, methods of using the glowing apoaequorins and encoding polypeptides to detect, for example, the presence of calcium ions in a sample, or to identify agents that effect the movement of calcium ions from one compartment to another are provided, as are methods for identifying functional changes in cells associated with changes in calcium ion concentrations.

WO 03/082904 A2

Modified Aequorins Having Enhanced Glowing Kinetics and Methods of Using Same

Background of the Invention

Field of the Invention

The present invention relates generally to compositions for measuring calcium ion levels in a cell, and more specifically to modified aequorins having prolonged bioluminescence as compared to wild type aequorins, and to methods of using the modified aequorins to measure calcium ion flux in a cell, including high throughput screening assays for identifying agents that modulate intracellular calcium ion levels.

Background Information

G-protein coupled receptors (GPCRs) represent one of the largest classes of cell surface receptors expressed in eukaryotic cells. GPCR polypeptides have seven transmembrane domains, and contain an extracellular ligand binding domain and an intracellular domain that specifically associates with a heterotrimeric complex of G-proteins, which are guanine nucleotide binding protein. Upon binding of a specific ligand by the GPCR, a signal is transduced across the cell membrane to the G-proteins, resulting in activation of G-protein and conversion of GDP to GTP. The signal is then propagated through the G-protein along one or more signal transduction pathways, resulting, for example, in production of second messengers such as cyclic AMP and calcium ion, and induction of gene transcription.

The particular signal transduction pathway activated by ligand binding to a GPCR depends, in part, on the particular G-protein that associates with the GPCR. For example, GPCR stimulation of the GÉ q/11 family of G-protein É subunits, which include the GÉ q, GÉ 11, and GÉ 14 subunits, as well as the promiscuous GÉ 15, and GÉ 16 subunits, leads to the activation of phospholipase C-é with the subsequent generation of 1,2-diacylglycerol and inositol (1,4,5)-triphosphate. The inositol (1,4,5)-triphosphate metabolite signals the release of the second messenger calcium from intracellular stores, leading to an amplified cascade of events that ultimately result in a physiological response of the cell to the stimulus. As such, GPCRs play a primary role in sensing and responding to a diverse repertoire of extracellular stimuli and, consequently, represent one of the most important families of targets for drug discovery. In fact, more than 20% of the top two hundred best selling drugs interact with GPCRs.

The extreme diversity in the GPCR signaling system is a function of the large number of different GPCRs, G-proteins and effector molecules. Although there is only minimal amino acid sequence identity across the entire GPCR superfamily, subfamilies of GPCRs share enough similarity to allow for the identification of novel receptors. However, it can be much more difficult to identify endogenous ligands for newly discovered GPCRs, and cognate ligands have not been identified for many newly discovered GPCRs. Such GPCRs, which are classified as orphan receptors, represent about 40% of the cloned human GPCRs. Substantial efforts have been directed to identifying ligands for orphan receptors, including orphan GPCRs.

Until recently, the only useful tools for identifying ligands and characterizing GPCRs were low throughput, technically difficult methods that often required the use of radioisotopes for measuring second messenger accumulation. More recently, *in vivo* assays have been developed using calcium reporters, including calcium sensitive fluorescent dyes such as Fura and calcium activated photoproteins

such as aequorin, which originally was isolated from the bioluminescent coelenterate, *Aequorea victoria*. Expression of recombinant aequorin in mammalian cells has a great advantage over the use of fluorescent dyes. For example, unlike fluorescent dyes, aequorin is nontoxic, does not interfere with normal cellular function, does not leak from cells, and is more sensitive with less background.
5 Unfortunately, aequorin flashes for only a few seconds after exposure to calcium. As such, aequorin cannot be used for characterizing and screening of GPCRs, for example, in high throughput format drug screening assays, because such assays would require injecting aequorin transfected cells into wells that contain a library of compounds, then simultaneously measuring light activity. However, such a method is plagued with problems, including, for example, difficulty in controlling the number of cells injected
10 per well, and an inability to use standard equipment and methodologies. In addition, because there is a pause of many seconds in the signal transduction and bioluminescent event after the cell comes into contact with the ligand, there is a requirement for long read-times (up to 30 seconds) per well.

Currently, the use of wild-type aequorin allows for the screening of about 10,000 compounds per day, and requires the use of expensive multiport injectable luminometers with
15 stirred cell bioreactors. However, methods of combinatorial chemistry allow the production of tens of millions of compounds or more. As such, even methods that allow for screening of 10,000 compounds per day would require years to screen a library of ten million compounds. Thus, a need exists for luminescent reagents that have a long read time, thus abrogating the requirement for injectable luminometers, and allowing screening of at least about 150,000 compounds or more per day. The
20 present invention satisfies this need and provides related advantages.

SUMMARY OF THE INVENTION

The present invention relates to modified apoaequorin polypeptides and functional fragments thereof, which, when charged with a luciferin, have enhanced glowing kinetics as compared to a corresponding wild type apoaequorin. The invention is based, in part, on the identification of a
25 "flash element" in the region of a calcium binding site of apoaequorin, including amino acid residues corresponding to residues 122 to 124 of SEQ ID NO:2. As such, the glowing aequorins of the invention comprise a modified apoaequorin containing at least one amino acid mutation (or deletion) corresponding to amino acid residues 122 to 124 of the wild type apoaequorin as set forth in SEQ ID NO:2, provided that when the modified apoaequorin has a mutation at (or deletion of) amino acid
30 residue 124, it further contains a mutation (or deletion) of amino acid residue 122, amino acid 123, or both.

The modified apoaequorins, which comprise a glowing aequorin, are defined, for convenience, with respect to the corresponding wild type apoaequorin polypeptide having an amino acid sequence as set forth in SEQ ID NO:2. However, it is well known that apoaequorins exhibit
35 substantial microheterogenity and, therefore, will be recognized that modified apoaequorin polypeptides of the invention also can be based on or derived from any of the numerous naturally occurring variants of SEQ ID NO:2.

A modified apoaequorin polypeptide of the invention can have an amino acid sequence corresponding to a full length wild type apoaequorin, such as amino acid residues 1 to 196 of SEQ ID
40 NO:2, or can comprise a functional fragment of SEQ ID NO:2, for example, a fragment comprising amino acid residues 1 to 124 of SEQ ID NO:2, amino acid residues 8 to 196 of SEQ ID NO:2, amino acid residues 8 to 124 of SEQ ID NO:2, amino acid residues 26 to 196 of SEQ ID NO:2, or amino acid residues 26 to 124 of SEQ ID NO:2, provided the fragment, when a component of an aequorin photoprotein, exhibits enhanced glowing kinetics, and provided the polypeptide comprises a mutation

as defined, or can comprise such a fragment, except wherein one, two or all three of amino acid residues 122 to 124 is deleted, for example, a fragment comprising amino acid residues 1 to 121 of SEQ ID NO:2, or a fragment comprising amino acid residues 1 to 121 of SEQ ID NO:2 operatively linked to amino acid residues 125 to 196 of SEQ ID NO:2.

5 In one embodiment, the amino acid residues corresponding to amino acid residues 122 to 124 of the wild type apoaequorin are Thr-Leu-Lys in a modified glowing apoaequorin. In another embodiment, a modified apoaequorin having enhanced glowing kinetics has an amino acid sequence as set forth in SEQ ID NO:4, or a functional fragment thereof, for example, a fragment comprising amino acid residues 8 to 171 of SEQ ID NO:4 or amino acid residues 26 to 171 of SEQ ID NO:4. In still 10 another embodiment, a modified glowing apoaequorin has an amino acid sequence as set forth in SEQ ID NO:8, or a functional fragment thereof, for example, a fragment comprising amino acid residues amino acid residues 8 to 196 of SEQ ID NO:8, or amino acid residues 26 to 196 of SEQ ID NO:8. In comparison, a modified apoaequorin having an amino acid sequence as set forth in SEQ ID NO:6, which contains a mutation of Asp to Ser at position 124, lacks glowing activity when a component of an 15 aequorin photoprotein.

A modified apoaequorin of the invention also can include a brightness element, which confers increased brightness on the glowing apoaequorin. For example, the glowing apoaequorin can include the brightness element comprising an amino acid sequence corresponding to amino acid residues 124 to 196 or SEQ ID NO:2, or a functional fragment thereof. Although reference is made 20 herein to a "glowing apoaequorin", it should be understood that glowing due to a modified apoaequorin occurs only when the apoaequorin is charged with a luciferin such as a coelenterazine, thus forming an aequorin photoprotein.

The present invention also relates to a fusion protein, which includes a modified glowing apoaequorin polypeptide, or functional fragment thereof, operatively linked to a heterologous 25 polypeptide. The heterologous polypeptide can be any polypeptide, including, for example, a cellular compartmentalization domain, a peptide that acts as a ligand for a receptor, a peptide useful as a tag for identifying or isolating the fusion protein, or a polypeptide that is naturally expressed in or secreted from a cell.

The present invention further relates to an antibody, or an antigen binding fragment thereof, 30 that specifically binds a modified glowing apoaequorin. Such antibodies of the invention are characterized, in part, in that they do not substantially bind to a wild type apoaequorin. As such, it will be recognized that an antibody of the invention, which can be a polyclonal antibody preparation, a monoclonal antibody, a single chain antibody, a humanized or human antibody, or the like, specifically binds to one or more epitopes that are substantially unique to the glowing apoaequorin. In one 35 embodiment, the antibodies of the invention include antibodies that, upon binding a modified glowing apoaequorin, enhance the glowing activity of an aequorin photoprotein comprising the apoaequorin or quench the glowing activity of the aequorin photoprotein, In another embodiment, an antibody of the invention specifically binds to a apoaequorin, but has no effect on the glowing activity of an aequorin photoprotein comprising the apoaequorin.

40 The present invention also relates to polynucleotides that encode a modified apoaequorin polypeptide having enhanced glowing kinetics, or a functional fragment of the glowing apoaequorin, as well as to polynucleotides that encode two or more polypeptides, including the glowing apoaequorin and one or more other proteins, and polynucleotides that encode a fusion protein comprising the

glowing apoaequorin. The polynucleotide can be single stranded or double stranded, and can be a deoxyribonucleic acid (DNA), a ribonucleic acid (RNA), or a hybrid or modified form thereof, for example, a DNA:RNA hybrid or DNA molecule containing nucleoside analogs. A polynucleotide of the invention is exemplified by the polynucleotides set forth in SEQ ID NO:3 or SEQ ID NO:7.

5 A polynucleotide of the invention can be contained in a vector, which can be a cloning vector or an expression vector, and can be a vector derived from a bacterial or plant cell plasmid, a virus, or a portion of a genomic nucleic acid sequence, such a vector being useful for introducing the polynucleotide of the invention into a genome by homologous recombination. In addition, a polynucleotide of the invention, or a vector containing the polynucleotide can be contained in a cell, for 10 example, a host cell, which allows propagation of a vector containing the polynucleotide, or a helper cell, which allows packaging of a viral vector containing the polynucleotide.

The polynucleotide can be transiently contained in the cell, or can be stably maintained due, for example, to integration into the cell genome or other stably maintained nucleic acid in the cell, e.g., mitochondrial DNA or chloroplast DNA. As such, the present invention also provides a transgenic non-15 human organism, which comprises cells containing a polynucleotide encoding a glowing apoaequorin stably maintained therein. The transgenic non-human organism can be a transgenic animal, including an invertebrate or vertebrate animal, or can be a transgenic plant, and the polynucleotide encoding the glowing apoaequorin can be expressed, either constitutively, inducibly, or in a developmental stage specific manner, in one particular cell type, i.e., in a tissue specific manner, or in a variety of cell types, 20 including in all of the cells of the transgenic non-human organism.

In addition, the present invention relates to a process for producing a modified apoaequorin polypeptide having enhanced glowing kinetics. In one embodiment, a process of the invention is practiced by expressing a polynucleotide encoding the glowing apoaequorin, for example, in a coupled transcription/translation reaction *in vitro*, or in a cell containing the polynucleotide. In another 25 embodiment, a process of producing a modified apoaequorin polypeptide having enhanced glowing kinetics is performed by mutating, either randomly, in a site-specific manner, or a combination thereof, a wild type apoaequorin polynucleotide, expressing mutant polynucleotides generated therefrom, identifying polynucleotides that encode a glowing apoaequorin, and producing the modified glowing apoaequorin from the mutated polynucleotide.

30 The present invention further relates to a process for producing a polynucleotide encoding a bioluminescent polypeptide having enhanced glowing kinetics as compared to a corresponding wild type bioluminescent polypeptide. Such a method can be performed, for example, by introducing mutations into a polynucleotide encoding a polypeptide corresponding to a wild type bioluminescent polypeptide, thereby generating mutant polynucleotides; contacting an expression library comprising 35 the mutant polynucleotides with a luciferin and divalent cations, under conditions sufficient to generate luminescent activity of a corresponding wild type bioluminescent polypeptide; and examining clones of the library for luminescent activity at a time after which the corresponding wild type bioluminescent polypeptide no longer exhibits luminescent activity, whereby luminescent activity of a clone identifies the clone as expressing a polynucleotide encoding a bioluminescent polypeptide having enhanced 40 glowing kinetics. Such a method can further include, after contacting the expression library with the luciferin, a step of examining clones of the library for luminescent activity within a time during which a corresponding wild type bioluminescent polypeptide exhibits luminescent activity.

By first examining clones during the time a corresponding wild type bioluminescent polypeptide is known to exhibit luminescent activity, and then again examining the clones at a time after which the flash period is expected to have ended, a clone can be confirmed as having enhanced glowing kinetics. In addition, inclusion of the additional examining step provides an internal control, in
5 that bioluminescent polypeptides encoded by polynucleotides that do not contain a mutation, or that encode mutant polypeptides that do not exhibit enhanced glowing, can be observed (i.e., luminesce) during the first examination, but not at the second examination.

By way of example, where the method is used to produce a polynucleotide encoding a modified glowing apoaequorin, one or a library of clones can be examined for luminescent activity
10 within about two to three minutes after being contacted with calcium ions, for example, by contacting the clones with a photographic film, then again examining the clones, using a second piece of film, at a time after at least about three minutes. Where the two films are positioned such that they can be compared to each other with respect to the library, clones that exhibit enhanced glowing can be identified. Alternatively, or in addition, individual clones can be isolated, for example, by transfer to a
15 well of a 96 well plate, and analyzed for enhanced glowing kinetics.

A method of preparing modified bioluminescent polypeptide having enhanced glowing kinetics can utilize, as a starting material, a polynucleotide encoding any wild type bioluminescent polypeptide that exhibits flash kinetics, including, as exemplified herein, an apoaequorin polypeptide, or a mnemiopsin, berovin, or obelin. The mutations can be introduced into the polynucleotide using
20 any method, and can include introducing random mutations, introducing mutations by a site directed mutagenesis method, or a both. Furthermore, the mutations can be any type of mutation, including, for example, an insertion, a deletion, a substitution, or a combination of such mutations. The clones of a library can be examined using any method that detects light of the wavelength generated by the bioluminescent polypeptide, including, for example, a photographic film or emulsion, or a luminometer.

25 A method of the invention also can include a step of isolating one or more clones, or the polynucleotide(s) contained therein, that are identified as expressing a bioluminescent polypeptide having enhanced glowing kinetics. Accordingly, the present invention provides polynucleotides encoding modified bioluminescent polypeptides having enhanced glowing kinetics, produced by such a process, and further provides isolated polynucleotides obtained by a method of the invention.

30 The present invention relates to a method of producing an aequorin photoprotein, which has enhanced glowing kinetics as compared to a corresponding wild type aequorin photoprotein. Such a method can be performed, for example, by contacting the modified glowing apoaequorin with a luciferin, particularly with coelenterazine or an analog thereof, under conditions sufficient for reconstituting an aequorin photoprotein. Such conditions can include, for example, contacting the
35 modified apoaequorin polypeptide and the coelenterazine in the presence of molecular oxygen. Such conditions also can include, where appropriate, contacting the modified apoaequorin polypeptide and the coelenterazine in the presence of a reducing agent such as é-mercaptoethanol. The method of producing a glowing aequorin photoprotein can further include a step of isolating the glowing aequorin photoprotein. Accordingly, the present invention also provides a modified aequorin photoprotein
40 produced by a such a method.

Methods for using a glowing apoaequorin, or encoding polynucleotide, to identify the presence of divalent cations, including calcium ions, strontium ions, and the like, in a sample, or to identify changes in cation concentration, which can be indicative, for example, of a physiologic

condition of or toxicologic exposure to a cell, also are provided. As disclosed herein, the methods of using a glowing apoaequorin polypeptide or an aequorin photoprotein reconstituted therefrom, or an encoding polynucleotide, can be an *in vitro* assay, for example, an enzyme-linked immunosorbent assay (ELISA), a lateral flow assay, or a post-polymerase chain reaction (PCR) assay; or can be a cell based assay, for example, a calcium influx assay. As such, the compositions are useful for functional biological assays, including, for example, muscle contraction assays, fertilization assays, G-protein coupled receptor assays, gap junction regulation assays, complement fixation/cytolysis assays, macrophage activation assays, oxidative burst/free radical assays, and apoptosis assays, as well as assays that detect fusions of membranes, for example, fusions of liposomes with each other or with target cells, and fusions of cells as can occur due to a viral infection. The compositions of the invention also can be used in an environmental assay to detect the presence of a divalent cation, for example, a contaminant such as strontium ion; or can be used as a component of a novelty item, for example, a glowing toy, a squirt guns, or a glowing food.

Accordingly, the present invention relates to a method of detecting the presence of divalent cations in a sample. Such a method can be performed, for example, by contacting the sample with a composition containing a modified glowing apoaequorin, or functional fragment thereof, under conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, and examining the sample for such luminescent activity, wherein detection of luminescent activity is indicative of the presence of divalent cations in the sample.

The method can be performed by contacting the sample with the glowing apoaequorin and with a coelenterazine, or analog thereof, such that an aequorin photoprotein is generated, or the aequorin photoprotein can be reconstituted prior to contacting the composition with the sample. The sample can be any sample containing or suspected of containing divalent cations that can activate the aequorin photoprotein, including a water or other aqueous sample, which can be examined, for example, for strontium contamination; a sample obtained from an organism, for example, a cell sample, a tissue sample, or an organ sample, or an extract thereof; and the like.

The present invention also relates to a method of identifying an agent that effects divalent cation flux, particularly calcium ion flux, in a sample comprising at least two compartments, wherein at least one compartment contains the cations. As such, the method is useful, for example, as a drug screening assay, which can be formatted for high throughput analysis. Such a method can be performed, for example, by contacting a compartment of the sample with a composition comprising a glowing apoaequorin polypeptide, or functional fragment thereof, under conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, examining the sample for such luminescent activity, and contacting the sample with a test agent, whereby a change in said luminescent activity identifies the test agent as an agent that effects the cation flux.

The compartment that is contacted with the modified apoaequorin, or functional fragment thereof, can be a compartment containing calcium ions, strontium ions, or the like, in which case, an agent that effects the cation flux is identified by detecting a decrease in luminescent activity due to efflux of the cation out of the compartment; or can be a compartment that lacks a sufficient cation concentration to generate luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, in which case, an agent that effects the divalent cation flux is identified by an increase in luminescent activity due to influx of the cation into the compartment. Such a method, which also allows the detection, for example, of recurring calcium ion flux into and out of a compartment, thus

provides a means for real time analysis of calcium movement in and out of cells, between cells, or among intracellular compartments.

In one embodiment of a method of detecting an agent that effect divalent cation flux, the sample includes a first compartment containing bound divalent cations and a second compartment having an insufficient cation concentration to generate luminescent activity mediated by the glowing apoaequorin, or functional fragment thereof. For example, the sample can be an aqueous solution, the first compartment can comprise calcium ions bound to a chelating compound such as EDTA or EGTA, and the second compartment comprises the aqueous medium, which contains a glowing aequorin photoprotein. Upon contact of the sample with a test agent, detection of luminescent activity identifies the test agent as an agent that results in release of calcium ions from the chelating agent, thus effecting calcium ion flux. Conversely, the method similarly can be used to determine that a toxic divalent cation, for example, a heavy metal ion is chelated out of otherwise potable water, thus confirming detoxification of the water.

In another embodiment, the sample comprises a cell, which can be a prokaryotic cell such as a bacterium, or a eukaryotic cell such as a plant cell, a cell of an invertebrate, or a cell of a vertebrate, for example, a mammalian cell. In one aspect of this embodiment, the cell delineates at least two compartments, including an intracellular compartment and an extracellular compartment, one of which contains calcium ions and one of which lacks calcium ions or has an insufficient concentration of calcium ions to generate the luminescent activity. As such, the method can be useful, for example, to identify an agent that effects calcium influx from the extracellular compartment into the cell or, conversely, that effects calcium efflux out of the cell. Similarly, the first compartment can be a first cell and the second compartment can be a second cell, wherein the method provides a means to detect intercellular flux of a divalent metal ion, for example, from the first cell to the second cell.

In another aspect of the embodiment, the cell comprises at least one intracellular compartment containing calcium ions and at least one intracellular compartment having an insufficient calcium ion concentration to generate luminescent activity mediated by the modified apoaequorin, or functional fragment thereof. The intracellular compartment containing calcium ions can be any intracellular compartment known to store calcium ions, including, for example, endoplasmic reticulum, sarcoplasmic reticulum in muscle cells, mitochondria, secretory vesicles such as the neuropeptide-containing dense core secretory vesicle present in neuronal cells, or a plant vacuole.

An agent that effects calcium ion flux in a cell can act directly, for example, by specifically binding to a calcium channel protein and modulating the activity of the channel, or can act indirectly, for example, by specifically binding a receptor that is linked to a pathway involving calcium ion flux. For example, the agent can effect calcium flux in a cell by binding specifically to a G-protein coupled receptor (GPCR) such as a thrombin receptor, an É-adrenergic receptor, or a é-adrenergic receptor, thus modulating a G-protein mediated signal transduction pathway associated with calcium ion flux. As such, the method provides a means to identify an agent that can act as an agonist or a partial agonist for a GPCR, or as an antagonist for the GPCR.

A screening method of the invention conveniently can be adapted to high throughput format, thus providing a means to examine a plurality of samples or agents or both in a single assay. For example, the method can be performed using a plurality of samples, each of which is the same, and contacting the samples with a plurality of test agents, which can be the same or different or a combination thereof, e.g., triplicates of a plurality of different test agents. The test agents can be

peptides, peptidomimetics, polynucleotides, small organic molecules, or the like, and can be obtained from naturally occurring sources, can be synthesized using, for example, a combinatorial method for generating libraries of molecules, or can be derived from a natural or known material and modified to identify derivatives having more desirable characteristics. Alternatively, or in addition, the samples can comprise a plurality of different samples. Where the method is performed in a high throughput format, the samples generally are arranged in an array, which can be an addressable array, on a microchip, a glass slide, or a plastic well.

The present invention also relates to a method of detecting a functional change associated with divalent cation flux across a membrane of a cell. Such a method can be performed, for example, subjecting a cell containing intracellular modified glowing apoaequorin polypeptide, or functional fragment thereof, under conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, to a stimulus, and examining the cell for said luminescent activity, whereby a change in said luminescent activity identifies a functional change associated with calcium ion flux across a cell membrane of a cell. The glowing apoaequorin can be loaded into the cells as a polypeptide (or as a glowing aequorin photoprotein) using a microinjection, macroinjection, or permeabilization method, or can be expressed from an encoding polynucleotide that is transfected, transformed, transduced, or the like into the cell. The cell containing the modified glowing apoaequorin, or functional fragment thereof, also can be a progeny of such a genetically modified cell, or can be a cell obtained from a transgenic organism of the invention.

The functional assays of the invention can be utilized to identify various functional or physiological changes. For example, the cell can be an oocyte, which is contacted with sperm such as in an *in vitro* fertilization procedure, whereby a change in luminescent activity identifies calcium influx into the oocyte, which is indicative of fertilization of the oocyte. The cell also can be a bacterial cell, which is contacted with complement fixing antibodies specific for an epitope of the bacterial cell, whereby a change in luminescent activity is indicative of loss of selective permeability of the bacterial cell wall and of cytolysis of the bacterial cell. By way of another example, the cell can be a mammalian cell that is exposed, or suspected of being exposed to a physical, chemical, or biological material that can induce apoptosis of a cell, whereby a change in luminescent activity is indicative of loss of selective permeability of the cell membrane and of apoptosis of the mammalian cell. The cell also can be a muscle cell, whereby a change in luminescent activity is indicative of a stimulus that can result in muscle cell contraction.

Brief description of the drawings

Figure 1 provides a schematic diagram of a G-protein coupled receptor (GPCR) assay showing the pathway of events upon agonist binding to a GPCR in a cell stably transfected with aequorin. Cells expressing aequorin and a GPCR, which upon activation by an agonist results in the dissociation of the GÉq subunit, and exchange of GDP for GTP. GÉq, which is associated with a Géγ subunit, then activates phospholipase C-é ("PLC-beta"), which generates IP_3, with the resulting IP_3 receptor-mediated transient release of calcium from intracellular stores (diacylglycerol, "DAG", also is generated and can activate protein kinase C, "PKC"). Coelenterazine associates with apoaequorin in the cell to generate aequorin. Calcium binds to the charged aequorin and photons of light are emitted and can be detected by a photomultiplier tube of a luminometer.

Figure 2 shows an alignment of the predicted amino acid sequences of two glowing aequorin mutants, K2.1 (SEQ ID NO:4) and 3K2 (SEQ ID NO:8) and a non-glowing modified apoaequorin, S3 (SEQ ID NO:6), with wild type ("WT") aequorin (SEQ ID NO:2). Numbers to right and left of

sequences indicate amino acid number. A consensus sequence is shown between each of the mutant and wild type sequences being compared. Identical amino acids are shown by the one letter amino acid code, and differences among the sequences are indicated by a space.

Figure 3 provides a comparison of the kinetics of the three aequorin mutant and wild type
5 aequorin over a five minute period after injecting with Ca^{++}. The signals produced by the 3K2 (diamond) and K2.1 (square) mutants are prolonged and persisted approximately 2.5 minutes longer than those for the wild type aequorin (X) and S3 (triangle) mutant. In addition, the 3K2, K2.1 and S3 mutant proteins had a sustained light emission that was about 100, 90 and 3 times greater, respectively, than the wild type aequorin.

10 Figure 4 provides a map showing the functional elements of aequorin. Relevant amino acid residues of wild type apoaequorin (SEQ ID NO:2) and the modified K2.1 (SEQ ID NO:4), S3 (SEQ ID NO:6) and 3K2 (SEQ ID NO:8) apoaequorins are shown. Because 3K2 had about 100 times more sustained light emission than unmodified wild type aequorin and S3 only had about 3 times more sustained light emission than unmodified aequorin, the flash element (FE) was mapped between amino
15 acid residues 121 and 125 (isoleucine, isoleucine, aspartic acid). The brightness element (BE) also is shown.

Figure 5 shows the stability of wild type or modified (3K2) aequorin signals generated from GPCR activation by thrombin in CHO cells over a 30 minute incubation. "Diamond" and "square" symbols indicate modified aequorin; "triangle" and "quote" symbols indicates wild type aequorin in the
20 presence (diamond and triangle) or absence (square and quote) of thrombin. The signal produced by the CHO cells transfected with wild type aequorin was less stable and exhausted after 3 minutes, whereas the signal in cells expressing the modified aequorin persisted after 30 minutes following incubation with thrombin.

25 **Detailed description of the invention**

The present invention provides modified photoproteins, including modified aequorin photoproteins, which glow for a longer period of time than naturally occurring aequorins. As such, the modified glowing aequorins of the invention provide an advantage over wild type and other previously described aequorins in that the modified glowing aequorins can be used in high throughput format
30 assays to detect intracellular calcium ion levels. For example, the glowing aequorins are useful as reagents for detecting activation of G-protein coupled receptors (GPCRs), particularly those coupled to G-proteins that effect calcium channel activity such as the thrombin receptor, thus providing a means to screen libraries of molecules to identify GPCR agonists, antagonists, and the like. The glowing aequorins of the invention provide the additional advantage in that, as compared to fluorescent dyes,
35 which also are used to detect calcium ion levels in cells, the glowing aequorins are nontoxic, and highly efficient reporter of calcium status.

As disclosed herein, a polynucleotide encoding a wild type apoaequorin polypeptide, which is a component of an aequorin photoprotein, was subjected to mutagenesis analysis, and mutants having enhanced glowing kinetics were identified. Using this method, modified aequorins having greater than
40 one hundred times the glowing capacity of natural aequorin were obtained (see Example 2). As such, the modified glowing aequorins of the invention are useful, for example, as sensors for detecting the presence of calcium, for example, intracellular calcium using a cell based assay, and as reagents for detecting biomolecular interactions in *in vitro* assays. *In vivo* calcium sensors can be used to evaluate

the movement of calcium from intracellular stores to the cytoplasm during signal transduction events, particularly those that involve calcium as a second messenger, including, for example, GPCR mediated events.

Based on the present disclosure that a bioluminescent protein such as apoaequorin can be modified to have enhanced glowing kinetics, it will be recognized that other wild type bioluminescent proteins that exhibit flash kinetics similarly can be modified and examined according the methods disclosed herein to obtain glowing photoproteins. As such, glowing photoproteins that emit light at various wavelengths can be obtained, thus providing, for example, reagents useful for performing multiplex types of analysis. Bioluminescent proteins are well known in the art and include, for example, photoproteins that catalyze the oxidation of luciferin to emit light, but do not release the oxidized substrate. Examples of such bioluminescent proteins include those isolated from the ctenophores *Mnemiopsis* (mnemiopsin) and *Beroe ovata* (berovin), those isolated from the coelenterates *Obelia* (obelin) and *Pelagia*; (see U.S. Pat. No. 5,486,455, which is incorporated herein by reference).

Aequorin is a photoprotein that naturally occurs in *Aequorea victoria*, a bioluminescent coelenterate. Aequorin was first isolated over 20 years ago and was determined to be a coelenterazine containing, calcium activated light-producing protein. Apoaequorin, which is a component of the aequorin photoprotein, binds coelenterazine, a luciferin, at a one-to-one ratio and catalyzes the oxidation of coelenterazine to coelenteramide, carbon dioxide and monochromatic light at approximately 469 nm, following a calcium induced conformational change. Because natural aequorin is unable to bind additional coelenterazine in the presence of calcium, the reaction results in a flash of light that is completely extinguished within a few seconds following exposure to calcium.

The term "aequorin" or "aequorin photoprotein" is used herein to refer a complex that is formed by the association of an apoaequorin polypeptide, a luciferin, and molecular oxygen and that, upon contact with calcium ions, undergoes a conformational change that catalyzes oxidation of the bound luciferin and results in the emission of visible light. Generally, the luciferin is coelenterazine or an analog thereof. Coelenterazine, and analogs thereof, are well known in the art and include, for example, those referred to as coelenterazine n, coelenterazine f, coelenterazine h, coelenterazine hcp, and coelenterazine cp (Molecular Probes, Inc.; Eugene OR; see, also, Intl. Publ. No. WO 01/68824, which is incorporated herein by reference). The term "apoaequorin" is used herein to refer to the polypeptide (or encoding polynucleotide) component of an aequorin photoprotein.

Apoaequorin polypeptides exhibit microheterogenity at several positions, including, for example, the presence of a Glu, Asp or Cys residue at position 99, or of a Val or Ile residue at position 123 (see Prasher et al., *Biochem. Biophys. Res. Comm.* 126:1259-1268, 1985; Prasher, et al. *Biochemistry* 26:1326-1332, 1987; U.S. Pat. Nos. 5,162,227 and 5,360,728, each of which is incorporated herein by reference). Apoaequorins have a molecular weight of about 22 kiloDaltons (kDa; Shimomura et al., *J. Cellular and Comp. Physiol.* 59:233-238, 1962; Shimomura et al., *Biochemistry* 11:1602-1608, 1972), and contains three calcium binding sites. Contact with calcium ions at concentrations as low as 1×10^{-6} M induces a conformational change in the photoprotein, and catalyzes the oxidation of the bound coelenterazine using the protein-bound oxygen, resulting in a flash of 469 nm blue light. Apoaequorins are exemplified by SEQ ID NO:2, as well as other naturally occurring apoaequorins that exhibit microheterogenity as compared to SEQ ID NO:2 (see, for example, U.S. Pat. No. 5,360,728, which is incorporated herein by reference; see, also, Intl. Publ. No. WO 01/68824), by previously described modified apoaequorins as described herein, for example, a modified apoaequorin known by the trade name "AQUALITE™" (U.S. Pat. No. 5,162,227, which is

incorporated herein by reference) or otherwise known in the art, and by the glowing apoaequorins of the invention.

Polynucleotides encoding isoforms of apoaequorin have been isolated (see, for example, U.S. Pat. No. 5,093,240, each of which is incorporated herein by reference; see, also, SEQ ID NO:1 and U.S. Pat. Nos. 5,162,227 and 5,360,782), and can be used for preparing a modified apoaequorin of the invention, which, when reconstituted into an aequorin photoprotein, exhibits enhanced glowing as compared to a naturally occurring aequorin. As used herein, the term "reconstitute" means that an apoaequorin polypeptide is mixed with a coelenterazine (or analog thereof) under conditions such that an aequorin photoprotein is produced. Conditions for reconstituting can be such oxygen is present, or the aequorin photoprotein can be reconstituted in the absence of molecular oxygen, and can be exposed to the oxygen at a later time, for example, at the time luminescence is desired (see, for example, U.S. Pat. No. 5,023,181, which is incorporated herein by reference). Conditions for reconstituting the aequorin photoprotein also can include the presence of a reducing agent, for example, é-mercaptoethanol (see U.S. Pat. No. 5,093,240), and can further include a calcium chelating agent such as EGTA.

In addition to the modifications disclosed herein for generating a glowing apoaequorin, the apoaequorin polypeptide can contain additional modifications as desired. For example, a glowing apoaequorin of the invention can further include substitutions of one or more of the three cysteine residues in the polypeptide with another amino acid such as serine, thus providing a modified apoaequorin that can be reconstituted into an aequorin photoprotein in the absence of a reducing agent (see U.S. Pat. No. 5,093,240, which is incorporated herein by reference).

Modified apoaequorins having greater bioluminescent activity than naturally occurring apoaequorins previously have been described, including, for example, a modified apoaequorin having a substitution of Asp to Ser at position 124 (see, for example, U.S. Pat. No. 5,360,728). Such modified apoaequorins are characterized, in part, in that they emit more photons per unit time and, therefore, are brighter than naturally occurring apoaequorin. However, the previously described modified apoaequorins have similar flash kinetics to the naturally occurring apoaequorins and, therefore, are distinguishable from the modified glowing apoaequorins of the invention, which have prolonged flash kinetics such that they glow.

The present invention provides modified apoaequorin polypeptides, which have a modified amino acid sequence as compared to wild type apoaequorins and, as a result, have enhanced glowing kinetics. It should be recognized that a modified apoaequorin of the invention does not luminesce when present as an isolated polypeptide, but only does so when part of an aequorin photoprotein. As such, reference to a modified apoaequorin having enhanced glowing kinetics (or to a glowing apoaequorin) will be understood to refer to an activity of the modified apoaequorin when it is a component of an aequorin photoprotein. As such, the present invention provides modified apoaequorin polypeptides, and further provides modified aequorin photoproteins, which include a modified apoaequorin polypeptide as a component and, therefore, exhibit enhanced glowing kinetics.

The term "luminesce" or "luminescent" or the like refers to an emission of visible light that is due to a chemical, physical, or biochemical reaction, and is not a result of thermal energy. The term "bioluminescent" refers to luminescence that occurs due to a biochemical reaction. Generally, bioluminescence occurs in a living organism, although the biochemical reaction responsible for the

bioluminescence also can be performed, for example, in an *in vitro* reaction, thus generating a bioluminescent signal.

The term "glowing" is used herein to refer to sustained luminescence, and particularly to luminescence that lasts for about two seconds or longer, when examined using a bacterial system or using purified reagents. An example of a bacterial system for measuring luminescence is provided in Example 2. In comparison, the term "flash" is used herein to refer to luminescence that peaks in less than about three seconds when examined using a bacterial system or using purified reagents. In general, naturally occurring apoaequorins and previously described modified apoaequorins, as well as the modified apoaequorin as set forth in SEQ ID NO:6, exhibit flash kinetics, wherein the luminescence quickly rises, peaks within about one to three seconds when examined, for example, in a bacterial system, then quickly dissipates.

For comparison purposes, the modified apoaequorin designated S3 (SEQ ID NO:6), which contains a mutation of amino acid residue 124 (D124S), but not of residues 122 and/or 123, is provided herein as a point of reference for determining whether a modified apoaequorin exhibits enhanced glowing kinetics. As disclosed herein, the S3 apoaequorin only exhibits a modest increase in luminescent activity (Figure 3), but does not exhibit glowing activity. As such, it will be recognized that a modified apoaequorin having luminescent activity longer than that of the S3 modified apoaequorin is considered to be a glowing apoaequorin and, therefore, encompassed within the present invention. In general, a glowing apoaequorin has luminescent activity that lasts at least about twice as long as that of the S3 apoaequorin, generally at least about three times as long, usually at least about four times as long, and particularly at least about five times as long as that of the S3 apoaequorin, when compared under substantially the same conditions.

With respect to the conditions under which luminescent activity is determined, it should further be recognized that, when luminescence due to an aequorin photoprotein is examined in eukaryotic cell, the flash kinetics of a wild type apoaequorin can be extended for a period much longer than two seconds due, for example, to a prolongation of a signal transduction or of release of calcium from an intracellular store. As such, the "flash" can appear to occur over a period of time longer than about three seconds (see, for example, Figure 5, triangles, showing the flash due to calcium release mediated by activation of the thrombin GPCR signal transduction pathway peaks at about 1 to 2 minutes). Nevertheless, enhanced glowing due to a modified apoaequorin of the invention is clearly identifiable when examined using the same system (Figure 5, diamonds; see, also, Example 3). As such, the term "enhanced glowing" or "enhanced glowing kinetics", when used in reference to a modified apoaequorin of the invention, is used in a relative sense to indicate that the luminescence of a modified glowing apoaequorin of the invention lasts for a longer period of time than does the luminescence of a reference apoaequorin, for example, a corresponding wild type apoaequorin or a corresponding previously described modified apoaequorin from which the modified apoaequorin of the invention is derived, when examined under substantially the same conditions. As disclosed herein, two exemplified modified apoaequorins, K2.1 (SEQ ID NO:4) and 3K2 (SEQ ID NO:8) glow 90 and 100 times longer, respectively, than wild type apoaequorins or modified apoaequorins such as S3 (SEQ ID NO:6; see Example 2).

Accordingly, the present invention provides modified apoaequorin polypeptides that, when reconstituted with a luciferin and oxygen, generate aequorin photoproteins that, when contacted with calcium ions, exhibit enhanced sustained light emission relative to unmodified aequorins. Such modified apoaequorins having enhanced glowing kinetics also are referred to herein as "glowing

apoaequorins" (or "glowing aequorins"). Also provided are polynucleotides that encode such glowing apoaequorins, and that can be introduced into an organism or a cell for the expression of a modified aequorin exhibiting enhanced glowing kinetics. In addition, modified aequorin polynucleotides and polypeptides having mutations derived randomly, or having site-directed mutations are provided.

5 The modified glowing apoaequorin polypeptides disclosed herein were produced using genetic engineering methodologies that result in mutations or alterations in the natural amino acid sequence of a wild type apoaequorin (SEQ ID NO:2). One example of an apoaequorin having a modified amino acid sequence that results in enhanced light emission kinetics is the polypeptide set forth as SEQ ID NO:4 (designated K2.1, and encoded by SEQ ID NO:3), where a deleted thymine 10 nucleotide at position 365 (nucleotide 1 is the adenine in the start codon of natural aequorin) changes the reading frame of the sequences and results in a polypeptide that has a substantially different amino acid sequence after amino acid residue 121 relative to the corresponding unmodified wild type apoaequorin (SEQ ID NO:2). Another example of a apoaequorin having a modified amino acid sequence that results in enhanced light emission kinetics is the polypeptide set forth as SEQ ID NO:8 15 (designated 3K2, and encoded by SEQ ID NO:7), where a deleted thymine nucleotide at position 365 of SEQ ID NO:7 changes the reading frame of the sequence and an inserted guanine nucleotide after position 375 reinstates the reading frame, such that SEQ ID NO:8 has a substantially different amino acid sequence between amino acid residues 121 and 125 relative to the corresponding unmodified apoaequorin (SEQ ID NO:2; see Figure 4). In comparison, an apoaequorin having a modified amino 20 acid sequence that results in slightly increased light emission, but not enhanced glowing, as compared to the wild type apoaequorin is set forth as SEQ ID NO:6 (designated S3, and encoded by SEQ ID NO:5), where a deleted nucleotide at position 370 changes the reading frame and results in a polypeptide that has a substantially different amino acid sequence beginning with amino acid residue 124 relative to the corresponding unmodified apoaequorin (SEQ ID NO:2).

25 A modified apoaequorin of the invention can contain modifications in addition to those exemplified herein for generating enhanced glowing kinetics, including, for example, substitutions of nucleotides encoding degenerate codons in a polynucleotide encoding the modified apoaequorin; by incorporating specific amino acid substitutions at positions of microheterogeneity (see, for example, U.S. Pat. No. 5,360,728); by altering non-essential amino acid residues, which do not substantially 30 change the enhanced glowing kinetics of the modified apoaequorin; or by making conservative amino acid changes, for example, one hydrophobic amino acid residue such as leucine for a different hydrophobic amino acid such as isoleucine or valine; or by making deletions or truncations of the apoaequorin polypeptide sequence to remove non-essential amino acids, for example, deletion of one, two, three, four, five, or more amino acid residues from the N-terminus or C-terminus (see, for 35 example, U.S. Pat. No. 5,360,728, describing a truncated apoaequorin from Val-8 to Pro-196; see SEQ ID NO:2, where the initiator Met is position 1); or by inserting one or a few amino acids into the apoaequorin or adding one or a few amino acids at one or both termini; or any combination of such modifications, provided the modified apoaequorin exhibits enhanced glowing kinetics. For example, a glowing apoaequorin polypeptide can contain one or more D-amino acids in place of a corresponding 40 L-amino acid; or can contain one or more amino acid analogs, for example, an amino acid that has been derivatized or otherwise modified at its reactive side chain, or a reactive group at the amino terminus or the carboxy terminus or both can be modified. Such modifications can be selected, for example, to engineer the glowing apoaequorin to have improved stability to a protease, an oxidizing agent or other reactive material the polypeptide may encounter in an environment to which it will be exposed, for 45 example, a biological environment, and, therefore, can be particularly useful in performing a method of

the invention. Of course, the glowing apoaequorins also can be modified to have decreased stability in a particular environment such that the period of time the polypeptide is available in the environment is reduced.

The ability to incorporate substantial changes in the amino acid sequence of a glowing apoaequorin of the invention is exemplified by the demonstration that a change in the reading frame of the encoding polynucleotide resulting the generation of a glowing apoaequorin having a completely different C-terminus of the flash element as compared to a corresponding wild type apoaequorin does not alter the function of the glowing apoaequorin (see, for example, SEQ ID NO:4; see, also, Figure 4). As such, it will be recognized that substantial changes can be made, for example, in the amino acids corresponding to amino acid residues 122 to the C-terminus, including the flash element, of SEQ ID NO:2 or a variant thereof.

Methods for detecting enhanced glowing kinetics of a modified apoaequorin are disclosed herein (see Examples 2 and 3), or otherwise known in the art. For example, a change in an apoaequorin polypeptide that results in sustained light emission (i.e., enhanced glowing) can be determined by reconstituting the apoaequorin with a luciferin in the presence of oxygen, introducing calcium or other divalent cations, exposing the reaction to a light detecting source, and taking light measurements over time. As such, any modification of an apoaequorin can be readily tested to determine the level of sustained light emission, thus allowing a determination as to whether the modified protein emits sustained light for a longer time than a corresponding unmodified aequorin, or allowing a determination that a further change to a glowing apoaequorin maintains the enhanced glowing activity, thus identifying the nucleotide or amino acid that is changed as non-essential, and the modified polypeptide as functionally equivalent to the modified apoaequorin.

As disclosed herein, the glowing apoaequorins of the invention comprise at least one amino acid mutation or deletion corresponding to amino acid residues 122 to 124 of the wild type apoaequorin as set forth in SEQ ID NO:2, except that when the modified apoaequorin has a mutation at or deletion of amino acid residue 124, it further contains a mutation or deletion of amino acid residue 122, amino acid 123, or both. For convenience of discussion, the modified glowing apoaequorins are described with respect to the corresponding wild type apoaequorin polypeptide having an amino acid sequence as set forth in SEQ ID NO:2 (encoded by SEQ ID NO:1). However, in view of the well known substantial microheterogenity exhibited among naturally occurring apoaequorins, it will be recognized that glowing apoaequorin polypeptides of the invention also can be based on or derived from any of the numerous naturally occurring variants of SEQ ID NO:2.

A modified apoaequorin polypeptide of the invention can have an amino acid sequence corresponding to (but modified with respect to positions 122 to 124, when present, as defined herein) a full length wild type apoaequorin, such as amino acid residues 1 to 196 of SEQ ID NO:2, or can comprise a functional fragment of SEQ ID NO:2, for example, a fragment comprising amino acid residues 1 to 121, or 1 to 122, or 1 to 123, or 1 to 124 of SEQ ID NO:2; amino acid residues 8 to 196 of SEQ ID NO:2; amino acid residues 8 to 121, or 8 to 122, or 8 to 123, or 8 to 124 of SEQ ID NO:2; amino acid residues 26 to 196 of SEQ ID NO:2; amino acid residues 26 to 124 of SEQ ID NO:2; or a deletion mutant such as comprising amino acid residues 1 to 121 operatively linked to residues 125 to 196 of SEQ ID NO:2, provided the fragment exhibits enhanced glowing kinetics. Such modified glowing apoaequorins are exemplified herein by the modified apoaequorin K2.1, which has an amino acid sequence as set forth in SEQ ID NO:4, or a functional fragment thereof, for example, a fragment comprising amino acid residues 1 to 121 of SEQ ID NO:4, or amino acid residues 8 to 171 of SEQ ID

NO:4, or amino acid residues 26 to 171 of SEQ ID NO:4; and by the glowing apoaequorin 3K2, which has an amino acid sequence as set forth in SEQ ID NO:8 or a functional fragment thereof, for example, a fragment comprising amino acid residues 1 to 122 of SEQ ID NO:8, amino acid residues 8 to 196 of SEQ ID NO:8, or amino acid residues 1 to 121 operatively linked to residues 125 to 196 of SEQ ID NO:8.

The apoaequorin flash element was determined to include at least amino acid residues 122 to 124 of SEQ ID NO:2, and a brightness element was localized to the C-terminal portion of apoaequorin beginning with about residue 124 (see Figure 4; see, also, U.S. Pat. No. 5,360,728). As such, a modified apoaequorin of the invention also can include a brightness element, which confers increased brightness on the glowing apoaequorin. For example, the glowing apoaequorin can include the brightness element comprising an amino acid sequence corresponding to amino acid residues 124 to 196 of SEQ ID NO:2, or a functional fragment thereof. It should be recognized that a single amino acid change at position 124 is sufficient to increase brightness of an aequorin photoprotein comprising amino acid residues 125 to 196 of a wild type apoaequorin, as indicated by the 3K2 modified apoaequorin (SEQ ID NO:8; see Figure 4; see, also, U.S. Pat. No. 5,369,728). In comparison, the S3 mutant apoaequorin, which, in addition to a mutation at amino acid residue 124, also contained a frame shift and, therefore, completely different C-terminus as compared to a corresponding wild type apoaequorin, exhibited somewhat less brightness than an aequorin photoprotein comprising the wild type apoaequorin (see Example 2).

As disclosed herein, the modified glowing apoaequorins of the invention are useful as a component of various compositions, and as a reagent in various assays. As such, a glowing apoaequorin polypeptide, or functional fragment thereof, can be operatively linked to a heterologous polypeptide to produce a fusion protein. As used herein, the term "operatively linked" or "operatively associated" means that two or more molecules are positioned with respect to each other such that they act as a single unit and effect a function attributable to one or both molecules or a combination thereof. For example, a polynucleotide encoding a glowing apoaequorin polypeptide can be operatively linked to a second (or more) coding sequence, such that a chimeric polypeptide can be expressed from the operatively linked coding sequences. The chimeric polypeptide can be a fusion protein, in which the two (or more) encoded polypeptides are translated into a single polypeptide, i.e., are covalently bound through a peptide bond; or can be translated as two discrete peptides that, upon translation, can operatively associate with each other to form a stable complex. Similarly, a polynucleotide sequence encoding a glowing apoaequorin of the invention can be operatively linked to a regulatory element, in which case the regulatory element confers its regulatory effect on the polynucleotide similarly to the way in which the regulatory element would effect a polynucleotide sequence with which it normally is associated with in a cell.

In addition, the term "operatively linked" is used to refer to a deletion mutant of an apoaequorin, wherein a first portion of the polypeptide is linked to a second part of the polypeptide. The term is used in such a manner because such a deletion mutant can be constructed, for example, by isolating or synthesizing a first polynucleotide sequence encoding amino acid residues 1 to 121 of SEQ ID NO:2 and linking it in frame with a second isolated or synthetic polynucleotide encoding residues 125 to 196 of SEQ ID NO:2, and because such molecules, which are not generally found in nature, can be considered heterologous with respect to each other. As such, a deletion mutant lacking, for example, amino acid residues corresponding to residues 122 to 124 of SEQ ID NO:2 is referred to as comprising amino acid residues 1 to 121 operatively linked to residues 125 to 196 of SEQ ID NO:2.

A fusion protein of the invention generally demonstrates some or all of the characteristics of each of its polypeptide components. As such, a chimeric polypeptide can be particularly useful in performing methods of the invention, as disclosed herein. For example, the fusion protein can include a glowing apoaequorin operatively linked to a cell compartment localization domain such that expression of the fusion protein in a cell or loading of the cell with fusion protein allows translocation of the apoaequorin to a desired intracellular compartment, wherein, upon reconstitution, it can glow in response to contact with a divalent metal ion such as calcium.

The heterologous polypeptide component of the fusion protein can be any polypeptide (or peptide) of interest, including, for example, a cell compartmentalization domain, a peptide that acts as a ligand for a receptor, a peptide useful as a tag for identifying or isolating the fusion protein, or a polypeptide that is naturally expressed in or secreted from a cell. Cell compartmentalization domains, for example, are well known and include a plasma membrane localization domain, a nuclear localization signal, a mitochondrial membrane localization signal, an endoplasmic reticulum localization signal, and the like, as well as signal peptides, which can direct secretion of a polypeptide from a cell (see, for example, Hancock et al., *EMBO J.* 10:4033-4039, 1991; Buss et al., *Mol. Cell. Biol.* 8:3960-3963, 1988; U.S. Pat. No. 5,776,689 each of which is incorporated herein by reference). Such a domain can be useful to target a glowing aequorin to a particular compartment in the cell, or to target the glowing aequorin for secretion from a cell.

A fusion protein comprising a modified glowing apoaequorin operatively linked to peptide tag such as a His-6 tag or the like, which can facilitate identification of expression of the agent in the target cell. A polyhistidine tag peptide such as His-6 can be detected using a divalent cation such as nickel ion, cobalt ion, or the like. Additional peptide tags include, for example, a FLAG epitope, which can be detected using an anti-FLAG antibody (see, for example, Hopp et al., *BioTechnology* 6:1204 (1988); U.S. Pat. No. 5,011,912, each of which is incorporated herein by reference); a c-myc epitope, which can be detected using an antibody specific for the epitope; biotin, which can be detected using streptavidin or avidin; and glutathione S-transferase, which can be detected using glutathione. As such, the peptide tags provide an alternative means of detecting the presence of an apoaequorin polypeptide, which, by itself (i.e., not a component of aequorin photoprotein), does not glow. Such tags provide the additional advantage that they can facilitate isolation of the operatively linked apoaequorin, or functional fragment thereof, or an aequorin photoprotein comprising the apoaequorin, for example, where it is desired to obtain a substantially purified glowing apoaequorin polypeptide or an isolated glowing aequorin photoprotein.

An glowing apoaequorin, or functional fragment thereof, or a glowing aequorin photoprotein, also can be a component of a novelty item. Such a use for apoaequorins is well known (see, for example, Intl. Appl. No. WO 01/68824). Use of a glowing aequorin can add significant enjoyment value to such items, including, for example, toys such as squirt guns, paint ball guns, balloons, and board games; clothes such as children's clothes, nightclub-wear, and costumes; foods and drinks such as gum, gelatins, cereals, sodas, and alcoholic beverages; and other items such as bath salts, paper and ink products, and body paints (see Intl. Appl. No. WO 01/68824).

The present invention also provides antibodies, or an antigen binding fragments thereof, that specifically bind a modified glowing apoaequorin. Such antibodies of the invention are characterized, in part, in that they do not substantially bind to a wild type apoaequorin. Furthermore, in one embodiment, the antibodies are characterized, in part, by an ability, when specifically bound to a glowing apoaequorin, to modulate the luminescent activity. As such, an antibody of the invention can

enhance or quench the glowing activity, including the length of time of glowing, the intensity (brightness) of the luminescence, or both.

As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. An antibody useful in
5 a method of the invention, or an antigen binding fragment thereof, is characterized, for example, by having specific binding activity for an epitope of a modified glowing apoaequorin. The term "binds specifically" or "specifically reacts" or the like, when used in reference to an antibody means that an interaction of the antibody and a particular epitope has a dissociation constant of at least about 1×10^{-6}, generally at least about 1×10^{-7}, usually at least about 1×10^{-8}, and particularly at least about 1×10^{-9} or
10 1×10^{-10} or less. As such, Fab, $F(ab')_2$, Fd and Fv fragments of an antibody that retain specific binding activity for an epitope of a glowing apoaequorin, are included within the definition of an antibody. For purposes of the present invention, an antibody that reacts specifically with an epitope of a glowing apoaequorin is considered to not substantially react with a wild type or other non-glowing apoaequorin if the antibody has at least a two-fold greater binding affinity, generally at least a five-fold greater
15 binding affinity, and particularly at least a ten-fold greater binding affinity for the glowing apoaequorin as compared to the non-glowing apoaequorin.

The term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-
20 naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains (see Huse et al., *Science* 246:1275-1281 (1989), which is incorporated herein by reference). These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in
25 the art (Winter and Harris, *Immunol. Today* 14:243-246, 1993; Ward et al., *Nature* 341:544-546, 1989; Harlow and Lane, *Antibodies: A laboratory manual* (Cold Spring Harbor Laboratory Press, 1988); Hilyard et al., *Protein Engineering: A practical approach* (IRL Press 1992); Borrabeck, *Antibody Engineering*, 2d ed. (Oxford University Press 1995); each of which is incorporated herein by reference).

Antibodies that bind specifically with a modified glowing apoaequorin can be raised using
30 the modified polypeptide as an immunogen and removing antibodies that crossreact, for example, with wild type apoaequorins, particularly that from which the modified glowing apoaequorin was derived, or using a peptide portion of the modified glowing apoaequorin that includes the modified amino acid residues. Where such a peptide is non-immunogenic, it can be made immunogenic by coupling the hapten to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin
35 (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art (see, for example, by Harlow and Lane, *supra*, 1988). If desired, a kit containing the modified glowing apoaequorin, as well as reagents such as a coelenterazine, or containing a reconstituted glowing aequorin photoprotein, or a polynucleotide encoding the glowing apoaequorin can be prepared, and can include such an antibody.
40 Accordingly, kits containing compositions of the invention are provided.

Methods for raising polyclonal antibodies, for example, in a rabbit, goat, mouse or other mammal, are well known in the art (see, for example, Green et al., "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed., Humana Press 1992), pages 1-5; Coligan et al., "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in *Curr. Protocols Immunol.*

(1992), section 2.4.1; each or which is incorporated herein by reference). In addition, monoclonal antibodies can be obtained using methods that are well known and routine in the art (see, for example, Kohler and Milstein, *Nature* 256:495, 1975, which is incorporated herein by reference; see, also, Coligan et al., *supra*, 1992, see sections 2.5.1-2.6.7; Harlow and Lane, *supra*, 1988). For example,
5 spleen cells from a mouse immunized with a glowing apoaequorin, or an epitopic fragment thereof, can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using labeled antigen to identify clones that secrete monoclonal antibodies having the appropriate specificity, and hybridomas expressing antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies.
10 Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well established techniques, including, for example, affinity chromatography with Protein-A SEPHAROSE, size exclusion chromatography, and ion exchange chromatography (Coligan et al., *supra*, 1992, see sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; see, also, Barnes et al., "Purification of Immunoglobulin G (IgG)," in *Meth. Molec. Biol.* 10:79-104 (Humana Press 1992), which is incorporated herein by
15 reference). The antibodies can be further screened for the inability to bind specifically with non-glowing apoaequorins. Such monoclonal antibodies can be useful, for example, for preparing standardized kits containing glowing apoaequorins.

Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human
20 variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are known (see, for example, Orlandi et al., *Proc. Natl. Acad. Sci., USA* 86:3833, 1989, which is hereby incorporated in its entirety by reference).
25 Techniques for producing humanized monoclonal antibodies also are known (see, for example, Jones et al., *Nature* 321:522, 1986; Riechmann et al., *Nature* 332:323, 1988; Verhoeyen et al., *Science* 239:1534, 1988; Carter et al., *Proc. Natl. Acad. Sci., USA* 89:4285, 1992; Sandhu, *Crit. Rev. Biotechnol.* 12:437, 1992; and Singer et al., *J. Immunol.* 150:2844, 1993; each of which is incorporated herein by reference).

30 Antibodies of the invention also can be derived from human antibody fragments isolated from a combinatorial immunoglobulin library (see, for example, Barbas et al., *METHODS: A Companion to Methods in Immunology* 2:119, 1991; Winter et al., *Ann. Rev. Immunol.* 12:433, 1994; each of which is incorporated herein by reference). Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from Stratagene
35 Cloning Systems (La Jolla, CA). An antibody of the invention also can be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic
40 mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green et al., *Nature Genet.* 7:13, 1994; Lonberg et al., *Nature* 368:856, 1994; and Taylor et al., *Intl. Immunol.* 6:579, 1994; each of which is incorporated herein by reference, and can be obtained from commercial sources (for example, Abgenix Inc.; Fremont CA).

Antibody fragments can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$.
5 This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see, for example, Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, each of which is incorporated by reference, and references contained therein; Nisonhoff et al., *Arch.*
10 *Biochem. Biophys.* 89:230. 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman et al., *Meth. Enzymol.*, 1:422 (Academic Press 1967), each of which is incorporated herein by reference; see, also, Coligan et al., *supra*, 1992, see sections 2.8.1-2.8.10 and 2.10.1-2.10.4; see, further, Whitlow et al., *METHODS: A Companion to Methods in Enzymology* 2:97, 1991; Bird et al., *Science* 242:423-426, 1988; Ladner et al., U.S. patent No. 4,946,778; Pack et al., *BioTechnology* 11:1271-1277, 1993; each of which is
15 incorporated herein by reference; for preparing Fv fragments).

The present invention also relates to polynucleotides that encode a modified apoaequorin polypeptide having enhanced glowing kinetics, or a functional fragment of the glowing apoaequorin, as well as to polynucleotides that encode two or more polypeptides, including the glowing apoaequorin and one or more other proteins, and polynucleotides that encode a fusion protein comprising the
20 glowing apoaequorin. Such polynucleotides of the invention are exemplified by those set forth in SEQ ID NO:3 or SEQ ID NO:7. The term "polynucleotide" is used broadly herein to mean a sequence of two or more deoxyribonucleotides or ribonucleotides that are linked together by a phosphodiester bond. As such, the term "polynucleotide" includes RNA and DNA, which can be a gene or a portion thereof, a cDNA, a synthetic polydeoxyribonucleic acid sequence, or the like, and can be single stranded or
25 double stranded, as well as a DNA/RNA hybrid. Furthermore, the term "polynucleotide" as used herein includes naturally occurring nucleic acid molecules, which can be isolated from a cell, as well as synthetic molecules, which can be prepared, for example, by methods of chemical synthesis or by enzymatic methods such as by the polymerase chain reaction (PCR). In various embodiments, a polynucleotide of the invention can contain nucleoside or nucleotide analogs, or a backbone bond other
30 than a phosphodiester bond, for example, a thiodiester bond, a phosphorothioate bond, a peptide-like bond or any other bond known to those in the art as useful for linking nucleotides to produce synthetic polynucleotides (see, for example, Tam et al., *Nucl. Acids Res.* 22:977-986 (1994); Ecker and Crooke, *BioTechnology* 13:351360 (1995), each of which is incorporated herein by reference). The incorporation of non-naturally occurring nucleotide analogs or bonds linking the nucleotides or analogs
35 can be particularly useful where the polynucleotide is to be exposed to an environment that can contain a nucleolytic activity, including, for example, a tissue culture medium, a cell or in a living subject, since the modified polynucleotides can be less susceptible to degradation.

In general, the nucleotides comprising a polynucleotide are naturally occurring deoxyribonucleotides, such as adenine, cytosine, guanine or thymine linked to 2'-deoxyribose, or
40 ribonucleotides such as adenine, cytosine, guanine or uracil linked to ribose. However, a polynucleotide also can contain nucleotide analogs, including non-naturally occurring synthetic nucleotides or modified naturally occurring nucleotides. Such nucleotide analogs are well known in the art and commercially available, as are polynucleotides containing such nucleotide analogs (Lin et al., *Nucl. Acids Res.* 22:5220-5234 (1994); Jellinek et al., *Biochemistry* 34:11363-11372 (1995); Pagratis et
45 al., *Nature Biotechnol.* 15:68-73 (1997), each of which is incorporated herein by reference).

A polynucleotide comprising naturally occurring nucleotides and phosphodiester bonds can be chemically synthesized or can be produced using recombinant DNA methods, using an appropriate polynucleotide as a template. In comparison, a polynucleotide comprising nucleotide analogs or covalent bonds other than phosphodiester bonds generally will be chemically synthesized, although an enzyme such as T7 polymerase can incorporate certain types of nucleotide analogs into a polynucleotide and, therefore, can be used to produce such a polynucleotide recombinantly from an appropriate template (Jellinek et al., *supra,* 1995).

A polynucleotide of the invention can be contained in a vector, which can facilitate manipulation of the polynucleotide, including introduction of the polynucleotide into a target cell. The vector can be a cloning vector, which is useful for maintaining the polynucleotide, or can be an expression vector, which contains, in addition to the polynucleotide, regulatory elements useful for expressing the polynucleotide and encoded glowing apoaequorin in a particular cell. An expression vector can contain the expression elements necessary to achieve, for example, sustained transcription of the encoding polynucleotide, or the regulatory elements can be operatively linked to the polynucleotide prior to its being cloned into the vector.

An expression vector (or the polynucleotide) generally contains or encodes a promoter sequence, which can provide constitutive or, if desired, inducible or tissue specific or developmental stage specific expression of the encoding polynucleotide, a poly-A recognition sequence, and a ribosome recognition site or internal ribosome entry site, or other regulatory elements such as an enhancer, which can be tissue specific. The vector also can contain elements required for replication in a prokaryotic or eukaryotic host system or both, as desired. Such vectors, which include plasmid vectors and viral vectors such as bacteriophage, baculovirus, retrovirus, lentivirus, adenovirus, vaccinia virus, semliki forest virus and adeno-associated virus vectors, are well known and can be purchased from a commercial source (Promega, Madison WI; Stratagene, La Jolla CA; GIBCO/BRL, Gaithersburg MD) or can be constructed by one skilled in the art (see, for example, *Meth. Enzymol.*, Vol. 185, Goeddel, ed. (Academic Press, Inc., 1990); Jolly, *Canc. Gene Ther.* 1:51-64, 1994; Flotte, *J. Bioenerg. Biomemb.* 25:37-42, 1993; Kirshenbaum et al., *J. Clin. Invest.* 92:381-387, 1993; each of which is incorporated herein by reference).

A tetracycline (tet) inducible promoter can be particularly useful for driving expression of a polynucleotide of the invention. Upon administration of tetracycline, or a tetracycline analog, to a subject containing a polynucleotide operatively linked to a tet inducible promoter, expression of the encoded glowing apoaequorin is induced. The polynucleotide also can be operatively linked to tissue specific regulatory element, for example, a muscle cell specific regulatory element, such that expression of the glowing apoaequorin is restricted to the muscle cells in an individual, or to muscle cells in a mixed population of cells in culture, for example, an organ culture. Muscle cell specific regulatory elements including, for example, the muscle creatine kinase promoter (Sternberg et al., *Mol. Cell. Biol.* 8:2896-2909, 1988, which is incorporated herein by reference) and the myosin light chain enhancer/promoter (Donoghue et al., *Proc. Natl. Acad. Sci., USA* 88:5847-5851, 1991, which is incorporated herein by reference) are well known in the art. Furthermore, the apoaequorin can be a fusion protein that includes a cell compartmentalization domain that directs translocation of the expressed polypeptide to sarcoplasmic reticulum in the muscle cells, thus providing a system useful for examining calcium ion flux in muscle cells, for example, in response to a muscle contracting stimulus. By including such components together, kits can be developed that are useful for specific or more general studies using methods as disclosed herein.

Viral expression vectors can be particularly useful for introducing a polynucleotide into a cell, particularly a cell in a subject. Viral vectors provide the advantage that they can infect host cells with relatively high efficiency and can infect specific cell types. For example, a polynucleotide encoding a glowing apoaequorin, or functional fragment thereof, can be cloned into a baculovirus vector, which then can be used to infect an insect host cell, thereby providing a means to produce large amounts of the encoded polypeptide. The viral vector also can be derived from a virus that infects cells of an organism of interest, for example, vertebrate host cells such as mammalian, avian or piscine host cells. Viral vectors can be particularly useful for introducing a polynucleotide useful in performing a method of the invention into a target cell. Viral vectors have been developed for use in particular host systems, particularly mammalian systems and include, for example, retroviral vectors, other lentivirus vectors such as those based on the human immunodeficiency virus (HIV), adenovirus vectors, adeno-associated virus vectors, herpesvirus vectors, vaccinia virus vectors, and the like (see Miller and Rosman, *BioTechniques* 7:980-990, 1992; Anderson et al., *Nature* 392:25-30 Suppl., 1998; Verma and Somia, *Nature* 389:239-242, 1997; Wilson, *New Engl. J. Med.* 334:1185-1187 (1996), each of which is incorporated herein by reference).

A polynucleotide, which can be contained in a vector, can be introduced into a cell by any of a variety of methods known in the art (Sambrook et al., *Molecular Cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press 1989); Ausubel et al., *Current Protocols in Molecular Biology,* John Wiley and Sons, Baltimore, MD (1987, and supplements through 1995), each of which is incorporated herein by reference). Such methods include, for example, transfection, lipofection, microinjection, electroporation and, with viral vectors, infection; and can include the use of liposomes, microemulsions or the like, which can facilitate introduction of the polynucleotide into the cell and can protect the polynucleotide from degradation prior to its introduction into the cell. The selection of a particular method will depend, for example, on the cell into which the polynucleotide is to be introduced, as well as whether the cell is isolated in culture, or is in a tissue or organ in culture or *in situ*.

Introduction of a polynucleotide into a cell by infection with a viral vector is particularly advantageous in that it can efficiently introduce the nucleic acid molecule into a cell *ex vivo* or *in vivo* (see, for example, U.S. Pat. No. 5,399,346, which is incorporated herein by reference). Moreover, viruses are very specialized and can be selected as vectors based on an ability to infect and propagate in one or a few specific cell types. Thus, their natural specificity can be used to target the nucleic acid molecule contained in the vector to specific cell types. As such, a vector based on an HIV can be used to infect T cells, a vector based on an adenovirus can be used, for example, to infect respiratory epithelial cells, a vector based on a herpesvirus can be used to infect neuronal cells, and the like. Other vectors, such as adeno-associated viruses can have greater host cell range and, therefore, can be used to infect various cell types, although viral or non-viral vectors also can be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A polynucleotide of the invention, or a vector containing the polynucleotide can be contained in a cell, for example, a host cell, which allows propagation of a vector containing the polynucleotide, or a helper cell, which allows packaging of a viral vector containing the polynucleotide. The polynucleotide can be transiently contained in the cell, or can be stably maintained due, for example, to integration into the cell genome or other stably maintained nucleic acid in the cell, e.g., mitochondrial DNA or chloroplast DNA.

As such, the present invention also provides a transgenic non-human organism, which comprises cells containing a polynucleotide encoding a glowing apoaequorin stably maintained therein.

The transgenic non-human organism can be a transgenic animal, including an invertebrate or vertebrate animal, or can be a transgenic plant, and the polynucleotide encoding the glowing apoaequorin can be expressed, either constitutively, inducibly, or in a developmental stage specific manner, in one particular cell type, i.e., in a tissue specific manner, or in a variety of cell types, including in all of the
5 cells of the transgenic non-human organism.

Various methods are known for producing a transgenic plant or a transgenic animal. For example, a transgenic non-human animal can be prepared beginning with an embryo at the pronuclear stage (a "one cell embryo") harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into the germ cells and somatic cells of
10 the resulting mature animal. In another method, embryonic stem cells are isolated and the transgene is incorporated into the stem cells by electroporation, plasmid transfection or microinjection; the stem cells are then reintroduced into the embryo, where they colonize and contribute to the germ line. Methods for microinjection of polynucleotides into mammalian species are described, for example, in U.S. Pat. No. 4,873,191, which is incorporated herein by reference. In yet another method, embryonic
15 animal cells are infected with a retrovirus containing the transgene, whereby the germ cells of the embryo have the transgene chromosomally integrated therein.

In the retroviral infection method, the developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, *Proc. Natl. Acad. Sci, USA* 73:1260-1264, 1976). Efficient infection of the blastomeres is
20 obtained by enzymatic treatment to remove the zona pellucida (Hogan et al., *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, 1986). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner_et al., *Proc. Natl. Acad. Sci., USA* 82:6927-6931, 1985; Van der Putten et al., *Proc. Natl. Acad. Sci, USA* 82:6148-6152, 1985). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of
25 virus producing cells (Van der Putten et al., *supra*, 1985; Stewart et al., *EMBO J.* 6:383-388, 1987). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al., *Nature* 298:623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic nonhuman animal. Further, the founder can contain various retroviral insertions of the transgene at
30 different positions in the genome, which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the mid-gestation embryo (Jahner et al., *supra*, 1982).

Embryonal stem cell (ES) also can be targeted for introduction of the transgene. ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al. *Nature*
35 292:154-156, 1981; Bradley et al., *Nature* 309:255-258, 1984; Gossler et al., *Proc. Natl. Acad. Sci., USA* 83:9065-9069, 1986; Robertson et al., *Nature* 322:445-448, 1986). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a nonhuman animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal
40 (see Jaenisch, *Science* 240:1468-1474, 1988).

The present invention also provides a process for producing a modified apoaequorin polypeptide having enhanced glowing kinetics. In one embodiment, a process of the invention is practiced by expressing a polynucleotide encoding the glowing apoaequorin, for example, in a coupled transcription/translation reaction *in vitro*, or in a cell containing the polynucleotide. In another

embodiment, a process of producing a modified apoaequorin polypeptide having enhanced glowing kinetics is performed by mutating, either randomly, in a site-specific manner, or a combination thereof, a wild type apoaequorin polynucleotide, expressing mutant polynucleotides generated therefrom, identifying polynucleotides that encode a glowing apoaequorin, and producing the modified glowing apoaequorin from the mutated polynucleotide. In addition, the method can further include a step of isolating the expressed glowing apoaequorin polypeptide. Accordingly, the present invention provides a modified apoaequorin polypeptide produced by such a method.

In addition, the invention provides a method of producing an aequorin photoprotein, which has enhanced glowing kinetics as compared to a corresponding wild type aequorin photoprotein. Such a method can be performed, for example, by contacting the modified glowing apoaequorin with a luciferin, particularly with coelenterazine or an analog thereof, under conditions sufficient for reconstituting an aequorin photoprotein. Such conditions can include, for example, contacting the modified apoaequorin polypeptide and the coelenterazine in the presence of molecular oxygen. Such conditions also can include, where appropriate, contacting the modified apoaequorin polypeptide and the coelenterazine in the presence of a reducing agent such as é-mercaptoethanol (see U.S. Pat. No. 5,093,240). The method of producing a glowing aequorin photoprotein can further include a step of isolating the glowing aequorin photoprotein. Accordingly, the present invention also provides a modified aequorin photoprotein produced by a such a method.

Use of the modified glowing aequorins of the invention provides substantial advantages over the use of wild type and previously described aequorins in that the flash reaction of the latter aequorins have a light emission duration of approximately 2 seconds and, therefore, is often complete before a sample can be analyzed. Use of a glowing aequorin in the methods as disclosed herein allows for the addition of multiple test agents, for example, while still providing sufficient time for a sustained luminescent read time. Thus, unlike naturally occurring and previously described modified aequorins, use of a glowing aequorin of the invention does not require specialized equipments such as an injectable luminometer to trigger the reaction and quickly read the light emitted in the sample. As such, the glowing aequorins of the invention are useful, for example, for detecting divalent metal ions such as calcium, strontium, magnesium, and the like in a sample, or to identify drug that can effect calcium ion flux in a cell.

Methods for using a glowing apoaequorin, or encoding polynucleotide, to identify the presence of divalent cations, including calcium ions, strontium ions, and the like, in a sample, or to identify changes in cation concentration, which can be indicative, for example, of a physiologic condition of or toxicologic exposure to a cell, also are provided. As disclosed herein, the methods of using a glowing apoaequorin polypeptide or an aequorin photoprotein reconstituted therefrom, or an encoding polynucleotide, can be an *in vitro* assay, for example, an enzyme-linked immunosorbent assay (ELISA), a lateral flow assay, or a post-polymerase chain reaction (PCR) assay; or can be a cell based assay, for example, a calcium influx assay. As such, the compositions are useful for functional biological assays, including, for example, muscle contraction assays, fertilization assays, GPCR assays, gap junction regulation assays, complement fixation/cytolysis assays, macrophage activation assays, oxidative burst/free radical assays, and apoptosis assays, as well as assays that detect fusions of membranes, for example, fusions of liposomes with each other or with target cells, and fusions of cells as can occur due to a viral infection; as well as for environmental, toxicological and forensic assays to detect the presence of a divalent cation in a sample, for example, to detect the presence of a contaminant such as strontium ion in a food or water sample.

Accordingly, the invention provides methods of detecting the presence of divalent cations in a sample. Such a method can be performed, for example, by contacting the sample with a composition containing a modified glowing apoaequorin, or functional fragment thereof, under conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, and examining the sample for such luminescent activity, wherein detection of luminescent activity is indicative of the presence of divalent cations in the sample. As used herein, reference to a method of the invention being performed "under conditions sufficient for luminescent activity" means that the apoaequorin is in, or can be reconstituted to be in, the form of an aequorin photoprotein such that, upon contact with an appropriate divalent metal ion such as calcium, luminescence occurs due to conversion, for example, coelenterazine to coelenteramide, and emission of light. As such, the method can be performed by contacting the sample with the glowing apoaequorin and with a coelenterazine, or analog thereof, such that an aequorin photoprotein is generated, or the aequorin photoprotein can be reconstituted prior to contacting the composition with the sample.

Luminescent activity mediated by the apoaequorin can be detected using any method as disclosed herein (see Examples 1 to 3), or otherwise known in the art and include, for example, visual examination, use of a photographic film or emulsion, or use of a luminometer, which can, but need not, be filtered to allow transmission only of light centered at about 469 nm. If desired, the luminescence also can be detected indirectly, for example, by further contacting a sample containing the aequorin photoprotein with a green fluorescent protein (GFP) such that the luminescence energy is transferred to the GFP, which then emits a fluorescent signal, which can be detected using a fluorimeter or other appropriate detector (see Intl. Publ. No. WO 01/68824).

The sample can be any sample containing or suspected of containing divalent cations that can activate the glowing aequorin photoprotein, including a water or other aqueous sample, which can be examined, for example, for strontium contamination; or a food, which can be examined for the presence of a heavy metal ion. In addition, the sample can be a biological sample, for example, a sample of a plant that is to be used as a foodstuff, or a sample of cell, tissue, or organ of an organism, including, for example, an extract or other fraction of such a biological sample.

The present invention also relates to a method of identifying an agent that effects divalent cation flux, particularly calcium ion flux, in a sample comprising at least two compartments, wherein at least one compartment contains the cations. As such, the method is useful, for example, as a drug screening assay, which can be formatted for high throughput analysis. A method of the invention can be performed, for example, by contacting a compartment of the sample with a composition comprising a glowing apoaequorin polypeptide, or functional fragment thereof, under conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, examining the sample for such luminescent activity, and contacting the sample with a test agent, whereby a change in said luminescent activity identifies the test agent as an agent that effects the cation flux.

As used herein, the term "compartment" is used broadly herein to refer to a defined portion of a particular sample. As such, the term is generally used in a relative sense to refer to at least one compartment of a sample containing at least two compartments. For example, a solution containing solutes can be considered to comprise at least two compartments, including the aqueous (solvent) compartment and the solute compartment. In addition, a solution separated by a selectively permeable barrier, particularly a barrier that is selectively permeable to divalent cations, can be considered to comprise at least two compartments, including one on each side of the barrier, which can be considered a third compartment, if desired. A cell is a particular example of a sample comprising compartments,

including intracellular and extracellular compartments, and further comprising two or more intracellular compartments.

For purposes of the methods of the invention, one compartment of a sample containing two or more compartments generally contains a higher divalent cation concentration that at least one other compartment, which generally either lacks divalent cations or has a concentration of cations that is not sufficient to activate an apoaequorin, e.g., less than about 10^{-6} M, usually less than about 10^{-7} M, and particularly less than about 10^{-8} M. However, a method of the invention also can be performed where, for example, both compartments of a two compartment sample contain sufficient concentrations of divalent cations to activate a glowing apoaequorin, but wherein the apoaequorin is restricted to only one of the compartments, thus providing a means to detect movement of the divalent metal ion out of the compartment containing the apoaequorin by detecting a decrease in luminescent activity.

The compartment that is contacted with the modified apoaequorin, or functional fragment thereof, can be a compartment containing calcium ions, strontium ions, or the like, in which case, an agent that effects the cation flux is identified by detecting a decrease in luminescent activity due to efflux of the cation out of the compartment; or can be a compartment that lacks a sufficient cation concentration to generate luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, in which case, an agent that effects the divalent cation flux is identified by an increase in luminescent activity due to influx of the cation into the compartment. Such a method, which also allows the detection, for example, of recurring calcium ion flux into and out of a compartment, thus provides a means for real time analysis of calcium movement in and out of cells, between cells, or among intracellular compartments.

The methods of the invention provide a means to identify an agent that effects divalent cation flux. As used herein, the term "effect" means that the agent causes or influences the cation movement. Generally, the agent effects the cation movement indirectly, for example, causing a conformational change in a cell channel protein such as a calcium channel so that movement of calcium through the channel is increased or decreased. However, an understanding of the mechanism by which an agent effects divalent cation flux is not necessary to practice the methods of the invention.

A method of detecting an agent that effects divalent cation flux can be performed using a sample that includes a first compartment containing bound divalent cations and a second compartment having an insufficient cation concentration to generate luminescent activity mediated by the glowing apoaequorin, or functional fragment thereof. For example, the sample can be an aqueous solution, including a first compartment comprising calcium ions bound to a chelating compound such as EDTA or EGTA, and a second compartment comprising the aqueous medium, which contains a glowing aequorin photoprotein. Upon contact of the sample with a test agent, detection of luminescent activity identifies the test agent as an agent that results in release of calcium ions from the chelating agent, thus effecting calcium ion flux. Similarly, the method can be used to determine that a toxic divalent cation, for example, a heavy metal ion is chelated out of otherwise potable water by detecting a decrease in luminescent activity due to chelation of the cation, thus confirming detoxification of the water.

A method of the invention also can be performed using a sample comprising a cell, which can be a prokaryotic cell such as a bacterium, or a eukaryotic cell such as a plant cell, a cell of an invertebrate, or a cell of a vertebrate, for example, a mammalian cell. As such, the cell can delineate at least two compartments, including an intracellular compartment and an extracellular compartment, one of which contains, for example, calcium ions and one of which lacks calcium ions or has an insufficient

concentration of calcium ions to generate the luminescent activity. As such, the method can be useful, for example, to identify an agent that effects calcium influx from the extracellular compartment into the cell or, conversely, that effects calcium efflux out of the cell. Similarly, the first compartment can be a first cell and the second compartment can be a second cell, wherein the method provides a means to
5 detect intercellular flux of a divalent metal ion, for example, from the first cell to the second cell.

The method also can be performed using a cell, wherein the compartments includes at least one intracellular compartment containing calcium ions and at least one intracellular compartment having an insufficient calcium ion concentration to generate luminescent activity mediated by the modified apoaequorin, or functional fragment thereof. The intracellular compartment containing
10 calcium ions can be any intracellular compartment known to store calcium ions, including, for example, endoplasmic reticulum, sarcoplasmic reticulum in muscle cells, mitochondria, secretory vesicles such as the neuropeptide-containing dense core secretory vesicle present in neuronal cells, or a plant vacuole.

An agent that effects calcium ion flux in a cell can act directly by specifically binding to a calcium channel protein, thereby modulating the activity of the channel, or can act by specifically
15 binding a receptor that is linked to a pathway involving calcium ion flux. For example, the agent can effect calcium flux in a cell by binding specifically to a G-protein coupled receptor (GPCR) such as a thrombin receptor, an É-adrenergic receptor, or a é-adrenergic receptor, thus modulating a G-protein mediated signal transduction pathway associated with calcium ion flux. As such, the method provides a means to identify an agent that can act as an agonist or a partial agonist for a GPCR, or as an antagonist
20 for the GPCR.

The GPCR superfamily represents the largest class of cell surface receptors. Activation of GPCRs leads to the activation of various G-protein mediated signal transduction pathways, and generation of second messengers such as cyclic AMP and calcium ions. As such, measurement of the second messengers, such as the level of calcium ion released into the cytoplasm, often is used to
25 determine whether a GPCR has been activated, for example, in assays for identifying whether an agent acts as an agonist, antagonist, or the like, of the GPCR and, therefore, of the G-protein mediated signal transduction pathway regulated by the GPCR. The glowing aequorins of the invention are particularly useful in such screening assays because, as disclosed herein, they allow for the use of readily available equipment and instrumentation for identifying agents that specifically bind a GPCR.

30 Accordingly, the screening methods of the invention conveniently can be adapted to high throughput format, thus providing a means to examine a plurality of samples or agents or both in a single assay. As such, the method can be performed using a plurality of samples, each of which is the same, and contacting the samples with a plurality of test agents, which can be the same or different or a combination thereof, e.g., triplicates of a plurality of different test agents. Alternatively, or in addition,
35 the samples can comprise a plurality of different samples. Where the method is performed in a high throughput format, the samples generally are arranged in an array, which can be an addressable array, on a microchip, a glass slide, or a plastic well.

The test agents can be peptides, peptidomimetics, polynucleotides, small organic molecules, or the like, and can be obtained from naturally occurring sources; can be synthesized using, for
40 example, a combinatorial method for generating libraries of molecules, which can be random, biased, or variegated; or can be derived from a natural or known material and modified to identify derivatives having more desirable characteristics. Methods for preparing a combinatorial library of molecules that can be tested for a desired activity are well known in the art and include, for example, methods of making a

phage display library of peptides, which can be constrained peptides (see, for example, U.S. Pat. No. 5,622,699; U.S. Pat. No. 5,206,347; Scott and Smith, Science 249:386-390, 1992; Markland et al., *Gene* 109:13-19, 1991; each of which is incorporated herein by reference); a peptide library (U.S. Pat. No. 5,264,563, which is incorporated herein by reference); a peptidomimetic library (Blondelle et al., *Trends Anal. Chem.* 14:83-92, 1995; a nucleic acid library (O'Connell et al., *Proc. Natl. Acad. Sci., USA* 93:5883-5887, 1996; Tuerk and Gold, *Science* 249:505-510, 1990; Gold et al., *Ann. Rev. Biochem.* 64:763-797, 1995; each of which is incorporated herein by reference); an oligosaccharide library (York et al., *Carb. Res.*, 285:99-128, 1996; Liang et al., *Science*, 274:1520-1522, 1996; Ding et al., *Adv. Expt. Med. Biol.*, 376:261-269, 1995; each of which is incorporated herein by reference); a lipoprotein library (de Kruif et al., *FEBS Lett.*, 399:232-236, 1996, which is incorporated herein by reference); a glycoprotein or glycolipid library (Karaoglu et al., *J. Cell Biol.*, 130:567-577, 1995, which is incorporated herein by reference); or a chemical library containing, for example, drugs or other pharmaceutical agents (Gordon et al., J. Med. Chem., 37:1385-1401, 1994; Ecker and Crooke, *BioTechnology*, 13:351-360, 1995; each of which is incorporated herein by reference). Polynucleotides can be particularly useful as agents that can modulate a specific interaction of myostatin and its receptor because nucleic acid molecules having binding specificity for cellular targets, including cellular polypeptides, exist naturally, and because synthetic molecules having such specificity can be readily prepared and identified (see, for example, U.S. Pat. No. 5,750,342, which is incorporated herein by reference).

The present invention also relates to a method of detecting a functional change associated with divalent cation flux across a membrane of a cell. Such a method can be performed, for example, subjecting a cell containing intracellular modified glowing apoaequorin polypeptide, or functional fragment thereof, under conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, to a stimulus, and examining the cell for said luminescent activity, whereby a change in said luminescent activity identifies a functional change associated with calcium ion flux across a cell membrane of a cell. The glowing apoaequorin can be loaded into the cells as a polypeptide (or as a glowing aequorin photoprotein) using, for example, a permeabilization method such as hypoosmotic shock (see, for example, Wu et al., *Clin. Med. Sci, J.* 11:49-52, 1996, which is incorporated herein by reference), a chemical method (see, for example, Urthaler et al., *Cardiovasc. Res.* 28:40-46, 1994, which is incorporated herein by reference), macroinjection into tissue (see, for example, Perreault et al., *Proc. Soc. Exp. Biol. Med.* 199:178-182, 1992, which is incorporated herein by reference), or a microinjection method, or can be expressed from an encoding polynucleotide that is transfected, transformed, transduced, or the like into the cell. The cell containing the modified glowing apoaequorin, or functional fragment thereof, also can be a progeny of such a genetically modified cell, or can be a cell obtained from a transgenic organism of the invention.

The functional assays of the invention can be utilized to identify various physiological changes of a cell, including measuring changes that generally occur in a specific cell type, for example, muscle contraction, or functions that are altered due, for example, to a physical, chemical or biological insult to a cell, or an organism containing the cell. For example, the cell can be an oocyte, which is contacted with sperm such as in an *in vitro* fertilization procedure, whereby a change in luminescent activity identifies calcium influx into the oocyte, which is indicative of fertilization of the oocyte. The cell also can be a bacterial cell, which is contacted with complement fixing antibodies specific for an epitope of the bacterial cell, whereby a change in luminescent activity is indicative of loss of selective permeability of the bacterial cell wall and of cytolysis of the bacterial cell. By way of another example, the cell can be a mammalian cell that is exposed, or suspected of being exposed to a physical, chemical,

or biological material that can induce apoptosis of a cell, whereby a change in luminescent activity is indicative of loss of selective permeability of the cell membrane and of apoptosis of the mammalian cell.

The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1
CHARACTERIZATION OF WILD TYPE AEQUORIN LUMINESCENCE

This example demonstrates that wild type aequorin luminescence occurs quickly after calcium activation and decays rapidly.

To determine if transfected cells that express wild type aequorin could be used to identify calcium release from intracellular stores into the cytoplasm during GPCR stimulation (see Figure 1), CHO cells, which endogenously express a thrombin GPCR were transfected with a plasmid containing a cDNA encoding apoaequorin (SEQ ID NO:1). The transfected cells were charged with coelenterazine and plated in a 96 well plate. Either thrombin diluted in a carrier solution, or the carrier solution alone (control), was injected into wells containing the cells, and light was measured over a period of time up to 120 seconds.

Thrombin, but not the carrier solution, interacted with the cells, resulting in increased levels of cytoplasmic calcium as reported by activation of the wild type aequorin. The maximal light output due to aequorin activation occurred 7 to 9 seconds after the addition of the thrombin. As a control, TRITON-X100 detergent was added to each well 60 seconds after the addition of the thrombin to disrupt the cell membranes, thus allowing the influx of calcium and contact with the cytoplasmic aequorin. The signal following the detergent addition was less in the cells exposed to thrombin relative to those cells that were not exposed to thrombin.

These results indicate that calcium released from intracellular stores triggered the light signal following thrombin treatment, and that the calcium was scavenged by the aequorin. Furthermore, based on the demonstration that the transduced signal peaked, on average, only about 8 seconds after the addition of thrombin, it was determined that the light emission kinetics of wild type aequorin were unsuitable for high throughput analysis.

EXAMPLE 2
PREPARATION AND CHARACTERIZATION OF MODIFIED AEQUORINS

This example provides a mutagenesis and screening method used to generate mutants of aequorin with glowing kinetics.

Aequorin mutants were developed using polymerase chain reaction (PCR) primers designed to modify specific nucleotide sequences. A combination of site directed and random mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene Cloning Systems; La Jolla CA) with increased concentrations of dNTPs to facilitate the production of random mutations. Mutagenic primers were designed to alter the wild type aequorin 1 cDNA sequence (SEQ ID NO:1) such that the aspartic acid in position 124 was changed to a basic residue (lysine), and random mutations, deletions and insertions were introduced by increasing the concentration of the dNTPs. The mutagenic primers were used according to the kit manual, except that the reaction was modified to include 2-fold more dNTPs, to amplify the entire sequence of the vector containing the coding region for aequorin 1, while

incorporating the specific single nucleic acid changes at the 124 amino acid site and random mutations throughout the cDNA.

The resulting PCR products were verified by 1% agarose gel, digested with Dpn I to remove parental template DNA, transformed into Top 10 cells (Invitrogen Corp.; Carlsbad CA) and plated onto LB plates containing 100 µg/ml ampicillin for selection. Plates were incubated for 18 hr at 37°C, then colonies where transferred to a nitrocellulose membrane and placed over a solution of 10 mM IPTG for 4 hr at 37°C to induce protein expression. The membranes were then transferred to a solution of coelenterazine (a luciferin) to allow charging of the apoaqeuorin overnight at 4°C. The membranes were then air-dried and placed between two sheets of clear plastic film with a paper towel backing. A solution of calcium was injected, and the membrane was immediately exposed to autoradiographic film for 15 sec to identify clones with bioluminescent activity. Following a 5 min rest period, the membranes were exposed to an additional piece of autoradiographic film for 15 sec to identify clones with sustained biolumenscent glow activity. Clones, including those that maintained light activity 2 min after triggering with calcium, were selected and the nucleotide sequence and deduced amino acid sequence were determined.

Transformed cells expressing two of the clones, AqLtK2.1 (K2.1; SEQ ID NO:4), which glowed, and AqLtS3 (S3; SEQ ID NO:6), which did not glow, or expressing wild type aequorin (SEQ ID NO:2) were grown in 1 ml LB medium containing 100 µg/ml ampicillin for 2 hr at 37°C with shaking. Expression was induced for 2 hr with 20 mM IPTG, then cultures were pelleted at 5000 rpm. Cell pellets were resuspended in 200 µl PBS, 10 mM EDTA, 2.5 mM é-mercaptoethanol, and sonicated for 10 sec on the lowest setting. Lysates were charged with 50 µM coelenterazine and oxygen at 4°C for 3 hr, then cell debris was pelleted and removed. For each sample, 10 µl of the lysate was added to a white microplate, and a 5 min kinetic curve was read on each sample upon addition of Ca^{++} using a microplate luminometer (Luminoskan).

Nucleotide sequence analysis revealed that the K2.1 nucleotide sequence (SEQ ID NO:3) contained a single thymine deletion at position 365 (nucleotide 1 is adenine in the start codon), which predicted an altered reading frame and a protein truncated at the C-terminus as compared to wild type aequorin (see Figure 2; SEQ ID NO:4). In comparison, the S3 nucleotide sequence (SEQ ID NO:5) had a single nucleotide deletion at position 370, five nucleotides downstream of that in the sequence encoding the K2.1 glowing apoaequorin. Comparative kinetic analysis revealed that, although K2.1 had approximately 40 fold less maximal light emission relative to wild type aequorin and 2 fold more maximal light than the modified S3 aequorin, K2.1 glowed for nearly 3 min longer than wild type and S3 (Figure 3). Because S3 contained a nucleotide deletion 5 bases downstream of the K2.1 deletion, resulting in the same truncated protein as K2.1, but showed only minimally enhanced glowing, the glowing (flash) element was mapped between the K2.1 and S3 deletions; i.e., amino acid residues 122 to 124 (isoleucine, isoleucine, aspartic acid; see Figure 4). In addition, because S3 was in a different frame at amino acid 124 relative to wild type protein, and had diminished maximal light activity, the light activity (brightness) element was mapped between amino acids 124 and the end of the protein (Figure 4).

The mapping of the glowing and light intensity elements of the protein using random deletion mutants led to the development of 3K2 (SEQ ID NO:8), which contained the deletion found in the K2.1 variant, and an insertion downstream (to place the reading frame back in frame) to reconstruct the light intensity element of the protein. The 3K2 glowing apoaequorin maintained glowing activity and had an increase in maximal light intensity relative to K2.1. The 3K2 glowing apoaequorin

represented a conversion of three amino acids of the wild type aequorin from isoleucine, isoleucine, aspartic acid at position 122 to 124 to threonine, leucine, lysine in the 3K2 mutant. The 3K2 apoaequorin glowed nearly 100 fold longer than wild type aequorin, and had approximately a 10 fold reduction in maximal light intensity relative to the wild-type aequorin.

5

EXAMPLE 3
GLOWING AEQUORIN EXPRESSION IN MAMMALIAN CELLS

CHO cells were transfected with 2 μg of purified DNA encoding either wild type aequorin or 3K2 glowing apoaequorin using the GenePORTER™ transfection reagent (GeneTherapy Systems, Inc.) according to the vendor's instructions, and stable transfectants were selected with 1 mg/ml G418.
10 Cells were harvested by scraping and concentrated to 1.4×10^7 cells/ml in F12 medium, without phenol red or serum. Two hundred μl of cell suspensions were transferred to a white plate and charged with 8 μM coelenterazine for 4 hr in the presence of oxygen. Aequorin activity was verified by injecting 150 μl of 0.1% TRITON-X100 detergent into wells and measuring relative luminescent units (RLU) using an injectable luminometer. GPCR activation by thrombin was measured by injecting 150 μl
15 1 μM thrombin in F12 medium (without serum or phenol red) into wells. RLU data was collected for each well using 5 sec integrated reads over the course of 30 min.

The light activity for the cells expressing wild type aequorin was completely extinguished after about 2 min following the addition of thrombin (Figure 5, triangles). In contrast, the light activity for the cells expressing the modified aequorin increased to a maximal signal after about 15 min, and
20 continued to glow for 30 min without a drop in signal (Figure 5, diamonds).

Although the photoprotein, aequorin, can be a useful reporter for assaying GPCR activity, the flash kinetics have hampered its widespread use in high throughput screening strategies. The results disclosed herein demonstrate that aequorin proteins can be modified to exhibit extended glowing kinetics, thus providing reagents suitable for high throughput analysis of GPCR activity.

25

EXAMPLE 4
BINDING ASSAY USING MODIFIED AEQUORINS

Glowing aequorin proteins can expand the utility of aequorin in *in vivo* assays. The current requirement for detection equipment that can simultaneously inject calcium is eliminated with a glowing aequorin.

30 Modified glowing aequorin is a useful replacement for other markers in assays that involve the detection of binding reactions such as immunoassays, nucleic acid binding assays and ligand binding assays. Enzymatic markers and isotopic tracers are cumbersome and insensitive relative to glowing aequorin. Modified glowing aequorin proteins can be conjugated to a wide variety of molecules, including, for example, haptens, DNA, RNA, PNA, antigens, antibodies and peptides, using
35 routine methods (see, for example, U.S. Pat. Nos. 5,486,455 and 5,648,218, each of which is incorporated herein by reference). Capture molecules can be immobilized to a solid surface for the capture of a specific analyte following contact with a sample. The captured analyte can then be detected by using a detection molecule bound to the modified glowing aequorin. The determination of the binding event can be made by contacting the reaction with a divalent cation such as calcium,
40 followed by exposure of the reaction to a light recording device.

A competitive analyte also can be conjugated to the modified glowing aequorin and mixed with the sample prior to contact with the analyte-binding molecule. If the sample contains the analyte,

binding of the conjugated analyte will be reduced, due to competition, as a function of the concentration of the analyte in the sample. The amount of unbound analyte conjugated to the modified glowing aequorin is determined by analyzing the amount of light emitted following the addition of a divalent cation such as calcium.

5 A glowing aequorin affords the ability to trigger the light reaction without the use of an instrument such as an injectable luminometer that injects calcium and immediately determines the amount of light in the reaction. By eliminating the need for injectable luminometers, the glowing aequorins will allow for the use of a broader range of light emission detection methods including autoradiographic film and solid state devices, which, in turn, will allow for the development of assays in 10 formats other than in tubes or in 96 well or 384 well plates, for example, lateral flow assays and microchip assays.

EXAMPLE 5
CELL BASED ASSAYS USING MODIFIED AEQUORINS

Many cellular functions are regulated by the influx of calcium from one subcellular 15 compartment to another. A modified glowing aequorin can be introduced into cells using many different methods including, for example, by microinjection, transient transfection or stable transfection. In addition, by fusing trafficking signal peptides to the modified aequorin the expressed protein can be localized to specific organelles. Because modified apoaequorin and the luciferin used to charge aequorin are not toxic to cells, the modified glowing aequorin has an advantage as a calcium 20 sensor over other calcium indicators such as Fura-2 and indo-1.

Enhanced glowing kinetics are particularly useful for high throughput screening of cells transfected with a modified aequorin. One example is in the use of aequorin as a sensor for second messenger calcium influx during the screening of GPCR agonist and antagonist. A modified glowing aequorin allows for the addition of multiple compounds to different wells in a multiple well system 25 (e.g., a 96 well or 384 well system) without the risk of missing the recording of the signal transduction event. Previous studies using wild type aequorin as a reporter of calcium flux in cells have shown that the light must be detected in less than 20 seconds post-addition of test compound. As such, wild type aequorin cannot conveniently be used for high throughput screening of multiple compounds, which yield light within the first few seconds following addition of the compound being screened.

30

EXAMPLE 6
RADIATIONLESS RESONANCE ENERGY TRANSFER

The natural photosystem for *Aequoria victoria* involves the radiationless transfer of energy from aequorin to green fluorescent protein (GFP). Both proteins can be used in a system for testing the interaction of fusion proteins in a homogeneous assay. For example, the modified glowing aequorin 35 can be fused to a "bait" peptide and mixed with a library of GFPs that are fused to a library of uncharacterized peptides. If the "bait"/aequorin fusion protein binds to a binding partner on the GFP, the interaction can be detected following the introduction of calcium and the analysis of a shift in light from 469nm (the wavelength of unbound aequorin) to the emission wave length of the green fluorescent protein due to the resonance energy transfer (see, for example, Intl. Publ. No. WO 01/68824). The use 40 of a modified glowing aequorin in such a system provides the advantage that the light emitted from the GFP due to resonance energy transfer does not have to be determined within the first few seconds of adding the calcium trigger, as would be the case with wild type aequorin.

Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A modified apoaequorin polypeptide having enhanced glowing kinetics as compared to a corresponding wild type apoaequorin, said modified apoaequorin comprising at least one amino acid mutation corresponding to amino acid residues 122 to 124 of the wild type apoaequorin as set forth in SEQ ID NO:2, or a functional fragment of said modified apoaequorin,

provided that when the modified apoaequorin has a mutation at amino acid residue 124, the modified apoaequorin also comprises a mutation of at least one of amino acid residues 122 and 123.

2. The modified apoaequorin polypeptide of claim 1, wherein the corresponding wild type apoaequorin polypeptide comprises an amino acid sequence as set forth in SEQ ID NO:2 or a naturally occurring variant thereof.

3. The modified apoaequorin polypeptide of claim 2, comprising an amino acid sequence corresponding to

amino acid residues 1 to 196 of SEQ ID NO:2,
amino acid residues 1 to 124 of SEQ ID NO:2,
amino acid residues 1 to 123 of SEQ ID NO:2,
amino acid residues 1 to 122 of SEQ ID NO:2,
amino acid residues 1 to 121 of SEQ ID NO:2,
amino acid residues 8 to 196 of SEQ ID NO:2,
amino acid residues 8 to 124 of SEQ ID NO:2,
amino acid residues 8 to 123 of SEQ ID NO:2,
amino acid residues 8 to 122 of SEQ ID NO:2,
amino acid residues 8 to 121 of SEQ ID NO:2,
amino acid residues 26 to 196 of SEQ ID NO:2,
amino acid residues 26 to 124 of SEQ ID NO:2,
amino acid residues 26 to 123 of SEQ ID NO:2,
amino acid residues 26 to 122 of SEQ ID NO:2, or
amino acid residues 26 to 121 of SEQ ID NO:2.

4. The modified apoaequorin polypeptide of claim 1, wherein, in said modified apoaequorin, the amino acid residues corresponding to amino acid residues 122 to 124 of the wild type apoaequorin are Thr-Leu-Lys.

5. The modified apoaequorin polypeptide of claim 1, comprising SEQ ID NO:4 or SEQ ID NO:8, or a functional fragment thereof.

6. The modified apoaequorin of claim 5, wherein said functional fragment comprises
amino acid residues 8 to 171 of SEQ ID NO:4,
amino acid residues 26 to 171 of SEQ ID NO:4,
amino acid residues 8 to 196 of SEQ ID NO:8, or
amino acid residues 26 to 196 of SEQ ID NO:8.

7. The modified apoaequorin of claim 5, wherein said functional fragment comprises
amino acid residues 1 to 121 operatively linked to residues 125 to 171 of SEQ ID NO:4,

amino acid residues 1 to 122 operatively linked to residues 125 to 171 of SEQ ID NO:4,

amino acid residues 1 to 123 operatively linked to residues 125 to 171 of SEQ ID NO:4,

5 amino acid residues 26 to 171 of SEQ ID NO:4,

amino acid residues 26 to 121 operatively linked to residues 125 to 171 of SEQ ID NO:4,

amino acid residues 26 to 122 operatively linked to residues 125 to 171 of SEQ ID NO:4, or

10 amino acid residues 26 to 123 operatively linked to residues 125 to 171 of SEQ ID NO:4.

8. The modified apoaequorin of claim 5, wherein said functional fragment comprises

amino acid residues 1 to 121 operatively linked to residues 125 to 196 of SEQ ID

15 NO:8,

amino acid residues 1 to 122 operatively linked to residues 125 to 196 of SEQ ID NO:8,

amino acid residues 1 to 123 operatively linked to residues 125 to 196 of SEQ ID NO:8,

20 amino acid residues 26 to 196 of SEQ ID NO:8,

amino acid residues 26 to 121 operatively linked to residues 125 to 196 of SEQ ID NO:8,

amino acid residues 26 to 122 operatively linked to residues 125 to 196 of SEQ ID NO:8, or

25 amino acid residues 26 to 123 operatively linked to residues 125 to 196 of SEQ ID NO:8.

9. The modified apoaequorin polypeptide of claim 1, wherein the mutation corresponding to amino acid residues 122 to 124 comprises a deletion.

30

10. The modified apoaequorin of claim 1, further comprising a brightness element.

11. The modified apoaequorin of claim 10, wherein the brightness element comprises an amino acid sequence corresponding to amino acid residues 124 to 196 or SEQ ID NO:2, or a functional

35 fragment thereof.

12. A fusion protein, comprising the modified apoaequorin polypeptide of claim 1 operatively linked to a second polypeptide.

40 13. The fusion protein of claim 12, wherein the modified apoaequorin polypeptide is operatively linked to a heterologous polypeptide.

14. The fusion protein of claim 13, wherein the heterologous polypeptide comprises a cell compartmentalization domain.

45

15. The fusion protein of claim 13, wherein the heterologous polypeptide comprises a peptide tag.

16. The fusion protein of claim 12, wherein the second polypeptide comprises a second modified apoaequorin having enhanced glowing kinetics.

5 17. An antibody that specifically binds the modified apoaequorin of claim 1, or an antigen binding fragment of said antibody, wherein said antibody does not substantially bind the corresponding wild type apoaequorin.

18. The antibody of claim 17, which is a monoclonal antibody.

10

19. The antibody of claim 17, wherein specific binding of said antibody modulates luminescent activity of an aequorin photoprotein comprising the modified apoaequorin.

20. The antibody of claim 19, which enhances the luminescent activity of the aequorin 15 photoprotein.

21. The antibody of 19, which decreases the luminescent activity of the aequorin photoprotein.

22. A polynucleotide encoding the modified apoaequorin polypeptide, or functional fragment 20 thereof, of claim 1.

23. The polynucleotide of claim 22, which is a double stranded deoxyribonucleic acid.

24. The polynucleotide of claim 22, which is a ribonucleic acid.

25

25. The polynucleotide of claim 22, which has a nucleotide sequence as set forth in SEQ ID NO:3 or SEQ ID NO:7.

26. A vector, comprising the polynucleotide of claim 22.

30

27. The vector of claim 26, which is an expression vector.

28. A host cell, which contains the polynucleotide of claim 22.

35 29. The host cell of claim 28, wherein the polynucleotide is stably maintained in the host cell.

30. The host cell of claim 29, wherein the polynucleotide is integrated in the host cell genome.

31. A transgenic non-human organism, comprising the polynucleotide of claim 22.

40

32. The transgenic non-human organism of claim 31, which is a transgenic animal or a transgenic plant.

33. The polynucleotide of claim 22, further comprising a nucleotide sequence encoding a 45 second polypeptide.

34. The polynucleotide of claim 33, which encodes a fusion protein comprising the second polypeptide operatively linked to the modified apoaequorin, or functional fragment thereof.

35. A process for producing a modified apoaequorin polypeptide having enhanced glowing kinetics, comprising expressing the polynucleotide of claim 22, thereby producing the modified apoaequorin polypeptide.

36. The process of claim 35, which is performed *in vitro*.

37. The process of claim 35, which is performed in a cell.

38. The process of claim 37, wherein the cell is a eukaryotic cell.

39. The process of claim 37, wherein the cell is a prokaryotic cell.

40. The process of claim 35, further comprising isolating the modified apoaequorin polypeptide.

41. A modified apoaequorin polypeptide produced by the method of claim 34.

42. A process for producing a polynucleotide encoding a bioluminescent polypeptide having enhanced glowing kinetics as compared to a corresponding wild type bioluminescent polypeptide, the method comprising

introducing mutations into a polynucleotide encoding a polypeptide corresponding to the wild type bioluminescent polypeptide, thereby generating mutant polynucleotides;

contacting an expression library comprising the mutant polynucleotides with a luciferin and divalent cations, under conditions sufficient to generate luminescent activity of a corresponding wild type bioluminescent polypeptide; and

examining clones of the library for luminescent activity at a time after which a corresponding wild type bioluminescent polypeptide no longer exhibits luminescent activity, whereby luminescent activity of a clone identifies the clone as expressing a polynucleotide encoding a bioluminescent polypeptide having enhanced glowing kinetics, thereby producing a polynucleotide encoding a bioluminescent polypeptide having enhanced glowing kinetics.

43. The method of claim 42, further comprising, after contacting the expression library with the luciferin and divalent cations, examining clones of the library for luminescent activity within a time during which the corresponding wild type bioluminescent polypeptide exhibits luminescent activity.

44. The method of claim 42, wherein the bioluminescent polypeptide is an apoaequorin.

45. The method of claim 42, wherein the wild type bioluminescent polypeptide is mnemiopsin, berovin, or obelin.

46. The method of claim 42, wherein the divalent cation is calcium ion.

47. The method of claim 42, wherein examining clones of the library is performed using a photographic film.

48. The method of claim 42, wherein examining clones of the library is performed using a luminometer.

5 49. The method of claim 42, wherein introducing mutations into the polynucleotide comprises introducing random mutations, introducing mutations by site directed mutagenesis, or a combination thereof.

50. The method of claim 42, wherein the mutations comprise an insertion, a deletion, a
10 substitution, or a combination thereof.

51. The method of claim 42, further comprising isolating the polynucleotide encoding a bioluminescent polypeptide having enhanced glowing kinetics.

15 52. A polynucleotide encoding a bioluminescent polypeptide having enhanced glowing kinetics as compared to a corresponding wild type apoaequorin, said polynucleotide produced by the method of claim 42.

53. An isolated polynucleotide encoding a bioluminescent polypeptide having enhanced glowing kinetics as compared to a corresponding wild type apoaequorin, said polynucleotide produced
20 by the method of claim 51.

54. A method of producing an aequorin photoprotein having enhanced glowing kinetics as compared to a corresponding wild type aequorin photoprotein, the method comprising contacting a modified apoaequorin polypeptide of claim 1 and a coelenterazine under conditions sufficient for
25 reconstituting an aequorin photoprotein.

55. The method of claim 54, wherein said conditions include contacting the modified apoaequorin polypeptide and the coelenterazine in the presence of molecular oxygen.

30 56. The method of claim 54, wherein said conditions include contacting the modified apoaequorin polypeptide and the coelenterazine in the presence of a reducing agent.

57. The method of claim 54, further comprising isolating the modified aequorin photoprotein.

35 58. A modified aequorin photoprotein produced by the method of claim 57.

59. A method of detecting the presence of divalent cations in a sample, the method comprising

contacting the sample with a composition comprising a modified apoaequorin polypeptide, or functional fragment thereof, having enhanced glowing kinetics, under
40 conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, and

examining the sample for said luminescent activity, whereby detecting said luminescent activity indicates the presence of divalent cations in the sample.

45 60. The method of claim 59, wherein said conditions include further contacting the sample with a coelenterazine, or analog thereof.

61. The method of claim 59, wherein the composition comprises a reconstituted aequorin photoprotein comprising the modified apoaequorin polypeptide, or functional fragment thereof.

62. The method of claim 59, wherein the divalent cations are calcium ions or strontium ions.

5

63. The method of claim 59, wherein the sample is a water sample.

64. The method of claim 59, wherein the sample is obtained from an organism.

10 65. The method of claim 64, wherein the sample is a cell sample, a tissue sample, or an organ sample, or an extract thereof.

66. A method of identifying an agent that effects divalent cation flux in a sample comprising at least two compartments, wherein at least one compartment contains divalent cations, the method

15 comprising

contacting a compartment of the sample with a modified apoaequorin polypeptide, or functional fragment thereof, having enhanced glowing kinetics, under conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof,

examining the sample for said luminescent activity, and

20 contacting the sample with a test agent, whereby a change in said luminescent activity identifies the test agent as an agent that effects divalent cation flux.

67. The method of claim 66, wherein the compartment contacted with the modified aequorin, or functional fragment thereof, is a compartment containing the divalent cations, and wherein an agent

25 that effects divalent cation flux is identified by a decrease in luminescent activity.

68. The method of claim 66, wherein the compartment contacted with the modified aequorin, or functional fragment thereof, is a compartment having an insufficient divalent cation concentration to generate luminescent activity mediated by the modified apoaequorin, or functional fragment thereof,

30 and wherein an agent that effects divalent cation flux is identified by an increase in luminescent activity.

69. The method of claim 66, wherein the sample comprises a first compartment comprising bound divalent cations and a second compartment having an insufficient divalent cation concentration

35 to generate luminescent activity mediated by the modified apoaequorin, or functional fragment thereof.

70. The method of claim 69, wherein the first compartment comprises calcium ions bound to a chelating compound.

40 71. The method of claim 66, wherein the sample comprises a cell.

72. The method of claim 71, wherein the cell is a prokaryotic cell or a eukaryotic cell.

73. The method of claim 71, wherein the cell is a cell of an invertebrate or a cell of a

45 vertebrate.

74. The method of claim 71, wherein the cell is a mammalian cell.

75. The method of claim 71, wherein the sample comprises an intracellular compartment and an extracellular compartment.

5 76. The method of claim 71, wherein the cell comprises at least one intracellular compartment containing calcium ions and at least one intracellular compartment having an insufficient calcium ion concentration to generate luminescent activity mediated by the modified apoaequorin, or functional fragment thereof.

10 77. The method of claim 76, wherein the intracellular compartment containing calcium ions is sarcoplasmic reticulum, endoplasmic reticulum, a mitochondrion, a secretory vesicle, or a plant vacuole.

78. The method of claim 66, wherein the agent specifically binds a receptor.

15

79. The method of claim 78, wherein the receptor is expressed on a cell surface.

80. The method of claim 78, wherein the receptor is a G-protein coupled receptor (GPCR).

20 81. The method of claim 80, wherein the GPCR is a thrombin receptor.

82. The method of claim 80, wherein the GPCR is an É-adrenergic receptor or a é-adrenergic receptor.

25 83. The method of claim 78, wherein the agent is an agonist or a partial agonist for the GPCR.

84. The method of claim 78, wherein the agent is an antagonist for the GPCR.

85. The method of claim 66, wherein the sample comprises one of a plurality of samples.

30

86. The method of claim 85, wherein each sample in the plurality is the same, said method further comprising samples of the plurality with at least one of a plurality of different test agents.

87. The method of claim 85, wherein samples in the plurality are different.

35

88. The method of claim 85, which is performed in a high throughput format.

89. The method of claim 88, wherein contacting a sample with an agent is performed on a microchip, a glass slide, or a plastic well.

40

90. A method of detecting a functional change associated with divalent cation flux across a membrane of a cell, the method comprising

subjecting a cell to a stimulus, said cell containing intracellular modified apoaequorin polypeptide, or functional fragment thereof, having enhanced glowing kinetics, under
45 conditions sufficient for luminescent activity mediated by the modified apoaequorin, or functional fragment thereof, to a stimulus, and

examining the cell for said luminescent activity, whereby a change in said luminescent activity is indicative of a functional change associated with divalent cation flux across the membrane of the cell.

5 91. The method of claim 90, wherein the divalent cation flux is calcium ion flux.

92. The method of claim 90, wherein the cell is an oocyte, and wherein the stimulus is exposure to sperm, whereby a change in luminescent activity is indicative of fertilization of the oocyte.

10 93. The method of claim 90, wherein the cell is a bacterial cell, and wherein the stimulus is exposure to complement fixing antibodies specific for an epitope of the bacterial cell, whereby a change in luminescent activity is indicative of cytolysis of the bacterial cell.

94. The method of claim 90, wherein the cell is a mammalian cell, and wherein the stimulus
15 comprises exposure to conditions that can induce apoptosis of a cell, whereby a change in luminescent activity is indicative of apoptosis of the mammalian cell.

95. The method of claim 90, wherein the cell is a muscle cell, and wherein the stimulus comprises exposure to conditions that can induce contraction of the muscle cell, whereby a change in
20 luminescent activity is indicative of a contraction event.

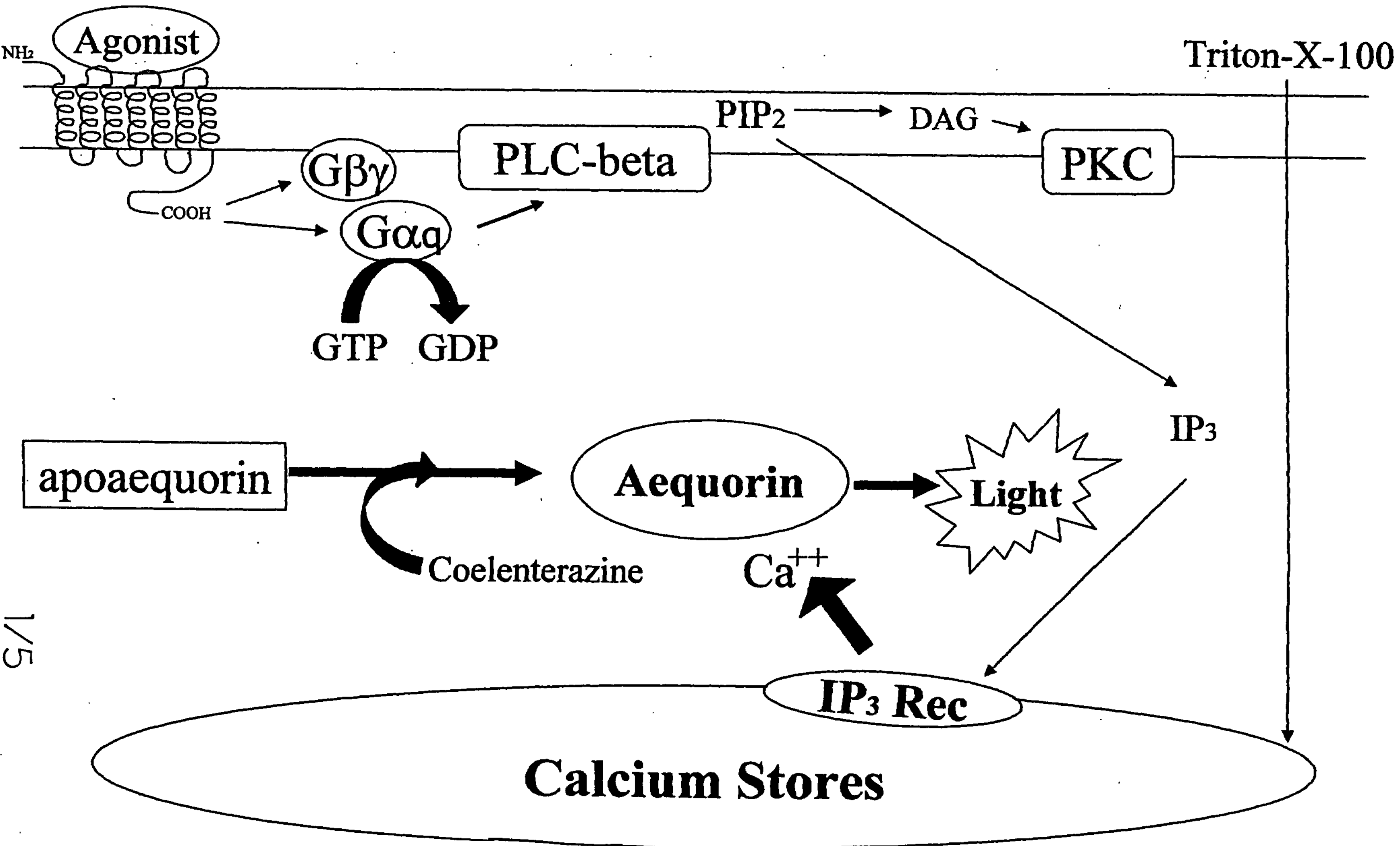


FIG. 1

Fig. 2

Sequences of Mutant Aequorin Proteins and Alignments to Wildtype

Wildtype vs. K2.1

```
WT  :   1  MTSEQYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE 60
           MTS+QYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE
K2.1:   1  MTSKQYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE 60

WT  :  61  QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF 120
           QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF
K2.1:  61  QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF 120

WT  : 121  DIIDKDQNGAISLDEWKAYTKSDGIIQSSEDCEETFRVCDIDESGQLDVDEMTRQHLGFW 180
           D + K +    + L  W      +  ++ SS                 D     R
K2.1: 121  DTLKKTK---MELFHWMNGKHTPNLLASSNRQKIARKHSECAILMKVDSSMLMR       171

WT  : 181  YTMDPACEKLYGGAVP 196
```

Wildtype vs. S3

```
WT    : 1   MTSEQYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE 60
            MTS+QYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE
S3    : 1   MTSKQYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE 60

WT    : 61  QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF 120
            QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF
S3    : 61  QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF 120

WT    : 121 DIIDKDQNGAISLDEWKAYTKSDGIIQSSEDCEETFRVCDIDESGQLDVDEMTRQHLGFW 180
            DII K +    + L  W      +  ++ SS                 D     R
S3    : 121 DIISKTK---MELFHWMNGKHTPNLLASSNRQKIARKHSECAILMKVDSSMLMR       171

WT    : 181 YTMDPACEKLYGGAVP 196
```

Wildtype vs. 3K2

```
WT    :   1 MTSEQYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE 60
            MTS+QYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE
3K2   :   1 MTSKQYSVKLTPDFDNPKWIGRHKHMFNFLDVNHNGRISLDEMVYKASDIVINNLGATPE 60

WT    :  61 QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF 120
            QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF
3K2   :  61 QAKRHKDAVEAFFGGAGMKYGVETEWPEYIEGWKRLASEELKRYSKNQITLIRLWGDALF 120

WT    : 121 DIIDKDQNGAISLDEWKAYTKSDGIIQSSEDCEETFRVCDIDESGQLDVDEMTRQHLGFW 180
            D + KDQNGAISLDEWKAYTKSDGIIQSSEDCEETFRVCDIDESGQLDVDEMTRQHLGFW
3K2   : 121 DTLKKDQNGAISLDEWKAYTKSDGIIQSSEDCEETFRVCDIDESGQLDVDEMTRQHLGFW 180

WT    : 181 YTMDPACEKLYGGAVP 196
            YTMDPACEKLYGGAVP
3K2   : 181 YTMDPACEKLYGGAVP 196
```

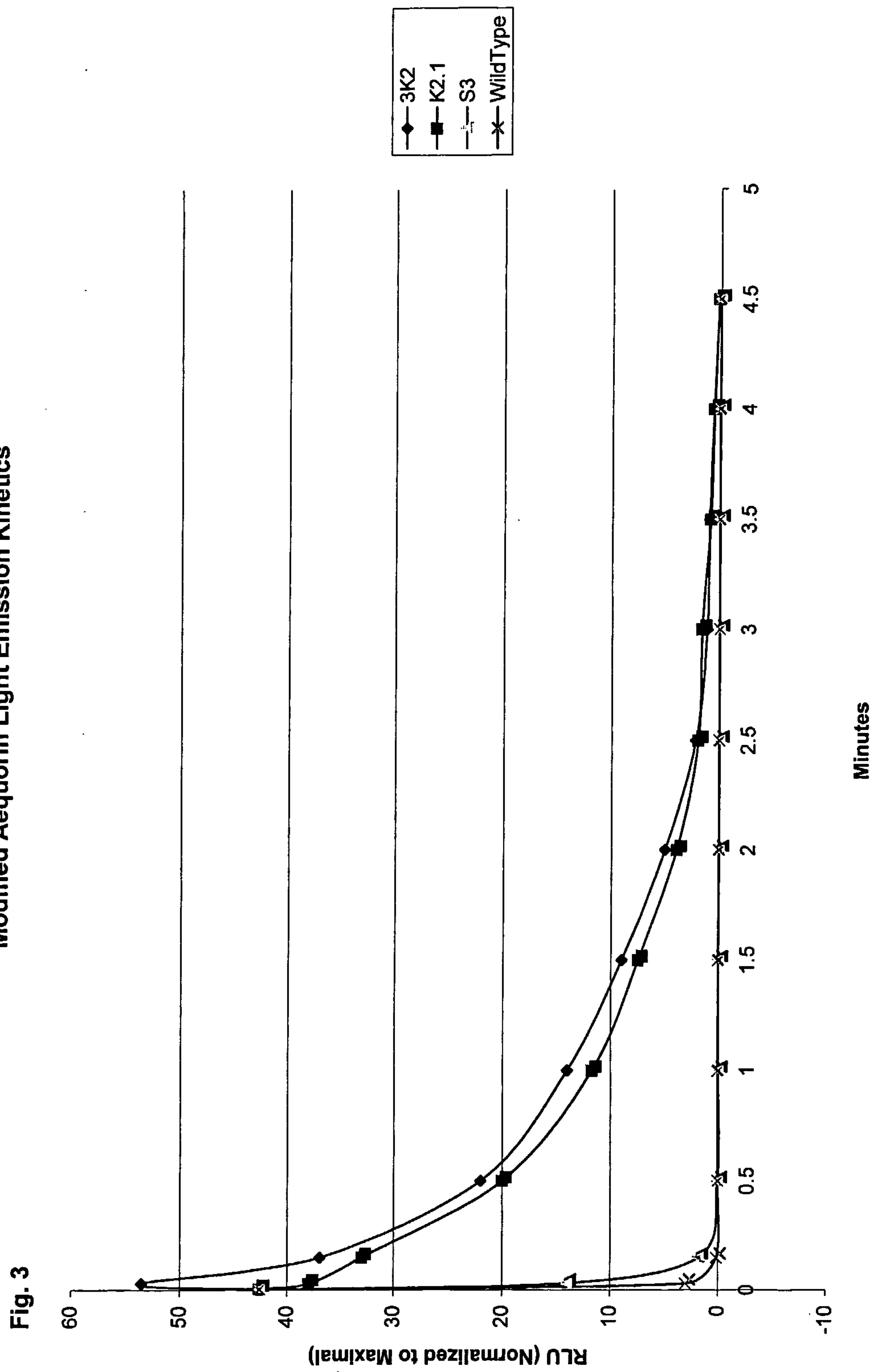
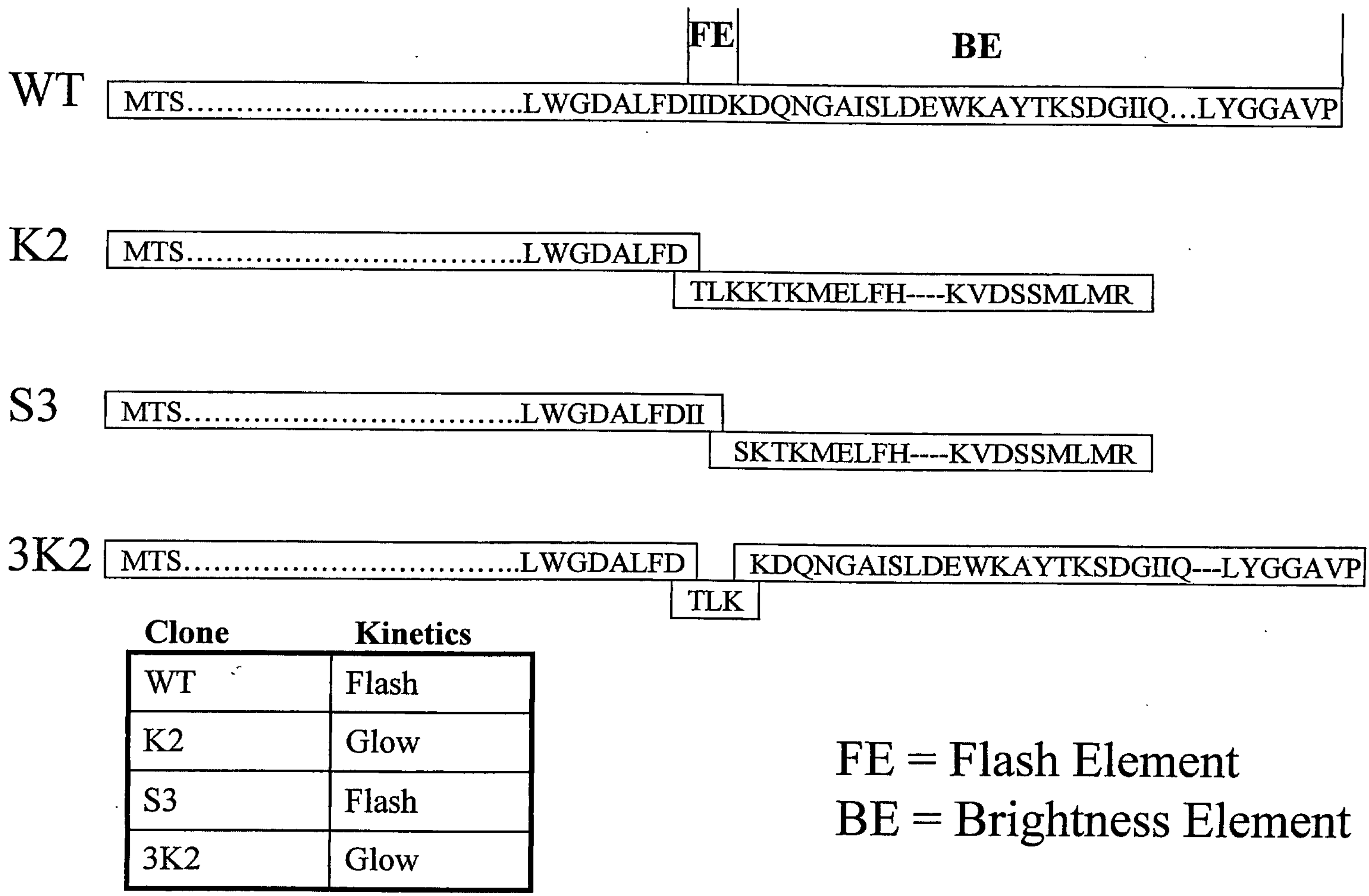


Fig. 3
Modified Aequorin Light Emission Kinetics
RLU (Normalized to Maximal)
60
50
40
30
20
10
0
-10
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Minutes
3K2
K2.1
S3
WildType

Fig. 4



Clone	Kinetics
WT	Flash
K2	Glow
S3	Flash
3K2	Glow

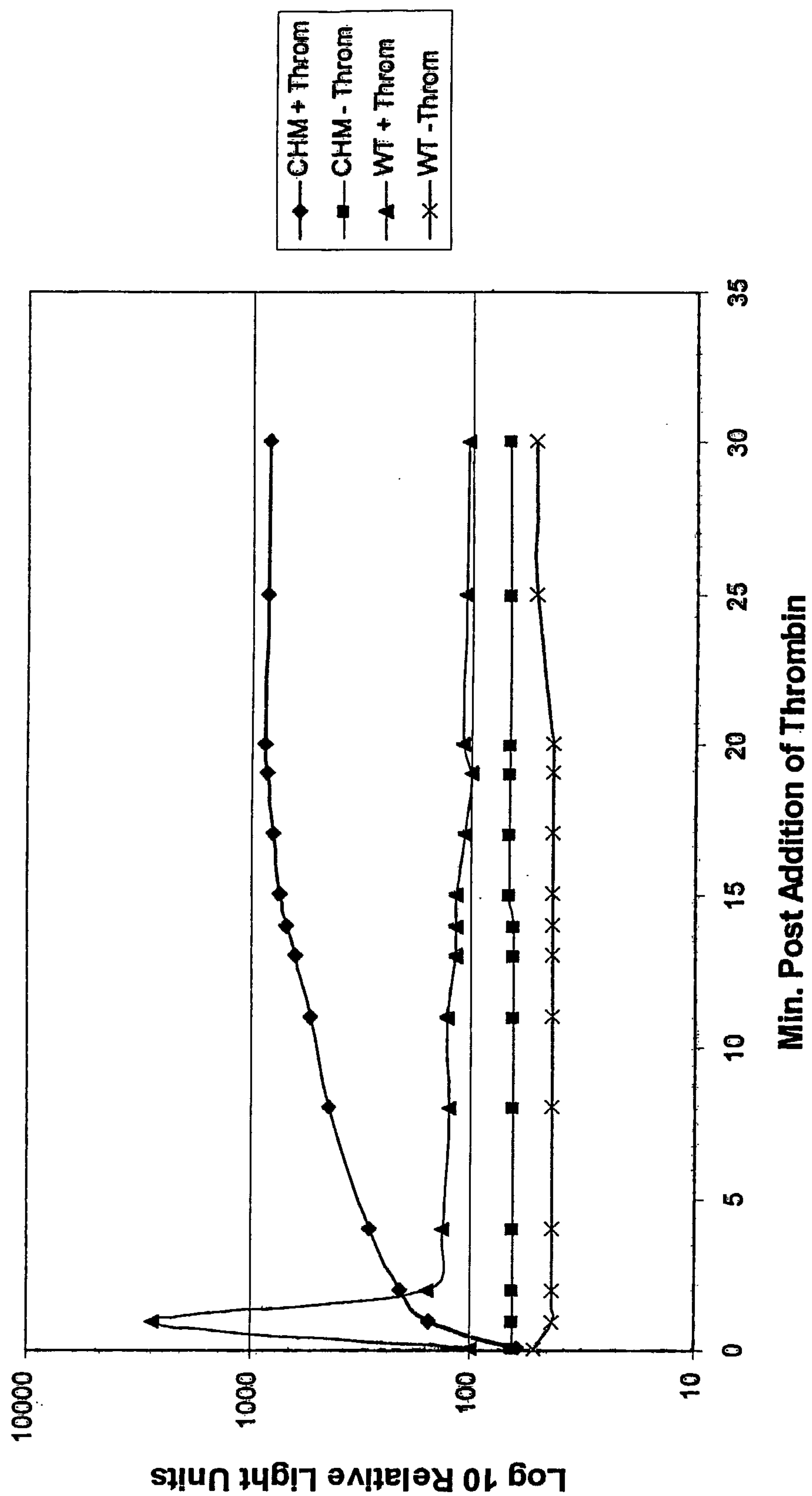


FIG. 5