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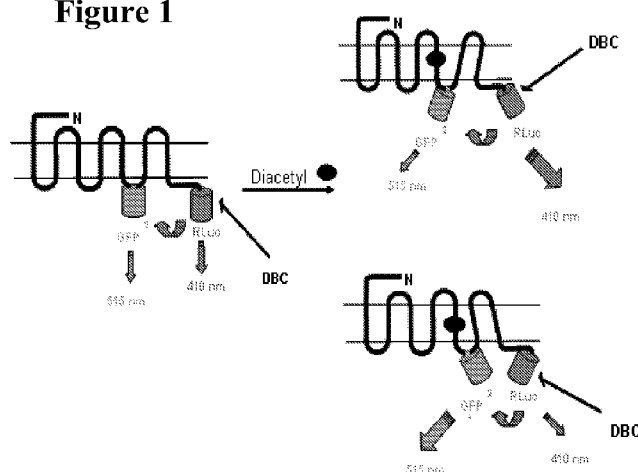
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(54) Title: MEASURING G PROTEIN COUPLED RECEPTOR ACTIVATION

Figure 1



(57) Abstract: The present invention relates to methods and polypeptides for detecting a compound in a sample. In particular, the present invention relates to the use of a cell-free composition comprising at least one G protein coupled receptor embedded in a lipid bilayer which when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus is inside the cell, and which is capable of binding the compound. Optionally, the composition also comprises at least one accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor. The G protein coupled receptor, and/or accessory molecule when present, in combination comprise a bioluminescent protein and an acceptor molecule, which enables bioluminescent resonance energy transfer (BRET) to be used to detect the compound binding the receptor.

MEASURING G PROTEIN COUPLED RECEPTOR ACTIVATION

FIELD OF THE INVENTION

The present invention relates to methods and polypeptides for detecting a compound in a sample. In particular, the present invention relates to the use of a cell-free composition comprising at least one G protein coupled receptor embedded in a lipid bilayer which when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus is inside the cell, and which is capable of binding the compound. Optionally, the composition also comprises at least one accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor. The G protein coupled receptor, and/or accessory molecule when present, in combination comprise a bioluminescent protein and an acceptor molecule, which enables bioluminescent resonance energy transfer (BRET) to be used to detect the compound binding the receptor.

BACKGROUND OF THE INVENTION

G protein coupled receptors (GPCRs) are a family of transmembrane receptors that sense molecules outside the cell and activate inside signal transduction pathways and, ultimately, cellular responses. GPCRs are found only in eukaryotes including yeast and animals. The ligands that bind and activate GPCRs include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. G protein-coupled receptors are involved in many diseases, and are also the target of therapeutic drugs.

Vertebrate and *C. elegans* odorant receptors (ORs) are members of the G-protein coupled receptor (GPCR) family (Buck et al., 1991; Troemel et al., 1995). GPCRs are characterised by their seven transmembrane spanning domains with ligand binding domains inferred to be on the extracellular side of the membrane and G-protein binding domains on the intracellular side. When a receptor binds the ligand, a conformational change occurs in the receptor allowing it to activate a heterotrimeric G-protein (Kobilka et al., 2007). The activated G-protein can then activate signal transduction pathways such as the guanyl cyclase or phospholipase C pathways, transducing the signal to higher processing centres (Gaillard et al., 2004).

Forster resonance energy transfer, or simply resonance energy transfer (RET), is the non-radiative transfer of energy from an excited state donor molecule to a ground state acceptor molecule (Ghanouni et al., 2001). Energy transfer efficiency is

dependent on the distance between the donor and acceptor, the extent of the spectral overlap and the relative orientation of the acceptor and donor dipoles. Previous cases where intramolecular RET has been used to monitor GPCR activation have employed a fluorescent donor and acceptor, a method referred to as fluorescence resonance energy transfer (FRET). In most cases both the fluorescent donor and acceptor are engineered variants of green fluorescent protein (GFP) from *Aequoria victoria* (Tsien, 1998). The most widely used FRET pair is cyan fluorescent protein (CFP) as the donor alongside yellow fluorescent protein (YFP) as the acceptor (Piston and Kremers, 2007) and this FRET system has previously been used to quantify direct ligand binding by a number of GPCRs (Lohse et al., 2003 and 2007; Vilardaga et al., 2003; Rochais et al., 2007; Lisenbee et al., 2007).

One method for monitoring receptor activation involves dual labelling a single GPCR with CFP and YFP at insertion sites within the third intracellular loop and C-terminus, respectively. Excitation of CFP with light at 436 nm causes CFP emission at 480 nm and FRET to YFP, which emits at 535 nm. The efficiency of FRET varies with the sixth power of the distance between donor and acceptor, providing an exquisitely sensitive indication of conformational changes in the GPCR. This was demonstrated with α 2AR, parathyroid hormone receptor (PTHr), β 1-AR and secretin receptors in intact cells. Interaction of the agonists noreadrenaline with α 2AR (Lohse et al., 2003), parathyroid hormone with PTHr (Vilardaga et al., 2003), norepinephrine with β 1-AR (Rochais et al., 2007) and secretin with secretin receptors (Lisenbee et al., 2007) changed the distance between CFP and YFP thus causing a change in FRET signal.

Replacement of the YFP acceptor with FLAsH, a fluorescein arsenical hairpin binder, in a FRET system (Hoffman et al., 2005; Nakanishi et al., 2006) resulted in a five-fold greater increase in agonist-induced FRET signal compared with the CFP/YFP system when used to monitor α 2-adrenergic receptor activation (Nakanishi et al., 2006). However, FLAsH involves a more difficult labelling and washing procedure which has limited use in the wider research community. The CFP/YFP system remains the most frequently reported FRET system for monitoring intramolecular conformational change. A major disadvantage associated with FRET is the need for a light source to energise the donor fluorophore (Piston and Kremers, 2007). This causes unwanted direct excitation of the acceptor at the donor excitation wavelength (a problem referred to as 'cross-talk').

In bioluminescence resonance energy transfer (BRET), the donor fluorophore of FRET is replaced with a luciferase and the acceptor can be any suitable fluorophore. The use of a luciferase avoids the need for illumination as the addition of a substrate

initiates bioluminescent emission and hence, RET. Two common implementations of BRET comprise *Renilla* luciferase (RLuc) with either coelenterazine *h* (BRET¹; $\lambda_{em} = \sim 475$ nm) or coelenterazine 400a (Clz400a) substrate (BRET²; $\lambda_{em} = \sim 395$ nm) as the donor system coupled to either of the GFP mutants, YFP (BRET¹; $\lambda_{em} = \sim 530$ nm) or GFP² (BRET²; $\lambda_{em} = \sim 510$ nm). The BRET² system offers superior spectral separation between the donor and acceptor emission peaks of ~ 115 nm compared to ~ 55 nm for the BRET¹ system at the expense of the quantum yield (Pfleger and Eidne et al., 2006).

FRET with odorant receptors has only previously been demonstrated for Class A (α_2 -adrenergic and parathyroid hormone, (Vilardaga et al., 2003)) and Class B (secretin, (Lisenbee et al., 2007)) GPCRs. Unlike mammalian ORs, which belong to GPCR Class A, nematode ORs (Robertson, 1998 and 2001) belong to neither of these classes and are evolutionarily and structurally distinct. Furthermore, all ORs, including mammalian ORs, which sit within Class A of the GPCR superfamily, are atypical in respect of their expression. Generally these proteins cannot be functionally expressed other than in neurons derived from the chemosensory lineage. A number of accessory proteins have been identified whose presence is required for proper expression of mammalian and nematode ORs.

Thus, there is a need for suitably sensitive methods and molecules which enable the detection of compounds which bind G protein coupled receptors for use in, for example, biosensors.

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that cell-free bioluminescence resonance energy transfer (BRET) using chimeric G protein coupled receptors (GPCRs), such as odorant receptors (ORs), to detect a target compound is more sensitive than other detection methods such as BRET using whole cells or FRET.

Thus, in a first aspect the present invention provides a method of detecting a compound, the method comprising,

- i) contacting a sample with a cell-free composition comprising
 - a) at least one G protein coupled receptor embedded in a lipid bilayer, and which is capable of binding the compound, and
 - b) optionally at least one accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor, wherein the G protein coupled receptor comprises one or more subunits that are the same or different, and wherein the G protein coupled receptor, and/or accessory

molecule when present, in combination comprise a bioluminescent protein and an acceptor molecule,

ii) simultaneously or sequentially with step i) providing a substrate of the bioluminescent protein, and allowing the bioluminescent protein to modify the substrate,

iii) determining if step ii) modulates bioluminescent resonance energy transfer (BRET) between the bioluminescent protein and the acceptor molecule,

wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the G protein coupled receptor, and wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.

In one embodiment, a subunit of the G protein coupled receptor which is capable of binding the compound comprises

- i) the bioluminescent protein, and
- ii) the acceptor molecule,

and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the subunit. For example, the subunit may comprise

- i) an amino acid sequence provided as SEQ ID NO:13, 15, 52 or 54, or
- ii) an amino acid sequence which is at least 40% identical to any one or more of SEQ ID NO:13, 15, 52 or 54.

In an alternate embodiment, the G protein coupled receptor comprises

- i) a first subunit comprising
 - a) the bioluminescent protein, and
 - b) the acceptor molecule, and

ii) a second subunit which is capable of binding the compound,

and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the second subunit.

In a further embodiment,

i) the bioluminescent protein forms part of the third non-transmembrane loop of the subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop, or

ii) the acceptor molecule forms part of the third non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop, or

iii) the bioluminescent protein forms part of the first non-transmembrane loop of the subunit, and the acceptor molecule forms part of the third non-transmembrane loop, or

iv) the acceptor molecule forms part of the first non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the third non-transmembrane loop, or

v) the bioluminescent protein forms part of the fifth non-transmembrane loop of the subunit, and the acceptor molecule forms part of the C-terminus, or

vi) the acceptor molecule forms part of the fifth non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the C-terminus.

In yet another alternate embodiment, the G protein coupled receptor comprises

i) a first subunit comprising the bioluminescent protein, and

ii) a second subunit comprising the acceptor molecule. For example, the first or second subunit may comprise:

i) an amino acid sequence provided as SEQ ID NO: 17 or 19, or

ii) an amino acid sequence which is at least 40% identical to SEQ ID NO: 17 and/or 19.

In an embodiment,

i) the bioluminescent protein forms part of the third non-transmembrane loop of the first subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop of the second subunit, or

ii) the acceptor molecule forms part of the third non-transmembrane loop of the first subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop of the second subunit, or

iii) the bioluminescent protein forms part of the first non-transmembrane loop of the first subunit, and the acceptor molecule forms part of the third non-transmembrane loop of the second subunit, or

iv) the acceptor molecule forms part of the first non-transmembrane loop of the first subunit, and the bioluminescent protein forms part of the third non-transmembrane loop of the second subunit, or

v) the bioluminescent protein forms part of the fifth non-transmembrane loop of the first subunit, and the acceptor molecule forms part of the C-terminus of the second subunit, or

vi) the acceptor molecule forms part of the fifth non-transmembrane loop of the first subunit, and the bioluminescent protein forms part of the C-terminus of the second subunit.

In a further alternate embodiment,

i) the G protein coupled receptor comprises a subunit comprising the bioluminescent protein, and the accessory molecule comprises the acceptor molecule, or

ii) the G protein coupled receptor comprises a subunit comprising the acceptor molecule, and the accessory molecule comprises the bioluminescent protein.

In another alternate embodiment, the accessory molecule comprises

i) the bioluminescent protein, and

ii) the acceptor molecule.

In a further alternate embodiment, the composition comprises at least two accessory molecules, and wherein a first accessory molecule comprises the bioluminescent protein and a second accessory molecule comprises the acceptor molecule.

The present inventors were particularly surprised to find how superior the present invention is when compared to FRET-cellfree and BRET-whole cell detection systems. Thus, in a preferred embodiment, the method of the invention is at least 2 fold, 3 fold or 4 fold more sensitive than if a non-bioluminescent protein is used as a donor molecule and a modulation of fluorescence resonance energy transfer (FRET) is determined. In a further preferred embodiment, the method of the invention provides at least a 10 fold, 20 fold, 30 fold or 40 fold more intense BRET signal than if the method used the same G protein coupled receptor present in an intact cell instead of being in a cell-free composition.

Given the atypical nature of Class A GPCRs, ORs generally and nematode ORs in particular, it was surprising that a chimera comprising a Class A GPCR with BRET donor and acceptor tags would a) retain structural integrity and b) be capable of transducing receptor activation by an odorant into an optical signal. Thus, in a preferred embodiment, the G protein coupled receptor is a Class A GPCR. In a further preferred embodiment, the class A GPCR is an odorant receptor. The odorant receptor can be from any source as long as when expressed in a cell the N-terminus of the receptor is outside the cell and the C-terminus is inside the cell. Examples include, but are not limited to, a chordate receptor, a nematode receptor, or a biologically active variant or fragment of any one thereof. Examples of chordate receptors include, but are not limited to mammalian receptors, avian receptors and fish receptors. In a preferred

embodiment, the odorant receptor is a nematode receptor or biologically active variant or fragment thereof. In an embodiment, the nematode receptor is a *Caenorhabditis elegans* receptor, or biologically active variant or fragment thereof.

In an embodiment, the odorant receptor comprises:

- i) an amino acid sequence as provided in any one of SEQ ID NOs 1 to 6, and
- ii) an amino acid sequence which is at least 40% identical to any one or more of SEQ ID NO:1 to 6.

In another embodiment, the subunit is a chimera of a portion of two or more different G protein coupled receptor subunits.

The accessory molecule can be any molecule that directly or indirectly associates with the G protein coupled receptor. Examples include, but are not limited to, G protein and arrestin.

The effect of changing compound concentrations on the amplitude of the change in BRET has provided the first demonstration that the response is dose-dependent. Thus, in a preferred embodiment the level of BRET is indicative of the relative amount of the compound in the sample.

Examples of bioluminescent proteins include, but are not limited to, a luciferase, a β -galactosidase, a lactamase, a horseradish peroxidase, an alkaline phosphatase, a β -glucuronidase or a β -glucosidase.

Examples of luciferases include, but are not limited to, a *Renilla* luciferase, a Firefly luciferase, a Coelenterate luciferase, a North American glow worm luciferase, a click beetle luciferase, a railroad worm luciferase, a bacterial luciferase, a *Gaussia* luciferase, Aequorin, a *Arachnocampa* luciferase, or a biologically active variant or fragment of any one, or chimera of two or more, thereof.

Examples of substrates include, but are not limited to, beetle luciferin, other luciferins, coelenterazine, or a derivative of coelenterazine. Also, in the case of some luminescent proteins such as aequorin, the substrate may be a cofactor such as calcium ions.

In one embodiment, the acceptor molecule is a protein, examples of which include, but are not limited to, green fluorescent protein (GFP), blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, t-dimer2, t-dimer2(12), mRFP1, pocilloporin, *Renilla* GFP, Monster GFP, paGFP, Kaede protein or a Phycobiliprotein, or a biologically active variant or fragment of any one thereof.

In an alternate embodiment, the acceptor molecule is a non-protein, examples of which include, but are not limited to, an Alexa Fluor dye, Bodipy dye, Cy dye, fluorescein, dansyl, umbelliferone, fluorescent microsphere, luminescent microsphere, fluorescent nanocrystal, Marina Blue, Cascade Blue, Cascade Yellow, Pacific Blue, Oregon Green, Tetramethylrhodamine, Rhodamine, Texas Red, rare earth element chelates, or any combination or derivatives thereof.

In another embodiment, the methods further comprise simultaneously or sequentially with step i) or step ii) providing a co-factor of the bioluminescent protein. Examples include, but are not limited to, ATP, magnesium, oxygen, FMNH₂, calcium, or a combination of any two or more thereof.

In an embodiment, the cell-free composition was obtained by producing the G protein coupled receptor in a recombinant cell and disrupting the membrane of the cell.

In an embodiment, the recombinant cell does not produce any non-endogenous proteins which associate with the G protein coupled receptor.

The cell from which the cell-free composition can be obtained is any cell type capable of expressing the G protein coupled receptor and incorporating the receptor into its cell membrane. In a particularly preferred embodiment, the recombinant cell is a yeast cell.

In an alternate embodiment, the G protein coupled receptor is embedded in the lipid bilayer of a liposome.

In a further embodiment, the method is preformed using microfluidics.

In another aspect, the present invention provides a purified and/or recombinant polypeptide for detecting a compound, the polypeptide comprising,

- i) a subunit of a G protein coupled receptor, and
- ii) a bioluminescent protein and/or an acceptor molecule,

wherein when expressed in a cell the N-terminus of the subunit is outside the cell and the C-terminus inside the cell.

In an embodiment, the polypeptide forms part of a protein complex which is a G protein coupled receptor comprising one or more different G protein coupled receptor subunits, and optionally one or more different accessory molecules.

In an embodiment,

- i) the bioluminescent protein forms part of the third non-transmembrane loop of the subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop, or

ii) the acceptor molecule forms part of the third non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop, or

iii) the bioluminescent protein forms part of the first non-transmembrane loop of the subunit, and the acceptor molecule forms part of the third non-transmembrane loop, or

iv) the acceptor molecule forms part of the first non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the third non-transmembrane loop, or

v) the bioluminescent protein forms part of the fifth non-transmembrane loop of the subunit, and the acceptor molecule forms part of the C-terminus, or

vi) the acceptor molecule forms part of the fifth non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the C-terminus, or

vii) the bioluminescent protein or the acceptor molecule forms part of the first non-transmembrane loop of the subunit, or

viii) the bioluminescent protein or the acceptor molecule forms part of the third non-transmembrane loop of the subunit, or

ix) the bioluminescent protein or the acceptor molecule forms part of the fifth non-transmembrane loop of the subunit, or

x) the bioluminescent protein or the acceptor molecule forms part of the C-terminus of the subunit.

In a further embodiment, the polypeptide comprises the bioluminescent protein and the acceptor molecule, and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the polypeptide.

In an alternate embodiment, the polypeptide comprises the subunit and the bioluminescent protein, and the polypeptide is directly or indirectly bound to a second polypeptide comprising an acceptor molecule, and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the polypeptide and/or the second polypeptide.

In a further alternate embodiment, the polypeptide comprises the subunit and the acceptor molecule, and the polypeptide is directly or indirectly bound to a second polypeptide comprising a bioluminescent protein, and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the polypeptide and/or the second polypeptide.

In the above two embodiments, the second polypeptide either comprises an G protein coupled receptor subunit (to form a homo- or hetero-dimer or higher multimer as defined herein) or an accessory molecule.

In a further aspect, the present invention provides a purified and/or recombinant polypeptide for detecting a compound, the polypeptide comprising,

- i) an accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor, and
- ii) a bioluminescent protein and/or an acceptor molecule,

wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.

As the skilled reader will appreciate, many of the above embodiments relating to the methods of detection also apply to the polypeptide aspects of the invention.

Also provided is an isolated and/or exogenous polynucleotide encoding a polypeptide of the invention.

In an embodiment, the polynucleotide comprises:

- i) a nucleotide sequence as provided in any one of SEQ ID NO's 7 to 12, 14, 16, 18, 20, 51 or 53, or
- ii) a nucleotide sequence which is at least 40% identical to any one or more of SEQ ID NO's 7 to 12, 14, 16, 18, 20, 51 or 53.

In another aspect, provided is a vector comprising a polynucleotide of the invention. In a preferred embodiment, the polynucleotide is operably linked to a promoter.

In a further aspect, provided is a host cell comprising the polynucleotide of the invention and/or the vector of the invention.

Preferably, the host cell produces, and hence comprises, a polypeptide of the invention.

The host cell can be any cell type.

The present inventors are also the first to show the functional expression of a nematode odorant receptor, which are evolutionarily different to mammalian odorant receptors, in yeast membranes. Thus, in a preferred embodiment the host cell is a yeast cell.

In a further aspect, the present invention provides a composition comprising the polypeptide of the invention, the polynucleotide of the invention, the vector of the invention, and/or the host cell of the invention.

Preferably, the polypeptide is embedded in a lipid bilayer.

Also provided is a cell-free composition comprising,

i) the polypeptide of the invention comprising a G protein coupled receptor subunit, wherein the polypeptide is embedded in a lipid bilayer, and/or

ii) the polypeptide of the invention comprising an accessory molecule which is directly or indirectly bound to an intracellular loop and/or the C-terminus of a G protein coupled receptor, wherein the G protein coupled receptor is embedded in a lipid bilayer, and wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.

Preferably, the lipid bilayer is a yeast lipid bilayer.

In an embodiment, the cell-free composition further comprises a substrate of the bioluminescent protein and/or a co-factor of the bioluminescent protein.

As the skilled reader will appreciate, many of the above embodiments relating to the methods of detection also apply to the cell-free aspect of the invention.

In a further aspect, the present invention provides a method of producing a cell-free composition of the invention, the method comprising obtaining a cell of the invention and disrupting the membrane of the cells.

In an embodiment, the cells are permeabilized or lysed.

In an embodiment, the cells are lysed in a French press.

In another aspect, the present invention provides a biosensor comprising a polypeptide of the invention, a host cell of the invention, a composition of the invention and/or a cell-free composition of the invention.

In a further aspect, the present invention provides a method for screening for a compound that binds a G protein coupled receptor, the method comprising,

i) contacting a candidate compound with a cell-free composition comprising

a) at least one G protein coupled receptor embedded in a lipid bilayer, and which is capable of binding the compound, and

b) optionally at least one accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor, wherein the G protein coupled receptor comprises one or more subunits that are the same or different, and wherein the G protein coupled receptor, and/or accessory molecule when present, in combination comprise a bioluminescent protein and an acceptor molecule,

ii) simultaneously or sequentially with step i) providing a substrate of the bioluminescent protein, and allowing the bioluminescent protein to modify the substrate,

iii) determining if step ii) modulates bioluminescent resonance energy transfer (BRET) between the bioluminescent protein and the acceptor molecule,

wherein a modulation of BRET indicates that the compound binds the G protein coupled receptor, and wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.

As the skilled reader will appreciate, many of the above embodiments relating to the methods of detection also apply to the method of screening aspect of the invention.

In a further aspect, the present invention provides a kit comprising a polypeptide of the invention, a polynucleotide of the invention, a vector of the invention, a host cell of the invention, a composition of the invention, a cell-free composition of the invention, and/or a biosensor of the invention.

As will be apparent, preferred features and characteristics of one aspect of the invention are applicable to many other aspects of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1. Principle of resonance energy transfer in ODR-10 receptor fused to GFP² and RLuc. GFP² is inserted in the third intracellular loop of the GPCR and RLuc to the C-terminus. Diacetyl binding could result in a conformational change resulting in a decrease (top right), or increase (bottom right), in the distance between RLuc and GFP² or a change in the relative orientations of their chromophore dipoles, which would alter the BRET² signal in a dose-dependent manner.

Figure 2. The response of OGOR to (A) μM (mean $\pm$ SEM, n=2) and (B) nM (mean $\pm$ SEM, n=6) concentration of ligand or water (* denotes a significant different ($P \leq 0.05$) compared to water).

Figure 3. The change in BRET² ratio (%) of OGOR to (A) μM (mean $\pm$ SEM, n=2) and (B) nM (mean $\pm$ SEM, n=6) concentration of ligand compared to water alone.

Figure 4. Diacetyl concentration dependence of the OGOR BRET² response (mean $\pm$ SEM, n=12)

Figure 5. Comparison of the response of OGOR (mean $\pm$ SEM, n=3) and the OGOR mutant (mean $\pm$ SEM, n=6) to water or one μ M of diacetyl in water.

Figure 6. Comparison of the response of OCOY (mean $\pm$ SEM, n=5) and OGOR (mean $\pm$ SEM, n=3) to water or one μ M of diacetyl in water.

Figure 7. Spectral scans of OCOY upon the addition of different concentrations of diacetyl. Top: Raw data. Bottom: Normalized (500 nm) data.

Figure 8. Normalized dose response curve for ODR-10 tagged with both FRET (mean $\pm$ SEM, n=5) and BRET² (mean $\pm$ SEM, n=12).

Figure 9. The response of TGTR to 10 μ L (mean $\pm$ SD, n=3) of OP50 bacterial supernatant (LB) or OP50 bacterial culture in LB compared to LB alone (* denotes a significant different ($P \leq 0.05$) compared to LB).

Figure 10. The response of TGTR to μ M (mean $\pm$ SD, n=3) concentration of ligand or water (* denotes a significant different ($P \leq 0.05$) compared to water).

Figure 11. ODR-10 transduction cassette. Dark – ODR-10 sequence, Light – variable receptor ligand binding domains. A) N-terminal fixed in cassette, B) N-terminal variable OR sequence. Rluciferase denoted as emitting at 410nm, and GFP² denoted as emitting at 515nm.

Figure 12. Co-expressed odr-10-Rluc and odr-10-GFP² yeast cells to show odr-10 receptor localization and tagged odr-10 expression level by confocal microscopy (Ex488 nm, Leica SP2 confocal laser scanning microscope).

Figure 13. Constitutive BRET² of nematode odr-10 in living Invscl yeast cells. All tested yeast strains were induced for expressing tagged odr-10 proteins for 24 hours at 15°C. Energy transfer measurements were performed in living cells by adding 10 μ M coelenterazine h (DeepBlueC) and measuring light emissions in a dual wavelength microplate reader with Rluc and GFP2 filter settings as described in the method. Data are means $\pm$ StD of two independent experiments.

Insvc1/odr-10-RLuc – negative control.

Invsc1/odr-10-RLuc + Invsc1/odr-10-GFP2 – OR and OG in separate cells. Cells mixed together for plate reading – BRET is not expected because the two constructs were expressed in separate cells.

Invsc1/odr-10-RLuc; odr-10-GFP2 – both constructs expressed in same cell. BRET is present, therefore dimerisation is occurring.

Figure 14. Detection of Odr-10 oligomers by immunoprecipitation. Crude membrane from Invsc1 yeast cells co-expressing the indicated receptors were solubilised and adjusted to the same amount of luciferase activity. Receptors were then immunoprecipitated with anti-GFP antibody and luciferase activity determined in precipitates. Values are presented as % of maximal amount of precipitated luciferase activity. Data are mean of two biological repeats.

Figure 15. Measured light output in the BRET² channels upon the addition of 5 μ M of coelenterazine 400a to 100 μ L of a whole-cell assay sample of 100 μ L of the cell-free membrane preparations following ultracentrifugation.

Figure 16. BRET² signal upon incubation (45 minutes) with 1 μ M of diacetyl (mean $\pm$ S.D., n=3) monitored using a whole-cell and cell-free assay systems.

KEY TO THE SEQUENCE LISTING

SEQ ID NO:1 – *C. elegans* Odr10 receptor.

SEQ ID NO:2 – *C. elegans* Str112 receptor.

SEQ ID NO:3 – *C. elegans* Str113 receptor.

SEQ ID NO:4 – *C. elegans* Str114 receptor.

SEQ ID NO:5 – *C. elegans* Str115 receptor.

SEQ ID NO:6 – *C. elegans* Str116 receptor.

SEQ ID NO:7 – ORF encoding *C. elegans* Odr10 receptor.

SEQ ID NO:8 – ORF encoding *C. elegans* Str112 receptor.

SEQ ID NO:9 – ORF encoding *C. elegans* Str113 receptor.

SEQ ID NO:10 – ORF encoding *C. elegans* Str114 receptor.

SEQ ID NO:11 – ORF encoding *C. elegans* Str115 receptor.

SEQ ID NO:12 – ORF encoding *C. elegans* Str116 receptor.

SEQ ID NO:13 – Polypeptide of the invention comprising *C. elegans* Odr10 receptor, RLuc inserted into the third intracellular loop (fifth non-transmembrane loop) and GFP inserted at the C-terminus.

SEQ ID NO:14 – Open reading frame encoding polypeptide of the invention comprising *C. elegans* Odr10 receptor, RLuc inserted into the third intracellular loop (fifth non-transmembrane loop) and GFP inserted at the C-terminus.

SEQ ID NO:15 – Polypeptide of the invention comprising *C. elegans* Odr10 receptor, GFP inserted into the third intracellular loop (fifth non-transmembrane loop) and RLuc inserted at the C-terminus.

SEQ ID NO:16 – Open reading frame encoding polypeptide of the invention comprising *C. elegans* Odr10 receptor, GFP inserted into the third intracellular loop (fifth non-transmembrane loop) and RLuc inserted at the C-terminus.

SEQ ID NO:17 – Polypeptide of the invention comprising *C. elegans* Odr10 receptor and RLuc inserted at the C-terminus.

SEQ ID NO:18 – Open reading frame encoding polypeptide of the invention comprising *C. elegans* Odr10 receptor RLuc inserted at the C-terminus.

SEQ ID NO:19 – Polypeptide of the invention comprising *C. elegans* Odr10 receptor and GFP2 inserted at the C-terminus.

SEQ ID NO:20 – Open reading frame encoding polypeptide of the invention comprising *C. elegans* Odr10 receptor GFP2 inserted at the C-terminus.

SEQ ID NO's 21 to 44 – Oligonucleotide primers.

SEQ ID NO:45 – mCitrine derivative.

SEQ ID NO:46 – mCFP derivative.

SEQ ID NO:47 - Open reading frame encoding mCitrine derivative.

SEQ ID NO:48 - Open reading frame encoding mCFP derivative.

SEQ ID NO:49 - Open reading frame encoding Odr10 FRET dual labelled fusion protein.

SEQ ID NO:50 - Odr10 FRET dual labelled fusion protein.

SEQ ID NO:51 – Open reading frame encoding Str112 BRET dual labelled fusion protein.

SEQ ID NO:52 - Str112 BRET dual labelled fusion protein.

SEQ ID NO:53 – Open reading frame encoding mouse α_{2A} adrenergic receptor BRET dual labelled fusion protein.

SEQ ID NO:54 - Mouse α_{2A} adrenergic receptor BRET dual labelled fusion protein.

SEQ ID NO:55 - Open reading frame encoding mouse α_{2A} adrenergic receptor.

SEQ ID NO:56 - Mouse α_{2A} adrenergic receptor.

DETAILED DESCRIPTION OF THE INVENTION**General Techniques and Definitions**

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, biosensors, G-coupled protein receptor biology, immunology, immunohistochemistry, protein chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, *A Practical Guide to Molecular Cloning*, John Wiley and Sons (1984), J. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), *Essential Molecular Biology: A Practical Approach*, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), *DNA Cloning: A Practical Approach*, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), *Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) *Antibodies: A Laboratory Manual*, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan *et al.* (editors) *Current Protocols in Immunology*, John Wiley & Sons (including all updates until present).

As used herein, unless specified otherwise, the term "G protein coupled receptor" refers to a seven transmembrane receptor which signals through G proteins. The receptor may be a single subunit, or two or more receptor subunits. When two or more receptor subunits are present they may be the same, different, or a combination thereof (for example, two of one subunit and a single of another subunit). Furthermore, unless specified or implied otherwise the terms "G protein coupled receptor" and "subunit of a G protein coupled receptor", or variations thereof, are used interchangeably.

As used herein, the term "odorant receptor", "olfactory receptor", "OR" or variations thereof refers to a polypeptide which, when present in a cell of an organism, is involved in chemosensory perception. In an embodiment, the cell is a neuron. Furthermore, the term "odorant receptor" or "olfactory receptor" refers to a polypeptide which binds an odorant ligand, or forms part of a protein complex that binds to an odorant ligand, resulting in a physiologic response.

As used herein, the term "bioluminescent protein" refers to any protein capable of acting on a suitable substrate to generate luminescence.

As used herein, the term "substrate" refers to any molecule that can be used in conjunction with a bioluminescent protein to generate or absorb luminescence.

As used herein, the phrase "allowing the bioluminescent protein to modify the substrate" refers to any enzymatic activity of the bioluminescent protein on the substrate that produces energy.

As used herein, the term "acceptor molecule" refers to any compound which can accept energy emitted as a result of the activity of a bioluminescent protein, and re-emit it as light energy.

As used herein, bioluminescent resonance energy transfer (BRET) is a proximity assay based on the non-radioactive transfer of energy between the bioluminescent protein donor and the acceptor molecule.

As used herein, the terms "modulate" or "modulation" or variations thereof refer to an alteration in the intensity and/or emission spectra of the bioluminescent protein and/or acceptor molecule.

As used herein, the term "spatial location" refers to the three dimensional positioning of the bioluminescent protein relative to the acceptor molecule which changes as a result of the compound binding a polypeptide defined herein comprising a G protein coupled receptor.

As used herein, the term "dipole orientation" refers to the direction in three-dimensional space of the dipole moment associated either with the bioluminescent protein and/or the acceptor molecule relative their orientation in three-dimensional space. The dipole moment is a consequence of a variation in electrical charge over a molecule.

As used herein, the term "more sensitive" refers to a greater change in resonance energy transfer ratio between the ligand unbound form to the ligand bound form of one reporter system (for example, BRET) to another reporter system (for example, FRET). As used herein, the term "reporter system" refers to a system comprising a polypeptide of the invention containing an isolated and/or recombinant polypeptide of the invention comprising a G protein coupled receptor subunit. Preferably, the cell-free composition comprises no live cells, whether they comprise a polypeptide of the invention or not.

As used herein, the term "contacting" refers to the addition of a sample comprising, or which may comprise, the compound to be detected in a way that the compound is capable of binding the G protein coupled receptor.

As used herein, the term "lipid bilayer" refers to two layers of, typically amphiphilic, molecular arrays arranged opposite to each other with a common

hydrophobic bilayer interior and two hydrophilic surfaces. The lipid bilayer can be naturally occurring or artificial. Most preferably, the lipid bilayer is a cellular or bio-membrane into which a polypeptide defined herein comprising an G protein coupled receptor is inserted, for example a mammalian, insect, plant or yeast cell membrane, most preferably of a yeast cell membrane.

As used herein, the term "yeast lipid bilayer" refers to the lipid bilayer being derived from a yeast cell expressing a polypeptide defined herein comprising an G protein coupled receptor. The skilled person can readily determine if a lipid bilayer is derived from a yeast by detecting proteins naturally occurring in yeast which are embedded in the membrane such as, but not limited to, Fus1p protein (Trueheart and Fink, 1989), SNARE complex which comprises the Sso1/2p, Sncip and Sec9p proteins (Strop et al., 2007), pheromone receptor Ste2p (Celic et al., 2003), and/or Alr1 (Graschopf et al., 2001).

As used herein, the term "simultaneously or sequentially" means that the substrate can be provided before, during or after the sample is contacted with a polypeptide as defined herein. Preferably, the substrate is provided at the same time or after the sample.

The "sample" can be any substance or composition suspected of comprising a compound to be detected. Examples of samples include air, liquid, biological material and soil. The sample may be obtained directly from the environment or source, or may be at least partially purified by a suitable procedure before a method of the invention is performed.

As used herein, the term "G protein coupled receptor, and/or accessory molecule when present, in combination comprise a bioluminescent protein and an acceptor molecule" means that the bioluminescent protein is associated, preferably covalently attached, more preferably produced as a fusion protein, with a subunit of the G protein coupled receptor or with an accessory molecule, and the acceptor molecule is associated, preferably covalently attached, more preferably produced as a fusion protein, with a subunit of the G protein coupled receptor or with an accessory molecule. The bioluminescent protein and accessory molecule may be associated, preferably covalently attached, more preferably produced as a fusion protein, with the same or different subunits of the G protein coupled receptor or the same or different accessory molecules. Furthermore, one of the bioluminescent protein and accessory molecule may be associated, preferably covalently attached, more preferably produced as a fusion protein, with a subunit of the G protein coupled receptor, and the other of the bioluminescent protein and accessory molecule may be associated with an accessory

molecule. In addition, multiple bioluminescent protein and accessory molecule pairs may be present in the combination for multiplexing to detect different ligands, or increase the sensitivity of the detection of the same ligand.

As used herein, the term "forms part of" refers to the bioluminescent protein or acceptor molecule being located within the specified region of the G protein coupled receptor, or subunit thereof. This term also includes the possibility that the bioluminescent protein and/or acceptor molecule is attached to or binds the G protein coupled receptor but does not form a continuous chain of amino acids. In one embodiment, the bioluminescent protein or acceptor molecule completely replaces the specified region of the G protein coupled receptor. In another embodiment, some, but not all, of the specified region of the G protein coupled receptor is replaced. In yet another embodiment, none of the specified region of the G protein coupled receptor is replaced. As the skilled addressee will appreciate, the bioluminescent protein or acceptor molecule will not be inserted such that it makes the G protein coupled receptor portion of a polypeptide of the invention incapable of binding the target compound to result in a spatial change to the location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule.

As used herein, the term "at least a majority" of a specified portion (domain) of a G protein coupled receptor, refers to at least 51%, more preferably at least 75% and even more preferably at least 90% of the specified region.

By "substantially purified" or "purified" we mean a polypeptide that has been separated from one or more lipids, nucleic acids, other polypeptides, or other contaminating molecules with which it is associated in its native state. It is preferred that the substantially purified polypeptide is at least 60% free, more preferably at least 75% free, and more preferably at least 90% free from other components with which it is naturally associated. However, at present there is no evidence that the polypeptides of the invention exist in nature.

The term "recombinant" in the context of a polypeptide refers to the polypeptide when produced by a cell, or in a cell-free expression system, in an altered amount or at an altered rate compared to its native state. In one embodiment, the cell is a cell that does not naturally produce the polypeptide. However, the cell may be a cell which comprises a non-endogenous gene that causes an altered, preferably increased, amount of the polypeptide to be produced. A recombinant polypeptide of the invention includes polypeptides which have not been separated from other components of the transgenic (recombinant) cell, or cell-free expression system, in which it is produced,

and polypeptides produced in such cells or cell-free systems which are subsequently purified away from at least some other components.

The terms "polypeptide" and "protein" are generally used interchangeably and refer to a single polypeptide chain which may or may not be modified by addition of non-amino acid groups. It would be understood that such polypeptide chains may associate with other polypeptides or proteins or other molecules such as co-factors. The terms "proteins" and "polypeptides" as used herein also include variants, mutants, biologically active fragments, modifications, analogous and/or derivatives of the polypeptides described herein.

The % identity of a polypeptide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. The query sequence is at least 25 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 25 amino acids. More preferably, the query sequence is at least 50 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 50 amino acids. More preferably, the query sequence is at least 100 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 100 amino acids. Even more preferably, the query sequence is at least 250 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 250 amino acids. Even more preferably, the GAP analysis aligns the two sequences over their entire length.

As used herein, a "biologically active fragment" is a portion of a polypeptide as described herein which maintains a defined activity of the full-length polypeptide. For example, a biologically active fragment of a G protein coupled receptor must be capable of binding the target compound resulting in a conformational change. Biologically active fragments can be any size as long as they maintain the defined activity. Preferably, biologically active fragments are at least 150, more preferably at least 250 amino acids in length.

As used herein, a "biologically active variant" is a molecule which differs from a naturally occurring and/or defined molecule by one or more amino acids but maintains a defined activity, such as defined above for biologically active fragments. Biologically active variants are typically least 50%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97%, and even more preferably at least 99% identical to the naturally occurring and/or defined molecule.

With regard to a defined polypeptide or polynucleotide, it will be appreciated that % identity figures higher than those provided above will encompass preferred

embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that the polypeptide or polynucleotide comprises an amino acid sequence which is at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

By an "isolated polynucleotide", including DNA, RNA, or a combination of these, single or double stranded, in the sense or antisense orientation or a combination of both, we mean a polynucleotide which is at least partially separated from the polynucleotide sequences with which it is associated or linked in its native state. Preferably, the isolated polynucleotide is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components with which they are naturally associated.

The term "exogenous" in the context of a polynucleotide refers to the polynucleotide when present in a cell, or in a cell-free expression system, in an altered amount compared to its native state. In one embodiment, the cell is a cell that does not naturally comprise the polynucleotide. However, the cell may be a cell which comprises a non-endogenous polynucleotide resulting in an altered, preferably increased, amount of production of the encoded polypeptide. An exogenous polynucleotide of the invention includes polynucleotides which have not been separated from other components of the transgenic (recombinant) cell, or cell-free expression system, in which it is present, and polynucleotides produced in such cells or cell-free systems which are subsequently purified away from at least some other components.

Furthermore, the term "polynucleotide" is used interchangeably herein with the term "nucleic acid". Polynucleotides of the present invention may possess, when compared to molecules provided herewith, one or more mutations which are deletions, insertions, or substitutions of nucleotide residues, or modified residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the nucleic acid). Usually,

monomers of a polynucleotide are linked by phosphodiester bonds or analogs thereof. Analogs of phosphodiester linkages include: phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate and phosphoramidate.

Compositions of the present invention may include an "acceptable carrier". Examples of such acceptable carriers include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. The exact nature of the "acceptable carrier" will depend on the use of the composition. Considering the uses described herein, and the nature of the component of the invention in the composition, the skilled person can readily determine suitable a "acceptable carrier(s)" for a particular use.

Bioluminescent Proteins

It is understood in the art that a bioluminescent protein is an enzyme which converts a substrate into an activated product which then releases energy as it relaxes. The activated product (generated by the activity of the bioluminescent protein on the substrate) is the source of the bioluminescent protein-generated luminescence that is transferred to the acceptor molecule.

There are a number of different bioluminescent proteins that can be employed in this invention (see, for example, Table 1). Given the size of bioluminescent proteins it was surprising that functional polypeptides useful for the present invention could be produced. Light-emitting systems have been known and isolated from many luminescent organisms including bacteria, protozoa, coelenterates, molluscs, fish, millipedes, flies, fungi, worms, crustaceans, and beetles, particularly click beetles of genus *Pyrophorus* and the fireflies of the genera *Photinus*, *Photuris*, and *Luciola*. Additional organisms displaying bioluminescence are listed in WO 00/024878, WO 99/049019 and Viviani (2002).

One very well known example is the class of proteins known as luciferases which catalyze an energy-yielding chemical reaction in which a specific biochemical substance, a luciferin (a naturally occurring fluorophore), is oxidized by an enzyme having a luciferase activity (Hastings, 1996). A great diversity of organisms, both prokaryotic and eukaryotic, including species of bacteria, algae, fungi, insects, fish and other marine forms can emit light energy in this manner and each has specific luciferase activities and luciferins which are chemically distinct from those of other organisms. Luciferin/luciferase systems are very diverse in form, chemistry and

function. Bioluminescent proteins with luciferase activity are thus available from a variety of sources or by a variety of means. Examples of bioluminescent proteins with luciferase activity may be found in US 5,229,285, 5,219,737, 5,843,746, 5,196,524, and 5,670,356. Two of the most widely used luciferases are: (i) *Renilla* luciferase (from *R. reniformis*), a 35 kDa protein, which uses coelenterazine as a substrate and emits light at 480 nm (Lorenz et al., 1991); and (ii) Firefly luciferase (from *Photinus pyralis*), a 61 kDa protein, which uses luciferin as a substrate and emits light at 560 nm (de Wet et al., 1987).

Table 1. Exemplary bioluminescent proteins.

<u>Species</u>	<u>Name</u>	<u>Organism</u>	<u>MW</u> <u>kDa x 10⁻³</u>	<u>Emission</u> <u>(nm)</u>	<u>Substrate</u>
Insect	FFluc	Photinus pyralis (North American Firefly)	~61	560	D-(-)-2-(6'-hydroxybenzothiazolyl)- Δ^2 -thiazoline-4-carboxylic acid, HBTTCa (C ₁₁ H ₈ N ₂ O ₃ S ₂) (luciferin)
Insect	FF'luc	Luciola cruciata (Japanese Firefly)		560-590 (many mutants)	Luciferin
Insect		Phengodid beetles (railroad worms)			
Insect		Arachnocampa sp.			Luciferin
Insect		Orphelia fultoni (North American glow worm)			
Insect	Cluc	Pyrophorus plagiophthalmus (click beetle)		546, 560, 578 and 593	Luciferin
Jellyfish	Aequorin	Aequorea	44.9	460-470	Coelenterazine
Sea pansy	Rluc	Renilla Reniformis	36	480	Coelenterazine
Sea pansy	Rluc8	Renilla reniformis	36	487	Coelenterazine

(modified)		(modified)		(peak)	/Deep Blue C
Sea pansy	Rmluc	Renilla mullerei	36.1	~480	Coelenterazine
Sea pansy		Renilla kollikeri			
Crustacea (shimp)	Vluc	Vargula hilgendorffii	~62	~460	coelenterazine *
Crustacea		Cypridina (sea firefly)	75	460	coelenterazine **
Dinofagellate (marine alga)		Gonyaulax polyedra	130	~475	Tetrapyrrole
Mollusc		Latia (fresh water limpet)	170	500	Enol formate, terpene, aldehyde
Hydroid		Obelia bicuspidata	~20	~470	Coelenterazine
Shrimp		Oplophorus gracilorostris	31	462	Coelenterazine
Others	Ptluc	Ptilosarcus		~490	Coelenterazine
	Gluc	Gaussia	~20	~475	Coelenterazine
	Plluc	Pleuromamma	22.6	~475	Coelenterazine

Gaussia luciferase (from *Gaussia princeps*) has been used in biochemical assays (Verhaegen et al., 2002). *Gaussia* luciferase is a 20 kDa protein that oxidises coelenterazine in a rapid reaction resulting in a bright light emission at 470 nm.

Luciferases useful for the present invention have also been characterized from *Anachnoca mpa* sp (WO 2007/019634). These enzymes are about 59 kDa in size and are ATP-dependent luciferases that catalyze luminescence reactions with emission spectra within the blue portion of the spectrum.

Alternative, non-luciferase, bioluminescent proteins that can be employed in this invention are any enzymes which can act on suitable substrates to generate a luminescent signal. Specific examples of such enzymes are β -galactosidase, lactamase, horseradish peroxidase, alkaline phosphatase, β -glucuronidase and β -glucosidase. Synthetic luminescent substrates for these enzymes are well known in the art and are commercially available from companies, such as Tropix Inc. (Bedford, MA, USA).

An example of a peroxidase useful for the present invention is described by Hushpulier et al. (2007).

In a preferred embodiment, a bioluminescent protein with a small molecular weight is used to prevent an inhibition of the interaction due to steric hindrance. The

bioluminescent protein preferably consists of a single polypeptide chain. Also the bioluminescent proteins preferably do not form oligomers or aggregates. The bioluminescent proteins *Renilla* luciferase, *Gaussia* luciferase and Firefly luciferase meet all or most of these criteria.

Substrates

The choice of the substrate can impact on the wavelength and the intensity of the light generated by the bioluminescent protein.

A widely known substrate is coelenterazine which occurs in cnidarians, copepods, chaetognaths, ctenophores, decapod shrimps, mysid shrimps, radiolarians and some fish taxa (Greer and Szalay, 2002). For *Renilla* luciferase for example, coelenterazine analogues/derivatives are available that result in light emission between 418 and 512 nm (Inouye et al., 1997). A coelenterazine analogue/derivative (400A, DeepBlueC) has been described emitting light at 400 nm with *Renilla* luciferase (WO 01/46691). Other examples of coelenterazine analogues/derivatives are EnduRen and ViviRen.

As used herein, the term “luciferin” refers to a class of light-emitting biological pigments found in organisms capable of bioluminescence, which are oxidised in the presence of the enzyme luciferase to produce oxyluciferin and energy in the form of light. Luciferin, or 2-(6-hydroxybenzothiazol-2-yl)-2-thiazoline-4-carboxylic acid, was first isolated from the firefly *Photinus pyralis*. Since then, various forms of luciferin have been discovered and studied from various different organisms, mainly from the ocean, for example fish and squid, however, many have been identified in land dwelling organisms, for example, worms, beetles and various other insects (Day et al., 2004; Viviani, 2002).

There are at least five general types of luciferin, which are each chemically different and catalysed by chemically and structurally different luciferases that employ a wide range of different cofactors. First, is firefly luciferin, the substrate of firefly luciferase, which requires ATP for catalysis (EC 1.13.12.7). Second, is bacterial luciferin, also found in some squid and fish, that consists of a long chain aldehyde and a reduced riboflavin phosphate. Bacterial luciferase is FMNH-dependent. Third, is dinoflagellate luciferin, a tetrapyrrolic chlorophyll derivative found in dinoflagellates (marine plankton), the organisms responsible for night-time ocean phosphorescence. Dinoflagellate luciferase catalyses the oxidation of dinoflagellate luciferin and consists of three identical and catalytically active domains. Fourth, is the imidazolopyrazine vargulin, which is found in certain ostracods and deep-sea fish, for example,

Porichthys. Last, is coelenterazine (an imidazolpyrazine), the light-emitter of the protein aequorin, found in radiolarians, ctenophores, cnidarians, squid, copepods, chaetognaths, fish and shrimp.

Acceptor Molecules

There are a number of different acceptor molecules that can be employed in this invention. The acceptor molecules may be a protein or non-proteinaceous. Examples of acceptor molecules that are protein include, but are not limited to, green fluorescent protein (GFP), blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, t-dimer2, t-dimer2(12), mRFP1, pocilloporin, *Renilla* GFP, Monster GFP, paGFP, Kaede protein or a Phycobiliprotein, or a biologically active variant or fragment of any one thereof. Examples of acceptor molecules that are not proteins include, but are not limited to, Alexa Fluor dye, Bodipy dye, Cy dye, fluorescein, dansyl, umbelliferone, fluorescent microsphere, luminescent microsphere, fluorescent nanocrystal, Marina Blue, Cascade Blue, Cascade Yellow, Pacific Blue, Oregon Green, Tetramethylrhodamine, Rhodamine, Texas Red, rare earth element chelates, or any combination or derivatives thereof.

One very well known example is the group of fluorophores that includes the green fluorescent protein from the jellyfish *Aequorea victoria* and numerous other variants (GFPs) arising from the application of molecular biology, for example mutagenesis and chimeric protein technologies (Tsien, 1998). GFPs are classified based on the distinctive component of their chromophores, each class having distinct excitation and emission wavelengths: class 1, wild-type mixture of neutral phenol and anionic phenolate: class 2, phenolate anion : class 3, neutral phenol : class 4, phenolate anion with stacked s-electron system: class 5, indole : class 6, imidazole : and class 7, phenyl.

A naturally occurring acceptor molecule which has been mutated (variants) can also be useful for the present invention. One example of an engineered system which is suitable for BRET is a *Renilla* luciferase and enhanced yellow mutant of GFP (EYFP) pairing which do not directly interact to a significant degree with one another alone in the absence of a mediating protein(s) (in this case, the G protein coupled receptor) (Xu et al., 1999).

In another embodiment, the acceptor molecule is a fluorescent nanocrystal. Nanocrystals, or “quantum dots”, have several advantages over organic molecules as fluorescent labels, including resistance to photodegradation, improved brightness, non-toxicity, and size dependent, narrow emission spectra that enables the monitoring of several processes simultaneously. Additionally, the absorption spectrum of nanocrystals is continuous above the first peak, enabling all sizes, and hence all colors, to be excited with a single excitation wavelength.

Fluorescent nanocrystals may be attached, or “bioconjugated”, to proteins in a variety of ways. For example, the surface cap of a “quantum dot” may be negatively charged with carboxylate groups from either dihydrolipoic acid (DHLA) or an amphiphilic polymer. Proteins can be conjugated to the DHLA–nanocrystals electrostatically, either directly or via a bridge consisting of a positively charged leucine zipper peptide fused to recombinant protein. The latter binds to a primary antibody with specificity for the intended target. Alternatively, antibodies, streptavidin, or other proteins are coupled covalently to the polyacrylate cap of the nanocrystal with conventional carbodiimide chemistry.

There are colloidal methods to produce nanocrystals, including cadmium selenide, cadmium sulfide, indium arsenide, and indium phosphide. These quantum dots can contain as few as 100 to 100,000 atoms within the quantum dot volume, with a diameter of 10 to 50 atoms. Some quantum dots are small regions of one material buried in another with a larger band gap. These can be so-called core-shell structures, for example, with CdSe in the core and ZnS in the shell or from special forms of silica called ormosil. The larger the dot, the redder (lower energy) its fluorescence spectrum. Conversely, smaller dots emit bluer (higher energy) light. The coloration is directly related to the energy levels of the quantum dot. Quantitatively speaking, the bandgap energy that determines the energy (and hence color) of the fluoresced light is inversely proportional to the square of the size of the quantum dot. Larger quantum dots have more energy levels which are more closely spaced. This allows the quantum dot to absorb photons containing less energy, i.e. those closer to the red end of the spectrum.

In an alternate embodiment, the acceptor molecule is a fluorescent microsphere. These are typically made from polymers, and contain fluorescent molecules (for example fluorescein GFP or YFP) incorporated into the polymer matrix, which can be conjugated to a variety of reagents. Fluorescent microspheres may be labelled internally or on the surface. Internal labelling produces very bright and stable particles with typically narrow fluorescent emission spectra. With internal labelling, surface groups remain available for conjugating ligands (for example, proteins) to the surface

of the bead. Internally-labelled beads are used extensively in imaging applications, as they display a greater resistance to photobleaching.

Carboxylate-modified fluorescent microspheres are suitable for covalent coupling of proteins using water-soluble carbodiimide reagents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). Sulfate fluorescent microspheres are relatively hydrophobic and will passively and nearly irreversibly adsorb almost any protein. Aldehyde-sulfate fluorescent microspheres are sulfate microspheres that have been modified to add surface aldehyde groups, and react with proteins.

In another embodiment, the acceptor molecule is a luminescent microsphere. These are typically made from polymers, which contain luminescent molecules (for example complexes of europium or platinum) incorporated into the polymer matrix, which can be conjugated to a variety of reagents.

Bioluminescent Protein and Acceptor Molecule Pairs

A criteria which should be considered in determining suitable pairings for BRET is the relative emission/fluorescence spectrum of the acceptor molecule compared to that of the bioluminescent protein. The emission spectrum of the bioluminescent protein should overlap with the absorbance spectrum of the acceptor molecule such that the light energy from the bioluminescent protein luminescence emission is at a wavelength that is able to excite the acceptor molecule and thereby promote acceptor molecule fluorescence when the two molecules are in a proper proximity and orientation with respect to one another. For example, it has been demonstrated that an *Renilla* luciferase/EGFP pairing is not as good as an *Renilla* luciferase/EYFP pairing based on observable emission spectral peaks (Xu, 1999; Wang, et al. (1997) in *Bioluminescence and Chemiluminescence : Molecular Reporting with Photons*, eds. Hastings et al. (Wiley, New York), pp. 419-422). To study potential pairing, protein fusions are prepared containing the selected bioluminescent protein and acceptor molecule and are tested, in the presence of an appropriate substrate.

It should also be confirmed that the bioluminescent protein and acceptor molecule do not spuriously associate with each other. This can be accomplished by separate co-expression of the bioluminescent protein and acceptor molecule in the same cells and then monitoring the luminescence spectrum in order to determine if BRET occurs. This may be achieved, for example, using the method of Xu et al. (1999). The selected bioluminescent protein and acceptor molecule form a suitable BRET pair if little or no BRET is observed.

The bioluminescent protein emission can be manipulated by modifications to the substrate. In the case of luciferases the substrate is coelenterazine. The rationale behind altering the bioluminescent protein emission is to improve the resolution between donor emission and acceptor emissions. The original BRET system uses the *Renilla* luciferase as donor, EYFP (or Topaz) as the acceptor and coelenterazine h derivative as the substrate. These components when combined in a BRET assay, generate light in the 475-480 nm range for the bioluminescent protein and the 525-530 nm range for the acceptor molecule, giving a spectral resolution of 45-55 nm.

Unfortunately, *Renilla* luciferase generates a broad emission peak overlapping substantially the GFP emission, which in turn contributes to decrease the signal to noise of the system. One BRET system of the present invention, using coel400a as the *Renilla* luciferase substrate, provides broad spectral resolution between donor and acceptor emission wavelengths (-105nm). *Renilla* luciferase with coel400a generates light between 390-400 nm and a GFP was prepared which absorbs light in this range and re-emits light at 505-508 nm. Because of this increase in spectral resolution between *Renilla* luciferase and GFP emissions, this BRET system provides an excellent biological tool to monitor small changes in conformation of a polypeptide of the invention. This is a significant improvement over the system described previously using the coelenterazine h derivative and EYFP, which has a wavelength difference between donor and acceptor of approximately 51 nm.

Various coelenterazine derivatives are known in the art, including coel400a, that generate light at various wavelengths (distinct from that generated by the wild type coelenterazine) as a result of *Renilla* luciferase activity. A worker skilled in the art would appreciate that because the light emission peak of the donor has changed, it is necessary to select an acceptor molecule which will absorb light at this wavelength and thereby permit efficient energy transfer. This can be done, for example by altering a GFP class 4 such that it becomes a class 3 or 1 GFP. Spectral overlapping between light emission of the donor and the light absorption peak of the acceptor is one condition among others for an efficient energy transfer. Class 3 and 1 GFPs are known to absorb light at 400 nm and re-emit between 505-511 nm. This results in a wavelength difference between donor and acceptor emissions of approximately 111 nm.

Examples of further bioluminescent proteins and acceptor molecule pairs are provided in Table 2.

Table 2. Exemplary BRET bioluminescent proteins and acceptor molecule pairs.

<u>BDP</u>	<u>Substrate</u>	<u>Substrate wavelength (peak)</u>	<u>Fluorescence acceptor molecule (FAM)</u>	<u>Wavelength of acceptor (Ex/Em)</u>
Rluc	Coelenterazine Wild type	470nm	Fluorescein	490/525nm
Rluc	Coelenterazine Wild type	470nm	Acridine yellow	470/550 nm
Rluc	Coelenterazine Wild type	470nm	Nile red	485/525 nm
Rluc	Coelenterazine cp	442nm	Lucifer yellow	428/540 nm
Rluc	Coelenterazine 400	400nm	Quin-2	365/490 nm
Rluc	Coelenterazine 400	400nm	Dansylchloride	380/475 nm
Firefly luciferase	Luciferin	560nm	Cyanine Cy3	575/605
Firefly luciferase	Luciferin	560nm	Texas red	590/615

G Protein Coupled Receptors

G protein-coupled receptors (GPCRs) are also known as seven transmembrane receptors, 7TM receptors, heptahelical receptors, and G protein linked receptors (GPLR). GPCRs are a large protein family of transmembrane receptors that sense molecules outside the cell and activate inside signal transduction pathways and, ultimately, cellular responses. The ligands that bind and activate these receptors include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. GPCRs are involved in many diseases, but are also the target of around half of all modern medicinal drugs. GPCRs can be grouped into at least 5 classes based on sequence homology and functional similarity:

Class A rhodopsin-like,

Class B secretin-like,

Class C metabotropic/pheromone,

Class D fungal pheromone, and

Class E cAMP receptors.

Class A Rhodopsin like receptors include: Amine receptors: Acetylcholine, Alpha Adrenoceptors, Beta Adrenoceptors, Dopamine, Histamine, Serotonin, Octopamine, and Trace amine; Peptide receptors: Angiotensin, Bombesin, Bradykinin, C5a anaphylatoxin, Fmet-leu-phe, APJ like, Interleukin-8, Chemokine receptors (C-C Chemokine, C-X-C Chemokine, BONZO receptors (CXCR6), C-X3-C Chemokine, and XC Chemokine), CCK receptors, Endothelin receptors, Melanocortin receptors, Neuropeptide Y receptors, Neurotensin receptors, Opioid receptors, Somatostatin receptors, Tachykinin receptors, (Substance P (NK1), Substance K (NK2), Neuromedin K (NK3), Tachykinin like 1, and Tachykinin like 2), Vasopressin-like receptors (Vasopressin, Oxytocin, and Conopressin), Galanin like receptors (Galanin, Allatostatin, and GPCR 54), Proteinase-activated like receptors (e.g., Thrombin), Orexin & neuropeptide FF, Urotensin II receptors, Adrenomedullin (G10D) receptors, GPR37/endothelin B-like receptors, Chemokine receptor-like receptors, and Neuromedin U receptors; Hormone protein receptors: Follicle stimulating hormone, Lutropin-choriogonadotropic hormone, Thyrotropin, and Gonadotropin; (Rhod)opsin receptors; Olfactory receptors; Prostanoid receptors: Prostaglandin, Prostacyclin, and Thromboxane; Nucleotide-like receptors: Adenosine and Purinoceptors; Cannabis receptors; Platelet activating factor receptors; Gonadotropin-releasing hormone receptors; Thyrotropin-releasing hormone & Secretagogue receptors: Thyrotropin-releasing hormone, Growth hormone secretagogue, and Growth hormone secretagogue like; Melatonin receptors; Viral receptors; Lysosphingolipid & LPA (EDG) receptors; Leukotriene B4 receptor: Leukotriene B4 receptor BLT1 and Leukotriene B4 receptor BLT2; and Class A Orphan/other receptors: Platelet ADP & KI01 receptors, SREB, Mas proto-oncogene, RDC1, ORPH, LGR like (hormone receptors), GPR, GPR45 like, Cysteinyl leukotriene, Mas-related receptors (MRGs), and GP40 like receptors.

Class B (the secretin-receptor family) of the GPCRs includes receptors for polypeptide hormones (Calcitonin, Corticotropin releasing factor, Gastric inhibitory peptide, Glucagon, Glucagon-like peptide-1,-2, Growth hormone-releasing hormone, Parathyroid hormone, PACAP, Secretin, Vasoactive intestinal polypeptide, Diuretic hormone, EMR1, Latrophilin), molecules thought to mediate intercellular interactions at the plasma membrane (Brain-specific angiogenesis inhibitor (BAI)) and a group of *Drosophila* proteins (Methuselah-like proteins) that regulate stress responses and longevity.

Class C Metabotropic glutamate/pheromone receptors include Metabotropic glutamate, Metabotropic glutamate group I, Metabotropic glutamate group II, Metabotropic glutamate group III, Metabotropic glutamate other, Extracellular calcium-sensing, Putative pheromone Receptors, GABA-B, GABA-B subtype 1, GABA-B subtype 2, and Orphan GPRC5 receptors.

GPCRs are involved in a wide variety of physiological processes, including the visual sense, the sense of smell, behavioral and mood regulation, regulation of immune system activity and inflammation, autonomic nervous system transmission, cell density sensing, and many others. It is known that the inactive G protein is bound to the receptor in its inactive state. Once the ligand is recognized, the receptor or a subunit thereof shifts conformation and thus mechanically activates the G protein, which detaches from the receptor. The receptor can now either activate another G protein, or switch back to its inactive state. It is believed that a receptor molecule exists in a conformational equilibrium between active and inactive biophysical states. The binding of ligands to the receptor may shift the equilibrium toward the active receptor states.

Polypeptides of the invention comprise G protein coupled receptors which, when expressed in a cell the N-terminus of the receptor is outside the cell and the C-terminus is inside the cell. The person skilled in the art is aware of suitable techniques for detecting the orientation of a transmembrane protein. Such techniques comprise but are not limited to crystallography, NMR-studies, modeling studies as well as microscopy techniques, like immunolabeling combined with detergent permeabilisation controls for light or electron microscopy preparation, fragment complementation tagging of two polypeptides and the like.

In a preferred embodiment, the G protein coupled receptor is a Class A GPCR. In a further preferred embodiment, the class A (rhodopsin-like) GPCR is an odorant receptor or an adrenergic receptor, more preferably an odorant receptor. The odorant receptor can be from any source as long as when expressed in a cell the N-terminus of the receptor is outside the cell and the C-terminus is inside the cell. Examples include, but are not limited to, a chordate receptor, a nematode receptor, or a biologically active variant or fragment of any one thereof. Examples of chordate receptors include, but are not limited to mammalian receptors, avian receptors and fish receptors. In a preferred embodiment, the odorant receptor is a nematode receptor or biologically active variant or fragment thereof. In an embodiment, the nematode receptor is a *Caenorhabditis elegans* receptor, or biologically active variant or fragment thereof. Examples of odorant receptors that can be used to produce polypeptides of the invention and/or used in the methods of the invention are described in Buck and Axel (1991), Robertson

(1998 and 2001), Aloni et al. (2006), Feldmesser (2006), Olender et al. (2004a and b), Glusman et al. (2000a, 2000b and 2001), Fuchs et al. (2001), Pilpel and Lancet (1999), Sharon et al. (1998), Zozulya et al. (2001), Niimura and Nei (2003), Lander et al. (2001), Zhang and Firestein (2002), Young et al. (2002). Furthermore, a comprehensive list of odorant receptors are available from the SenseLab website (<http://senselab.med.yale.edu>).

In other embodiments, the GPCR is a Class B or Class C receptor, with Class C being more preferred of these two embodiments.

In a particularly preferred embodiment, the G protein coupled receptor comprises seven transmembrane domains.

The bioluminescent protein can form part of the first, third, fifth non-transmembrane loops (domains) or the C-terminus of the G protein coupled receptor (or polypeptide of the invention). The acceptor molecule also can form part of the first, third, fifth non-transmembrane loops (domains) or the C-terminus of the G protein coupled receptor (or polypeptide of the invention). Each of these regions is intracellular when the G protein coupled receptor is expressed and present in a cell.

The acceptor molecule cannot be in the same region as the bioluminescent protein when part of the same molecule (namely, the same single polypeptide chain), however, the acceptor molecule can be in the equivalent region as the bioluminescent protein when the G protein coupled receptor is present as a dimer or higher multimer. For example, the bioluminescent protein can form part of the C-terminus of one subunit of the receptor, and the acceptor molecule can form part of the C-terminus of another subunit of the receptor. In this example, the subunit to which the label is associated can be the same or different, for instance the two subunits can be identical apart from one labelled with the bioluminescent protein and the other labelled with the acceptor molecule. In one embodiment, the bioluminescent protein forms part of the third non-transmembrane loop of the GPCR subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop. In an alternate embodiment, the acceptor molecule forms part of the third non-transmembrane loop of the GPCR subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop.

In another embodiment, the bioluminescent protein forms part of the first non-transmembrane loop of the GPCR subunit, and the acceptor molecule forms part of the third non-transmembrane loop. In another embodiment, the acceptor molecule forms part of the first non-transmembrane loop of the GPCR subunit, and the bioluminescent protein forms part of the third non-transmembrane loop.

In a preferred embodiment, the bioluminescent protein forms part of the fifth non-transmembrane loop of the GPCR subunit, and the acceptor molecule forms part of the C-terminus. In an alternate embodiment, the acceptor molecule forms part of the fifth non-transmembrane loop of the GPCR subunit, and the bioluminescent protein forms part of the C-terminus.

In another embodiment, the G protein coupled receptor comprises at least two subunits, where the bioluminescent protein forms part of the third non-transmembrane loop of a first subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop of a second subunit. In an alternate embodiment, the acceptor molecule forms part of the third non-transmembrane loop of a first subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop of a second subunit.

In another embodiment, the G protein coupled receptor comprises at least two subunits, where the bioluminescent protein forms part of the first non-transmembrane loop of a first subunit, and the acceptor molecule forms part of the third non-transmembrane loop of a second subunit. In another embodiment, the acceptor molecule forms part of the first non-transmembrane loop of a first subunit, and the bioluminescent protein forms part of the third non-transmembrane loop of a second subunit.

In another embodiment, the G protein coupled receptor comprises at least two subunits, where the bioluminescent protein forms part of the fifth non-transmembrane loop of a first subunit, and the acceptor molecule forms part of the C-terminus of a second subunit. In an alternate embodiment, the acceptor molecule forms part of the fifth non-transmembrane loop of a first subunit, and the bioluminescent protein forms part of the C-terminus of a second subunit.

In another embodiment, the G protein coupled receptor comprises at least two subunits and the donor and acceptor molecule are in the same site of the first and second subunits respectively.

In an embodiment, the bioluminescent protein or acceptor molecule is located after the second amino acid of the fifth transmembrane domain and before the second amino acid before the beginning of sixth transmembrane domain. In another embodiment, the bioluminescent protein or acceptor molecule is located after about amino acid 8 after the fifth transmembrane domain or after about amino acid 22 after the fifth transmembrane domain. In a further embodiment, the bioluminescent protein or acceptor molecule is inserted about 10 or 12 amino acids before the sixth

transmembrane domain. Most preferably, the bioluminescent protein or acceptor molecule is located in the middle of the third non-transmembrane loop (domain).

With regard to the C-terminus, it is preferred that about 5 to 25 amino acids of the natural C-terminus remain at the end of seventh transmembrane domain. Preferably, the bioluminescent protein or acceptor molecule is inserted after about the 16 or 20 amino acids after the seventh transmembrane.

Turning to the location of the bioluminescent protein or acceptor molecule in the first non-transmembrane loop (domain), it is preferred that said label is inserted about two amino acids after the end of first transmembrane domain and about two amino acids before the beginning of the second transmembrane domain. Most preferably, the bioluminescent protein or acceptor molecule is located in the middle of the first non-transmembrane loop (domain).

In a further embodiment, the bioluminescent protein can form part of the N-terminus, second, fourth, or sixth non-transmembrane loops (domains) of the G protein coupled receptor (or polypeptide of the invention). The acceptor molecule also can form part of the N-terminus, second, fourth, or sixth non-transmembrane loops (domains) of the G protein coupled receptor (or polypeptide of the invention), however, it cannot be in the same region as the bioluminescent protein when part of the same molecule. Each of these regions is extracellular when the G protein coupled receptor is expressed and present in a cell.

The GPCR may be a non-naturally occurring chimera of two or more different GPCRs. In particular, this enables a transduction cassette to be produced where portions of one receptor are always present in the chimera into which other portions of a wide variety of GPCRs are inserted depending on the compound to be detected.

In one embodiment, the subunit comprises the N-terminus and at least a majority of the first transmembrane domain of a first G protein coupled receptor subunit, at least a majority of the first non-transmembrane loop through to at least a majority of the fifth transmembrane domain of a second G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of the first G protein coupled receptor subunit.

In another embodiment, the subunit comprises the N-terminus through to at least a majority of the fifth transmembrane domain of a first G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of a second G protein coupled receptor subunit.

The skilled person can readily determine the N-terminal end, transmembrane domains, non-transmembrane loops (domains) and C-terminus of a G protein coupled.

For example, a variety of bioinformatics approaches may be used to determine the location and topology of transmembrane domains in a protein, based on its amino acid sequence and similarity with known transmembrane domain of G protein coupled receptors. Alignments and amino acid sequence comparisons are routinely performed in the art, for example, by using the BLAST program or the CLUSTAL W program. Based on alignments with known transmembrane domain-containing proteins, it is possible for one skilled in the art to predict the location of transmembrane domains. Furthermore, the 3 dimensional structures of some membrane-spanning proteins are known, for example, the seven transmembrane G-protein coupled rhodopsin photoreceptor structure has been solved by x-ray crystallography. Based on analyses and comparisons with such 3D structures, it may be possible to predict the location and topology of transmembrane domains in other membrane proteins. There are also many programs available for predicting the location and topology of transmembrane domains in proteins. For example, one may use one or a combination of the TMPred (Hofmann and Stoffel, 1993), which predicts membrane spanning proteins segments; TopPred (von Heijne et al., 1992) which predicts the topology of membrane proteins; PREDATOR (Frishman and Argos, 1997), which predicts secondary structure from single and multiple sequences; TMAP (Persson and Argos, 1994), which predicts transmembrane regions of proteins from multiply aligned sequences; and ALOM2 (Klien et al., 1984), which predicts transmembrane regions from single sequences.

In accordance with standard nomenclature, the numbering of the transmembrane domains and non-transmembrane loops (domains) is relative to the N-terminus of the polypeptide.

Amino acid sequence mutants/variants of naturally occurring G protein coupled receptors can be prepared by introducing appropriate nucleotide changes into the encoding polynucleotide, or by *in vitro* synthesis of the desired polypeptide. Such mutants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final polypeptide product possesses the desired characteristics.

Mutant (variant) polypeptides can be prepared using any technique known in the art. For example, a polynucleotide described herein can be subjected to *in vitro* mutagenesis. Such *in vitro* mutagenesis techniques may include sub-cloning the polynucleotide into a suitable vector, transforming the vector into a "mutator" strain such as the *E. coli* XL-1 red (Stratagene) and propagating the transformed bacteria for a suitable number of generations. In another example, the polynucleotides encoding G

protein coupled receptors are subjected to DNA shuffling techniques as broadly described by Harayama (1998). Products derived from mutated/variant DNA can readily be screened using techniques described herein to determine if they are useful for the methods of the invention.

In designing amino acid sequence mutants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting other residues adjacent to the located site.

Amino acid sequence deletions generally range from about 1 to 15 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Substitution mutants have at least one amino acid residue in the G protein coupled receptor removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as important for function. Other sites of interest are those in which particular residues obtained from various strains or species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are preferably substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 3.

Furthermore, if desired, unnatural amino acids or chemical amino acid analogues can be introduced as a substitution or addition into a polypeptide described herein. Such amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α -amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, C α -methyl amino acids, N α -methyl amino acids, and amino acid analogues in general.

Also included within the scope of the invention are polypeptides which are differentially modified during or after synthesis, e.g., by biotinylation, benzylation, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. These modifications may serve to increase the stability and/or bioactivity of the polypeptide.

Table 3. Exemplary substitutions.

Original Residue	Exemplary Substitutions
Ala (A)	val; leu; ile; gly
Arg (R)	lys
Asn (N)	gln; his
Asp (D)	glu
Cys (C)	ser
Gln (Q)	asn; his
Glu (E)	asp
Gly (G)	pro, ala
His (H)	asn; gln
Ile (I)	leu; val; ala
Leu (L)	ile; val; met; ala; phe
Lys (K)	arg
Met (M)	leu; phe
Phe (F)	leu; val; ala
Pro (P)	gly
Ser (S)	thr
Thr (T)	ser
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu; met; phe; ala

Polypeptides described herein can be produced in a variety of ways, including production and recovery of recombinant polypeptides, and chemical synthesis of the polypeptides. In one embodiment, an isolated polypeptide of the present invention is produced by culturing a cell capable of expressing the polypeptide under conditions effective to produce the polypeptide, and recovering the polypeptide. A preferred cell to culture is a recombinant cell of the present invention. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit polypeptide production. An effective medium refers to any

medium in which a cell is cultured to produce a polypeptide of the present invention. Such medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Accessory Molecules

An accessory molecule is a protein which is involved in G coupled protein receptor signalling. Examples include G proteins and arrestins. Any labelled accessory molecule can be used in the methods of the present invention as long as they directly or indirectly associate with the G protein coupled receptor (and/or polypeptide defined herein) and following binding of the compound to the G protein coupled receptor there is a spatial alteration in the relative position of the two labels to allow for BRET detection.

The skilled person can readily identify accessory molecules that associate with a specific G protein coupled receptor using standard techniques in the art.

Details of mutants (variants) described above for G protein coupled receptors also apply to the accessory molecules.

The bioluminescent protein or acceptor molecule can be located anywhere in the accessory molecule as long as the accessory molecule is still able to directly or indirectly bind the receptor, and following binding of a compound of interest a change in BRET can be detected. In an embodiment, the bioluminescent protein or acceptor molecule is located at the C-terminus or N-terminus of the accessory molecule. The accessory molecule indirectly binds the receptor if it requires at least one other accessory molecule to form a complex with the GPCR, for instance a first accessory molecule binds the receptor and a second accessory molecule indirectly binds the receptor by directly binding to the first accessory molecule.

G proteins

G-proteins are a family of proteins involved in second messenger cascades for intracellular signaling. G proteins function as "molecular switches," alternating between an inactive guanosine diphosphate (GDP) bound state and an active guanosine triphosphate (GTP) bound state. Ultimately, G proteins regulate downstream cell

processes by initiating cascades of signal transduction networks Hofmann et al., 2009; Oldham and Hamm, 2008).

There are two distinct families of G proteins: Heterotrimeric G proteins, sometimes referred to as the "large" G proteins, that are activated by G protein-coupled receptors and made up of alpha (α), beta (β), and gamma (γ) subunits; and "small" G proteins (20-25kDa) that belong to the Ras superfamily of small GTPases. These proteins are homologous to the alpha (α) subunit found in heterotrimeric G proteins, and also bind GTP and GDP and are involved in signal transduction. In order to associate with the plasma membrane, many G proteins are covalently modified with lipids, for example heterotrimeric G protein subunits may be myristoylated, palmitoylated, or prenylated, while small G proteins may be prenylated.

As the skilled addressee will be aware, there are many known G-proteins. A number of heterotrimeric G proteins have been identified in nematodes, for example *C. elegans* has 21 $G\alpha$, 2 $G\beta$ and 2 $G\gamma$ genes (Jansen et al., 1999; Cuppen et al., 2003). Based on sequence similarity, mammalian $G\alpha$ subunits have been divided into four families: Gs, Gi/o, Gq and G12 (Neves et al., 2002). *C. elegans* expresses one ortholog of each of the mammalian families: GSA-1 (Gs), GOA-1 (Gi/o), EGL-30 (Gq) and GPA-12 (G12). The remaining *C. elegans* α subunits (GPA-1-11, GPA-13-17 and ODR-3) do not share sufficient homology to allow classification. The conserved $G\alpha$ subunits, with the exception of GPA-12, are expressed broadly while 14 of the new $G\alpha$ genes are expressed in subsets of chemosensory neurons.

The $G\beta$ subunit, GPB-1, as well as the $G\gamma$ subunit, GPC-2, appear to function along with the α subunits in the classic G protein heterotrimer. The remaining $G\beta$ subunit, GPB-2, is thought to regulate the function of certain RGS proteins, while the remaining $G\gamma$ subunit, GPC-1, has a restricted role in chemosensation. The functional difference for most G protein pathways in *C. elegans*, therefore, resides in the α subunit.

Arrestins

Arrestins are one of the key proteins for the termination of G protein signaling. Activated GPCRs are specifically phosphorylated by G protein-coupled receptor kinases (GRKs) and then bind to arrestins to preclude the receptor/G protein interaction, resulting in quenching of the following signal transduction. Arrestins bind GPCRs, including those that have been agonist-activated and bind more tightly to those that have been phosphorylated by GRKs than those that are not.

Two distinct patterns of arrestin trafficking within the cell have been delineated resulting in the classification of GPCRs as follows: Class A where arrestin interacts with the receptor at the cell surface but does not endocytose into vesicles, thus showing a transient interaction with the receptor, and class B in which R-arrestins and receptor traffic together from the cell membrane to endocytic vesicles. These two classes of receptors also differ with regard to their affinity for different arrestin isoforms. In addition, Class A receptors preferentially bind R-arrestin2 whereas class B receptors bind to β -arrestin1 and H-arrestin2 with equal affinity.

R-arrestin-binding leads to the uncoupling of the receptor from its cognate G-proteins, causing dampening or desensitization of GPCR signaling via the downstream second messenger molecules.

While terminating G-protein signals, arrestin binding can initiate new signaling from GPCRs. For example, R-arrestins serve as adaptors, which bring non-receptor tyrosine kinases, such as Src, to form signaling complexes with the internalizing receptor. H-arrestins function as GPCR-regulated scaffolds for MAPK modules such as ASK-MKK4-JNK3 and RAF-MEK- ERK1/2. In addition, arrestins interact with proteins of the endocytic machinery, such as clathrin, β -adaptin subunit2 of the AP2 complex, and Arf-6 and thus promote internalization of receptors via clathrin-coated vesicles.

An example of a nematode arrestin is ARR-1 from *C. elegans*, which is primarily expressed in the nervous system, including the HSN neuron and various chemosensory neurons, involved in detecting soluble and volatile odorants. Over 20 putative arrestins have now been identified in the nematode *C. elegans*. Such arrestins are expressed throughout the nervous system and support receptor internalization, similarly to the vertebrate non-visual arrestins, yet participate in olfaction and vision, similarly to the visual/sensory subtypes.

Recombinant Vectors

One embodiment of the present invention includes a recombinant vector, which comprises at least one isolated/exogenous polynucleotide encoding a polypeptide as described herein, inserted into any vector capable of delivering the polynucleotide molecule into a host cell. Such a vector contains heterologous polynucleotide sequences, that are polynucleotide sequences that are not naturally found adjacent to polynucleotide molecules of the present invention and that preferably are derived from a species other than the species from which the polynucleotide molecule(s) are derived.

The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a transposon (such as described in US 5,792,294), a virus or a plasmid.

One type of recombinant vector comprises the polynucleotide(s) operably linked to an expression vector. The phrase operably linked refers to insertion of a polynucleotide molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified polynucleotide molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors include any vectors that function (i.e., direct gene expression) in recombinant cells, including in bacterial, fungal, endoparasite, arthropod, animal, and plant cells. Vectors of the invention can also be used to produce the polypeptide in a cell-free expression system, such systems are well known in the art.

"Operably linked" as used herein refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of transcriptional regulatory element to a transcribed sequence. For example, a promoter is operably linked to a coding sequence, such as a polynucleotide defined herein, if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell and/or in a cell-free expression system. Generally, promoter transcriptional regulatory elements that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are *cis*-acting. However, some transcriptional regulatory elements, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of polynucleotide molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of

such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, arthropod, nematode, plant or animal cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, *oxy-pro*, *omp/lpp*, *rrnB*, bacteriophage lambda, bacteriophage T7, T7lac, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha-mating factor, *Pichia* alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells.

Host Cells

Another embodiment of the present invention includes a host cell transformed with one or more recombinant molecules described herein or progeny cells thereof. Transformation of a polynucleotide molecule into a cell can be accomplished by any method by which a polynucleotide molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed polynucleotide molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Suitable host cells to transform include any cell that can be transformed with a polynucleotide of the present invention. Host cells of the present invention either can be endogenously (i.e., naturally) capable of producing polypeptides described herein or can be capable of producing such polypeptides after being transformed with at least one polynucleotide molecule as described herein. Host cells of the present invention can be any cell capable of producing at least one protein defined herein, and include bacterial, fungal (including yeast), parasite, nematode, arthropod, animal and plant cells. Examples of host cells include *Salmonella*, *Escherichia*, *Bacillus*, *Listeria*, *Saccharomyces*, *Spodoptera*, *Mycobacteria*, *Trichoplusia*, BHK (baby hamster kidney) cells, MDCK cells, CRFK cells, CV-1 cells, COS (e.g., COS-7) cells, and Vero cells. Further examples of host cells are *E. coli*, including *E. coli* K-12 derivatives;

Salmonella typhi; *Salmonella typhimurium*, including attenuated strains; *Spodoptera frugiperda*; *Trichoplusia ni*; and non-tumorigenic mouse myoblast G8 cells (e.g., ATCC CRL 1246). Particularly preferred host cells are yeast cells such as, but not limited to, *Saccharomyces spp.*, in particular, *Saccharomyces cerevisiae*, *Candida spp.*, *Hansenula spp.* or *Pichia spp.*

Recombinant DNA technologies can be used to improve expression of a transformed polynucleotide molecule by manipulating, for example, the number of copies of the polynucleotide molecule within a host cell, the efficiency with which those polynucleotide molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of polynucleotide molecules of the present invention include, but are not limited to, operatively linking polynucleotide molecules to high-copy number plasmids, integration of the polynucleotide molecule into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of polynucleotide molecules of the present invention to correspond to the codon usage of the host cell, and the deletion of sequences that destabilize transcripts.

Cell-Free Compositions

Methods for preparing cell-free compositions from cells are well-known in the art and include repeated cycles of freezing and thawing, grinding, treatment of cells with ultrasound in a sonicator device, homogenization, the use of a French press, the addition of detergent and/or enzymes, glass-bead lysis, differential centrifugation, several density gradient procedures using a variety of gradient media. These techniques are familiar to the skilled worker and are dealt with in detail for example in "Current Protocols in Protein Science"; John E. Caligan; Ben M. Dunn; Hidde L. Ploegh; David W. Speicher; Paul T. Wingfield; Wiley and Sons).

For isolating or preparing cell membrane extracts, a combination of these methods is usually employed (Rieder and Emr, 2001). Generally, cells are lysed either by mechanical means, or using detergents, and the membrane fractions isolated via differential centrifugation, for example, as outlined in US 7,393,934. The method outlined in Moore (1994) is commonly used. An example of such a method can be found in US 7,320,875. Other methods are also available for the preparation of

membrane fractions, for example, aqueous two-phase affinity partitioning (Persson and Jergil, 1994).

There are also many commercial kits available for purifying cell membrane fractions, for example, Qiagen's Qproteome Plasma Membrane Protein Kit. Cells are incubated in a hypotonic buffer, causing them to swell. A mild detergent is added and the resulting cell suspension is homogenized by mechanical disruption. Intact cells, cell debris, nuclei and major organelles are removed by centrifugation. The resulting supernatant contains cytosolic proteins and microsomes, Golgi vesicles, and plasma membranes. A ligand specific for molecules on the cell membrane is added to the supernatant. The ligand binds to the cell membrane vesicles and the ligand-vesicle complexes are precipitated using magnetic beads that bind to the ligand. After washing, plasma membrane vesicles are eluted under native conditions and the ligand remains bound to the beads.

For yeast cells, a commonly used method for preparing cell membranes is to spheroplast the cells. This gives high yields, and is ideally suited to the large-scale isolation of plasma membranes. Such a method generally entails coating the negatively charged surfaces of the spheroplasts with dense cationic silica beads, which makes the membrane denser than any other membranous organelle of the cell. A washing procedure removes the excess cationic beads, followed by addition of polyacrylic acid to block the free cationic groups on the beads. The coated spheroplasts are subsequently lysed by hand homogenization in an EGTA-containing lysis buffer to prevent aggregation of membrane components. Centrifugation of the spheroplast lysate pellets the heavy plasma membrane-microbead assemblies, leaving intracellular membranous organelles in the supernatant.

Further examples of the production of cell-free composition comprising G protein coupled receptor-related polypeptides which can be used for the present invention are described in Kaiser et al. (2008).

In certain embodiments, a polypeptide of the invention comprising a GPCR are embedded in the lipid bilayer of a liposome preparation. As used herein, the term "liposome" refers to a closed vesicle comprising bilayers of amphiphilic phospholipids, for example phosphatidyl ethanolamine and cholesterol. Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (like phosphatidylethanolamine), or of pure surfactant components like DOPE (dioleoylphosphatidylethanolamine).

Liposomes containing polypeptides described herein may be created by disrupting the phospholipid membrane of cells expressing the protein in water, for

example by sonication. The phospholipids would reassemble into liposomal spheres containing a core of aqueous solution. Low shear rates would create multilamellar liposomes, which have many layers. Continued high-shear sonication would form smaller unilamellar liposomes, more suited to the application of the present invention. Experimentation to produce the optimal liposome size with optimal orientation and accessibility is within the routine capabilities of one of ordinary skill in the art.

Polypeptides described herein may also be produced and incorporated into liposomes by membrane reconstitution techniques involving dissociation in detergents, followed by their reconstitution in the presence of lipids and removal of the detergents. The liposome composition would be adjusted so as to ensure optimal orientation and accessibility of the polypeptides contained in the liposomes with binding partners (compounds). Such adjustments to achieve optimal liposome compositions are routine in the art.

Assays for BRET

In a preferred embodiment, the energy transfer occurring between the bioluminescent protein and acceptor molecule is presented as calculated ratios from the emissions measured using optical filters (one for the acceptor molecule emission and the other for the bioluminescent protein emission) that select specific wavelengths (see equation 1).

$$E_a/E_d = \text{BRET ratio} \quad (1)$$

where E_a is defined as the acceptor molecule emission intensity (emission light is selected using a specific filter adapted for the emission of the acceptor) and E_d is defined as the bioluminescent protein emission intensity (emission light is selected using a specific filter adapted for the emission of the bioluminescent protein).

It should be readily appreciated by those skilled in the art that the optical filters may be any type of filter that permits wavelength discrimination suitable for BRET. For example, optical filters used in accordance with the present invention can be interference filters, long pass filters, short pass filters, etc. Intensities (usually in counts per second (CPS) or relative luminescence units (RLU)) of the wavelengths passing through filters can be quantified using either a photo-multiplier tube (PMT) or a CCD camera. The quantified signals are subsequently used to calculate BRET ratios and represent energy transfer efficiency. The BRET ratio increases with increasing intensity of the acceptor emission.

Generally, a ratio of the acceptor emission intensity over the donor emission intensity is determined (see equation 1), which is a number expressed in arbitrary units

that reflects energy transfer efficiency. The ratio increases with an increase of energy transfer efficiency (see Xu et al., 1999).

Energy transfer efficiencies can also be represented using the inverse ratio of donor emission intensity over acceptor emission intensity (see equation 2). In this case, ratios decrease with increasing energy transfer efficiency. Prior to performing this calculation the emission intensities are corrected for the presence of background light and auto-luminescence of the substrate. This correction is generally made by subtracting the emission intensity, measured at the appropriate wavelength, from a control sample containing the substrate but no bioluminescent protein, acceptor molecule or polypeptide of the invention.

$$E_d/E_a = \text{BRET ratio} \quad (2)$$

where E_a and E_d are as defined above.

The light intensity of the bioluminescent protein and acceptor molecule emission can also be quantified using a monochromator-based instrument such as a spectrofluorometer, a charged coupled device (CCD) camera or a diode array detector. Using a spectrofluorometer, the emission scan is performed such that both bioluminescent protein and acceptor molecule emission peaks are detected upon addition of the substrate. The areas under the peaks represent the relative light intensities and are used to calculate the ratios, as outlined above. Any instrument capable of measuring lights for the bioluminescent protein and acceptor molecule from the same sample, can be used to monitor the BRET system of the present invention.

In an alternative embodiment, the acceptor molecule emission alone is suitable for effective detection and/or quantification of BRET. In this case, the energy transfer efficiency is represented using only the acceptor emission intensity. It would be readily apparent to one skilled in the art that in order to measure energy transfer, one can use the acceptor emission intensity without making any ratio calculation. This is due to the fact that ideally the acceptor molecule will emit light only if it absorbs the light transferred from the bioluminescent protein. In this case only one light filter is necessary.

In a related embodiment, the bioluminescent protein emission alone is suitable for effective detection and/or quantification of BRET. In this case, the energy transfer efficiency is calculated using only the bioluminescent protein emission intensity. It would be readily apparent to one skilled in the art that in order to measure energy transfer, one can use the donor emission intensity without making any ratio calculation. This is due to the fact that as the acceptor molecule absorbs the light transferred from

the bioluminescent protein there is a corresponding decrease in detectable emission from the bioluminescent protein. In this case only one light filter is necessary.

In an alternative embodiment, the energy transfer efficiency is represented using a ratiometric measurement which only requires one optical filter for the measurement. In this case, light intensity for the donor or the acceptor is determined using the appropriate optical filter and another measurement of the samples is made without the use of any filter (intensity of the open spectrum). In this latter measurement, total light output (for all wavelengths) is quantified. Ratio calculations are then made using either equation 3 or 4. For the equation 3, only the optical filter for the acceptor is required. For the equation 4, only the optical filter for the donor is required.

$$E_a/E_o - E_a = \text{BRET ratio or } = E_o - E_a/E_a \quad (3)$$

$$E_o - E_d/E_d = \text{BRET ratio or } = E_d/E_o - E_d \quad (4)$$

where E_a and E_d are as defined above and E_o is defined as the emission intensity for all wavelengths combined (open spectrum).

It should be readily apparent to one skilled in the art that further equations can be derived from equations 1 through 4. For example, one such derivative involves correcting for background light present at the emission wavelength for bioluminescent protein and/or acceptor molecule.

In performing a BRET assay, light emissions can be determined from each well using the BRETCount. The BRETCount instrument is a modified TopCount, wherein the TopCount is a microtiterplate scintillation and luminescence counter sold by Packard Instrument (Meriden, CT). Unlike classical counters which utilise two photomultiplier tubes (PMTs) in coincidence to eliminate background noise, TopCount employs single- PMT technology and time-resolved pulse counting for noise reduction to allow counting in standard opaque microtiterplates. The use of opaque microtiterplates can reduce optical crosstalk to negligible level. TopCount comes in various formats, including 1, 2, 6 and 12 detectors (PMTs) which allow simultaneous reading of 1, 2, 6 or 12 samples, respectively. Beside the BRETCount, other commercially available instrument are capable of performing BRET: the Victor 2 (Wallac, Finland (Perkin Elmer Life Sciences)) and the Fusion (Packard Instrument, Meriden). BRET can be performed using readers that can detect at least the acceptor molecule emission and preferably two wavelengths (for the acceptor molecule and the bioluminescent protein) or more.

In an embodiment of the invention, BRET is detected using a microfluidics device. Microfluidics devices conveniently require only an aliquot of the sample, generally not more than about 50 μL , to be transferred to the sample reservoir of the

microfluidics device. This is performed either manually or by pneumatic injection via a syringe, capillary or the like.

An automated luminescence biochip device using microfluidics may be used to perform all the necessary BRET reaction steps. Automating BRET reactions in a microfluidic biochip platform is desirable as this avoids multiple manual handling steps and reduces human time and effort in performing experiments. The microfluidics device may contain a self-contained disposable biochip with patterned microchannels and compartments having storage means for storing a plurality of samples, reagents, and substrates. The steps of transferring sequentially at least one of the samples, or reagents, and then luminescent substrate from compartments through microchannels to the reaction sites could be automated. The luminescent substrates would then react with the donor molecules resulting in luminescence, which would be detected by an optical detector. An example of a microfluidics device for detecting luminescence is described in US 6,949,377.

Uses

The present invention can be used to detect a wide variety of compounds, particularly odorants. Typically, the odorant will be a volatile organic or inorganic compound or inorganic gas that may be detected by chemosensory odorant receptors of at least one organism. These may include amine- and/or sulphydryl-containing compounds, carboxylic acids, alcohols, aldehydes, alkanes, alkenes, aromatic compounds, esters, terpenes or terpene-derivatives, ethers, CO₂ etc. as well as compounds bearing combinations of these features.

Odorants may be indicative of some biological or chemical state of value or of interest to humans. Such indications may include:

- The sensory appeal, quality or safety of food and beverages, pharmaceuticals or related materials.
- The health, nutritional or exercise status of humans or animals.
- The presence or absence of hazardous substances, including pathogens.
- The progress or status of industrial processes.
- An environmental contamination or state.
- The sensory appeal, quality or safety of perfumes, fragrances or other cosmetics.

In a particularly preferred embodiment, the compound only binds the receptor portion of a polypeptide described herein.

In a particularly preferred embodiment, the polypeptide defined herein comprising an odorant receptor is used in a biosensor to detect a compound(s) of

interest. A biosensor is a device for the detection of an analyte that combines a biological component with a physicochemical detector component. It typically consists of three parts, firstly at least one polypeptide defined herein comprising a G protein coupled receptor. Second, a transducer or detector element is required, which works in a physicochemical way (eg. optical, electrochemical) that transforms the signal resulting from the interaction of the compound with the polypeptide into another signal (i.e. transducers) that can be more easily measured and quantified. Third, an associated electronic or signal processor is required, which then displays the results of the interaction in a user-friendly way. An example of a biosensor involving BRET is described in Charest et al. (2005).

In another embodiment, the methods of screening are used for drug discovery and/or development. More specifically, the receptor is a target for potential therapeutics. Thus, in this embodiment it is preferred that the receptor is a clinically important molecule such as, but not limited to, an adrenergic receptor, a serotonin receptor, a dopamine receptor, metabotropic/glutamate receptor, a GABA receptor, a vomeronasal receptor, a taste receptor, or a secretin-like receptor.

Within the scope of the present invention are also methods for identifying, characterizing and for screening of molecules which are capable of interacting with an G protein coupled receptor which comprise so-called high-throughput screening methods and similar approaches which are known in the art (Spencer, 1998; Oldenburg, 1998; Milligan, 1999) carried out using 96-well, 384-well, 1536-well (and other) commercially available plates. Further methods to be employed in accordance with the present invention comprise, but are not limited to, homogenous fluorescence readouts in high-throughput screenings (as described, *inter alia*, in Pope, 1999).

As the skilled person would be aware, the present invention can also be multiplexed. In this system, two or more polypeptides comprising different G protein coupled receptors are provided which bind different compounds. Each different G protein coupled receptor is labelled with a different bioluminescent protein and/or acceptor molecule such that they emit at different wavelengths to enable the detection and quantification of different target compounds.

EXAMPLES**EXAMPLE 1 - DUAL LABELLED BRET ODR-10 (OGPR) CONSTRUCTS****MATERIALS AND METHODS****Design and Construction of Dual Labelled BRET OR Constructs**

BRET² receptor constructs were generated with BRET² components inserted into the third intracellular loop (IC3) and at the C-terminus. Two BRET constructs were made with; (1) GFP² in the middle of IC3 and RLuc(h) on the C-terminus; (2) OR with RLuc(h) in the middle of IC3 and GFP² on the C-terminus. The position of the third intracellular loop (IC3) was determined using TMAP on The Biology Workbench a web-based tool for prediction of transmembrane segments (<http://seqtool.sdsc.edu>). For ODR-10, the middle of the IC3 is amino acids 240-241. The amino acid sequence for this construct referred to as OGPR, is provided as SEQ ID NO:15 (encoding nucleotide sequence provided as SEQ ID NO:16).

ODR-10 was amplified from *C. elegans* cDNA, which was prepared with standard techniques using superscript II (Invitrogen). PCR conditions were as follows 94°C 2mins, 30 cycles of 94°C 15secs, 59°C 30secs, 68°C 70sec, and a final extension step of 68°C for 5min. Standard Pfx50 PCR ingredients were used with primers ODR-10Xba1F 5'-AGTCTAGAATGTCGGGAGAATTGTGGATTA-3' (SEQ ID NO: 21) and ODR-10-attb1-R 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATCACGTCGGAAGTTGAG-3' (SEQ ID NO:22). Amplified cDNA was cloned into pGEM-T (Promega) and sequenced.

The BRET components, GFP² and *Renilla* Luciferase, RLuc(h), were sourced from a commercially available plasmid pGFP²-MCS-RLuc(h) (PerkinElmer; Lot#6310051-6D1).

BRET constructs were constructed by PCR amplification of individual fragments of the construct (see Table 4 for primers and annealing temperatures). PCR was performed with Pfx50TM standard ingredients (Invitrogen) and conditions were as follows 94°C 2min, 30 cycles of 94°C 15sec, 59°C 30sec, 68°C 70sec, and a final extension step of 68°C for 5min. Separate components of the constructs were amplified using overlapping primers.

Table 4. Primers for dual tagged ODR-10 receptor constructs.

Construct	Fragment	cDNA Fragment size (bp)	Primers	Sequence	Oligo Anneal Temp (°C)
ODR-10::GFP ² @AA240-241::RLuc(h) (Cterm) (OGOR)	(5') ODR-10::GFP ²	1452	F: attB1 ODR-10Forward	AACCATGTCGGGAGAATTGTG (SEQ ID NO:23)	57.7
			R: ODR-10-MidIC3GFP ² Jt2R	TGTAGCGCTCGCTTGTACAGCTCGTCCAT (SEQ ID NO:24)	
	(3') ODR-10	321	F: ODR-10-MidIC3GFP ² Jt2F	GAGCTGTACAAGCGAGCGCTACAGAAACAA (SEQ ID NO:25)	57.3
			R: ODR-10-CtermRLuc(h)JtR	CTTGCTGGTCATCGTCGGAACCTTGAGACA (SEQ ID NO:26)	
	RLuc(h) (Cterm)	949	F: ODR-10-CtermRLuc(h)JtF	CAAGTCCGACGATGACCAGCAAGGTGTA (SEQ ID NO:27)	56.5
			R: attB2 RLuc(h)Reverse	GTTACTGCTCGTTCTTCA (SEQ ID NO:28)	
	(3') ODR-10::RLuc(h) (Cterm)	1246	F: ODR-10-MidIC3GFP ² Jt2F	GAGCTGTACAAGCGAGCGCTACAGAAACAA (SEQ ID NO:29)	55.3
			R: attB2 RLuc(h)Reverse	GTTACTGCTCGTTCTTCA (SEQ ID NO:30)	
	ODR-10::GFP ² @AA240-241::RLuc(h) (Cterm)	2675	F: attB1 ODR-10Forward	AACCATGTCGGGAGAATTGTG (SEQ ID NO:31)	53.7
			R: attB2 RLuc(h)Reverse	GTTACTGCTCGTTCTTCA (SEQ ID NO:32)	
ODR-10::RLuc(h)@AA240-241::GFP ² (Cterm) (OROG)	(5') ODR-10::RLuc(h)	1669	F: attB1 ODR-10Forward	AACCATGTCGGGAGAATTGTG (SEQ ID NO:33)	58

			R: ODR-10-MidIC3RLuc(h)Jt2R	CTGTAGCGCTCGCTGCTCGTTCTTCAG (SEQ ID NO:34)	
	(3') ODR-10	321	F: ODR-10-MidIC3RLuc(h)Jt2F	AAGAACGAGCAGCGAGCGCTACAGAAACA (SEQ ID NO:35)	57.9
			R: ODR-10-CtermGFP ² JtR	CTTGCTCACCATCGTCGGAACCTTGAGACA (SEQ ID NO:36)	
	GFP ² (Cterm)	723	F: ODR-10-CtermGFP ² JtF	CAAGTTCCGACGATGGTGAGCAAGGGCGA (SEQ ID NO:37)	56.3
			R: attB2 GFP ² Reverse	GTTACTTGTACAGCTCGTC (SEQ ID NO:38)	
	(3') Odr10::GFP ² (Cterm)	1030	F: ODR-10-MidIC3RLuc(h)Jt2F	AAGAACGAGCAGCGAGCGCTACAGAAACA (SEQ ID NO:39)	54.7
			R: attB2 GFP ² Reverse	GTTACTTGTACAGCTCGTC (SEQ ID NO:40)	
	Odr10::RLuc(h)@AA240-241::GFP ² (Cterm)	2675	F: attB1 ODR-10Forward	AACCATGTCGGGAGAATTGTG (SEQ ID NO:41)	53.0
			R: attB2 GFP ² Reverse	GTTACTTGTACAGCTCGTC (SEQ ID NO:42)	

Components were then purified and placed in a paired reaction to denature at 94°C, anneal 52°C (at overlapping primer sites) and extend 68°C for 5min for the required pairs. This was then used as template for PCR using the most 5' and 3' primers of the pairs. This was repeated until the full length constructs were made. They were then cloned into pGEM-T for sequencing. Error free clones were subcloned into pDONR201 (Invitrogen) using ApaLI. These were then subcloned into pYES-DEST52 (Invitrogen) using Gateway® technology (attB sites were included in original 5' and 3' primers) for subsequent expression in *S. cerevisiae*.

Construction of OGOR Mutant (H110Y)

Site-directed mutagenesis was carried out with OGOR in pDONR201 vector (see above) as the double stranded template. The histidine 110 to tyrosine (H110Y) mutation was performed using the Stratagene Quickchange site directed mutagenesis kit according to standard protocols using the following primers; 5'-CACCAGTTTTGTTGTCTCAGGAGTTTATTTGTTTATCGATATTTTGCAACTT-3' (WT) (SEQ ID NO:43) and 5'-AAGTTGCAAAATATCGATAAACAATAAACTCCTGAGACAACAAAACCTG GTG-3' (H110Y antisense) (SEQ ID NO:44).

PCR was performed with Pfx50™ standard ingredients (Invitrogen) and conditions were as follows 94°C 2mins, 16 cycles of 94°C 30secs, 55°C 1 minute, 68°C 6.8 minutes. Following sequencing the OGOR mutant was then subcloned into pYES-DEST52 (Invitrogen) using Gateway® technology (5' and 3' attB sites were included in original OGOR sequence) for subsequent expression in *S. cerevisiae*.

Solution Preparations

20 % (w/v) Glucose - Add 20 g of glucose to 100 mL of deionized water, heat on hot-plate (50 °C) until dissolved and filter purify (0.2 µm filters) solution.

20 % (w/v) Raffinose - Add 20 g of glucose to 100 mL of deionized water, heat on hot-plate (50 °C) until dissolved and filter purify (0.2 µm filters) solution.

20 % (w/v) Galactose - Add 20 g of galactose to 100 mL of deionized water, heat on hot-plate (50 °C) until dissolved and filter purify (0.2 µm filters) solution.

YPD medium - Add 20g of bacteriological peptone, 10g of yeast extract to 1 L of deionized water. Autoclave for 15 minutes.

YPD plates - Add 20g of bacteriological peptone, 10g of yeast extract and 20 g of agar to 1 L of deionized water. Autoclave for 15 minutes. Following autoclaving add 20 mL of 20 % glucose and pour agar plates.

Yeast Synthetic Drop-Out Media without Uracil (SCMM-U) medium - Add 6.7 g of yeast nitrogen base without amino acids (Product no. Y 0626, Sigma) and 1.92 g of yeast synthetic drop-out media supplement without uracil (Product no. Y 1501) to 1 L of deionized water. Autoclave for 15 minutes.

SCMM-U plates - Add 1.34 g of yeast nitrogen base without amino acids (Product no. Y 0626, Sigma), 0.384 g of yeast synthetic drop-out media supplement without uracil (Product no. Y 1501, Sigma) and 4 g of agar to 180 mL of deionized water. Autoclave for 15 minutes. Following autoclaving add 20 mL of 20% glucose and pour agar plates.

1 × Sodium phosphate buffer (1 × PBS, pH 7.4.) – A 10 × stock solution was prepared by dissolving 82.33 g of sodium phosphate dibasic (0.58 M), 23.45g of sodium phosphate monobasic (0.17 M) and 40 g sodium chloride (NaCl) in deionized water. The stock solution was diluted to a 1 × PBS solution in deionized water.

Coelenterazine 400a - Dissolve 1 mg of Coelenterazine 400a (Clz400a, Biosynth) in 10.20 mL of pure ethanol to make a 250 µM stock solution. Pipette 40 or 400 µL aliquots of Coelenterazine 400a into microcentrifuge tubes and then dry down with a SpeedVac[®] Plus SC110A (Savant). Store aliquots at –80°C. Reconstitute samples in 100 or 1000 µL (40 or 400 µL aliquots, respectively) of absolute ethanol to give a concentration of 50 µM.

Odorant Preparation - A 1 % stock solution of the odorants diacetyl, benzaldehyde and octanal were made up in the respective solvents water, ethanol and DMSO. The odorants were serially diluted in water to give the desired final concentration. The same dilutions of solvents without the addition of the odorants were also prepared.

Plasmid Transformation Protocol

Yeast transformations were carried out using a yeast transformation kit (YEAST-1, Sigma). A YPD plate was streaked with INVSC1 (Invitrogen) *S. cerevisiae*

strain and incubated at 28 °C for 2-3 days. Scrape a loop of InVSC1 cells from the YPD plate into a microcentrifuge tube. Resuspend cells in 0.5 mL of transformation buffer (Product code T 0809) and spin for 5 seconds. Remove the supernatant, leaving 50 - 100 µL of the buffer in the tube. Add 10 µL of 10 mg/mL salmon testes DNA (Product code D 9156) to the tube. Add 1 µg of the pYES-DEST52 – OR plasmid DNA to be studied and vortex for 10 seconds. Add 600 µL of PLATE buffer (product code 8966) and vortex. Incubate for 4 hours at room temperature. Heat shock the sample for 15 minutes in a 42 °C heat block. Spin for 3 seconds in a microcentrifuge and remove the supernatant. Resuspend cells in 500 µL of sterile water. Plate 100 µL on SCMM-U plate. Incubate at 28 °C for 2-3 days until colonies appear.

Expression Protocol

Inoculate a single colony containing the pYES-DEST52 – OR construct into 15 mL of SCMM-U media containing 2 % glucose. Grow overnight at 28 °C with shaking (200 rpm).

The OD₆₀₀ of the overnight culture was determined. The amount of overnight culture required to obtain an OD₆₀₀ of 0.4 in 30 mL of induction medium (SCMM-U, 2% galactose, 2% raffinose) was removed and the cells pelleted at 1500 x g for 5 minutes. The cells were resuspended in 1 mL of induction medium and inoculated into 29 mL of induction medium. Grow at 15 °C with shaking (200 rpm) for 72 hours. The culture was removed from the flask and the cells were centrifuged at 1500 x g for 5 minutes at 4 °C. Decant the supernatant and resuspend cells in 1 mL of sterile water. Transfer cells to a sterile microcentrifuge tube and centrifuge samples for 1 minute at 10,000 x g. Remove the supernatant and carry out membrane isolation.

Membrane Preparations

Cells were harvested by centrifugation at 1500 x g (4 °C) for 5 minutes, washed in water and resuspended in 4 mL of Dulbecco's phosphate buffered saline (D-PBS) or 1 x PBS, containing 1000 mg/L glucose, 36 mg/L sodium pyruvate, calcium and magnesium (Invitrogen). The cells were lysed by French press (~ 18000 psi) and the soluble protein fractions were isolated by centrifugation at 9300 x g (4 °C) for 15 minutes. Following this the soluble protein fraction was transferred to 13.15 mL polycarbonate thick wall centrifuge tubes and ultracentrifuged at 40,000 rpm (Beckman Coulter L-80 ultra-centrifuge) for 1 hour at 4 °C. The supernatant was decanted and the membrane pellet resuspended in 1 mL of D-PBD or 1 x PBS, and left overnight at 4 °C to solubilise.

Spectral Measurements

All spectral scans were recorded with a plate-reading SpectraMax M2 spectrofluorimeter (Molecular Devices). 100 μ L aliquots of membrane preparations sample were scanned using 96-well plates (Perkin-Elmer). Fluorescence spectral scans recorded from 450 to 600 nm with an excitation wavelength of 420 nm using a 455 nm emission cut-off filter.

Simultaneous Dual Emission Detection

Simultaneous dual emission RET measurements were carried out with a POLARstar OPTIMA microplate reader (BMG LabTech). Simultaneous emission measurements used a BRET² filter set (410-80/515-30) with the set respective gains for each channel (3300/4095).

RET Analysis Protocol

Normalization of Sample Concentration

To assess the relative concentrations of BRET² tagged receptor samples the fluorescence intensity of the sample was determined from spectral scans and the sample amount normalized by the OGOR fluorescence intensity. To convert GFP² intensity into concentration purified GFP² protein was assayed at different concentrations to produce a calibration graph relating concentration to fluorescence intensity. GFP² proteins were purified using cobalt affinity chromatography according to the supplied instructions (BD Talon (BD Biosciences, Clontech)). Rearranging the line of best fit equation ($y = 271x + 233.31$) results in conversion of fluorescence intensity to concentration. An estimated concentration of 5 or 10 nM was used for assays in a final volume of 100 μ L.

Selectivity

RET analysis was carried out in 96-well plates (Perkin-Elmer) and involved incubating the specified dual tagged receptor protein (OGOR) for 45 minutes with 1 μ M of each particular odorant. The final volume was 100 μ L and included 10 μ L of odorant, receptor protein sample (see '*Normalization of sample concentration*' section), D-PBS (or 1 x PBS) and 5 μ L of Clz400a (5 μ M). A background signal was recorded by assaying a sample of D-PBS (or 1 x PBS). During the incubation time the 96-well plate wells were sealed with TopsealTM - A (Packard) with each sample area individually sealed. Non odorants samples (water, pyruvate, citric acid, lactic acid)

were prepared and assayed first. Following this odorants solutions were prepared in the fume hood and assayed in the following order: 2-butanone, 2,3 – pentanedione, 2,3-butanediol and diacetyl.

For BRET² measurements Clz400a substrate (5 μ M) was added following the 45 minute incubation time and the signal recorded using a 0.50s integration time over a 5 second period. Following addition of Clz400a the well was re-sealed while the signal was recorded to reduce cross-contamination of samples. The assays were sequentially carried out in the order the samples were added to the wells.

BRET² ratio is calculated as emission ratio measured as ((emission at 515 nm for receptor sample)-(emission at 515 nm for D-PBD (or 1 x PBS)))/(emission at 410 nm for receptor sample)- (emission at 410 nm for D-PBD) (or 1 x PBS)).

Dose Response Curve

The OGOR concentration was normalized to 10 nM by GFP² intensity. A dose response curve was constructed by varying odorant concentrations over the concentration range as indicated in the text. The lowest concentration was always assayed first and the highest last.

A sigmoidal dose response curve was fitted, and EC₅₀ calculated, by non-linear regression analysis using GraphPad Prism for Windows XP.

RESULTS

Ligand Response and Selectivity

The constructs produced have the BRET² donor and acceptor inserted within the third intracellular loop and at the C-terminus of the odorant receptor (Figure 1). Diacetyl binding by ODR-10 would cause an increase in the BRET² ratio if the donor and acceptor components moved apart or an increase in the signal if the components moved closer together.

The BRET² signal was recorded following incubation of OGOR with 10 nM or 1 μ M of the following ligands: water, pyruvate, citric acid, lactic acid, 2-butanone, 2,3 – pentanedione, 2,3-butanediol and diacetyl (Figures 2a and b). The largest change in BRET² signal occurred upon incubating OGOR with μ M concentrations of diacetyl, with a 37.5 % difference in the signal compared to the solvent (water) response (Figure 3a). This is the first example of monitoring ligand binding using a dual-labelled BRET² tagged receptor in a cell-free assay format.

The change in the BRET² ratio upon addition of both nM and μ M concentrations of diacetyl to OGOR was significantly different ($P > 0.05$) to the control

response (water) (Figures 3a and b). The response of OGOR to diacetyl stimulation was the only response which was significantly different to the control response compared to all other ligands tested at both nM and μ M concentrations. These results confirm that ODR-10 selectively binds diacetyl. Diacetyl was previously confirmed to be the only volatile compound to produce a Ca^{2+} elevation response in ODR-10 expressing HEK 293 cells (Zhang et al., 1997).

The largest change in the BRET^2 response to ligand binding of 37.5% is substantially greater than the observed change in the FRET signal ($\sim 5\%$) upon binding of 10 μ M norepinephrine by the α_{2A} receptor (Lohse et al., 2003) and 1 μ M PTH by PTHR ($\sim 20\%$) (Villardaga et al., 2003). The decrease in BRET^2 signal upon addition of diacetyl suggests that the BRET^2 components move apart upon odorant binding. This is the first demonstration that this BRET^2 transduction system exhibits superior sensitivity compared to a FRET transduction system for monitoring intramolecular changes.

Dose Response

The effect of changing odorant concentrations on the amplitude of the change in the BRET^2 signal of OGOR (Figure 4) provides the first demonstration of a dose-dependent response. The EC_{50} value was calculated to be 3.55 fM diacetyl, which is equivalent to 0.31 parts per quadrillion (ppq). The apparent affinity of ODR-10 for diacetyl when expressed in human cells, and monitored by calcium imaging technique, was calculated to be 2.3 μ M (~ 0.2 part per million (ppm)), an EC_{50} value consistent with chemotaxis results (Zhang et al., 1997).

The cell free assay presented here is more than nine orders of magnitude more sensitive for diacetyl quantification compared to whole cell assays and existing chemical detection systems such as fluorescence detection (Li et al., 2009) and a gas chromatographic technique with flame ionization detection (GC-FID) (Macciola et al., 2008).

Negative Control - OGOR Mutation (H110Y)

An ideal negative control for the OGOR response to diacetyl would place the BRET^2 components the same distance apart as in the OGOR receptor but the receptor would be unresponsive to diacetyl itself. Replacing the histidine with a tyrosine at position 110 in the 3rd membrane spanning residue of ODR-10 resulted in a strongly defective chemotaxis response to diacetyl (Troemel et al., 1995; Sengupta et al., 1996).

The introduction of a histidine 110 to tyrosine (H110Y) mutation to the OGOR construct reduced the response to μM concentrations of diacetyl from 32.4% for OGOR to 4.1% (Figure 5). This reduced response to diacetyl was not significantly different ($P < 0.05$) from the solvent (water) response indicating the mutation caused a loss of function. This infers that the response of OGOR to diacetyl is due to diacetyl interacting with the ODR-10 receptor and not non-specifically with the BRET² components themselves.

EXAMPLE 2 - COMPARISON OF DUAL-LABELLED FRET ODR-10 (OCOY) TO DUAL-LABELLED BRET² ODR-10 (OGOR)

To compare the sensitivity of the BRET² system to the FRET system for monitoring GPCR activation the BRET² components used to tag the ODR-10 receptor (OGOR) were replaced with FRET components resulting in the insertion of CFP into the 3rd intracellular loop and YFP at the C-terminus (OCOY). The CFP (mCFP) (SEQ ID NO:45) and YFP (mCitrine) (SEQ ID NO:46) derivatives were both monomeric and codon optimized (EUROSCARF) for yeast expression (SEQ ID NOs 47 and 48 respectively).

MATERIALS AND METHODS

The OCOY sequence flanked by *KpnI* and *XhoI* restriction sites was synthesised by Genscript. The OCOY sequence was inserted into the *KpnI* and *XhoI* sites of the pENTR11 vector. These were then recombined into pYES-DEST52 (Invitrogen) using Gateway[®] technology for subsequent expression in *S. cerevisiae*. The OCOY coding sequence is provided as SEQ ID NO:49, whereas the amino acid sequence is provided as SEQ ID NO:50.

OCOY Expression

The same expression protocol was used for the expression of OCOY in InVSC1 as for OGOR expression.

Concentration Normalization

To assess the relative concentrations of dual-tagged receptor samples the fluorescence intensity of the sample was determined from spectral scans and the sample amount normalized by the OCOY fluorescence intensity. The YFP intensity (530 nm) was normalized to 721.32 a.u. for OCOY. 50 μL of OCOY was pipetted into the appropriate well and the volume made up to 100 μL with $1 \times \text{PBS}$.

RESULTS

Ligand Response

There was a 7.6% reduction in FRET ratio (intensity at 520 nm/ intensity at 480 nm) of OCOY in response to μM concentrations of diacetyl compared to a 32.4% reduction for OGOR (Figure 6). The OCOY response was shown to be significantly different ($P < 0.05$) to the solvent (water) response. OGOR was shown to be more than four times more sensitive for monitoring diacetyl binding than OCOY.

The Förster distance (R_0), the RET probe separation corresponding to 50% of the maximum RET efficiency, for the BRET² system was recently determined to be 7.5 nm, the largest R_0 value determined for any genetically encoded RET pair (Dacres et al., 2009). The R_0 value for the FRET system was previously determined to be 4.8 nm (Evers et al., 2006) indicating that the BRET² system is able to probe a larger distance range (3.8 – 11.3 nm) than the FRET system (2.4 – 7.2 nm). The measured transfer efficiency for OGOR changed from 64.3% to 47% upon the addition of diacetyl (1 μM) indicating a distance change of 6.8 nm to 7.6 nm. A system with a Förster distance of 4.8 nm, such as the FRET system, would only exhibit a transfer efficiency change of 11.3% to 6.0% upon addition of diacetyl for this distance change. This confirms that the BRET² system is more suitable, in terms of detection sensitivity, for the measurement of this distance change in this distance range.

OCOY Dose Response Curve

Following the confirmation that the OCOY response to diacetyl was significantly different to the solvent (water) response a dose response was plotted. To generate this data spectral scans were recorded upon the addition of different concentrations of diacetyl (Figure 7). As the concentration of diacetyl increased from 1×10^{-21} M to 1×10^{-10} M there was a decrease in the YFP emission intensity and an accompanying increase in CFP emission intensity (Figure 7). Scaling the OCOY response with respect to the normalized OGOR response curve (Figure 8) confirmed that BRET² detection is more sensitive than FRET detection for monitoring diacetyl binding by ODR-10. The calculated log EC50 value for the FRET detection system was -16.43 ± 0.9741 M (mean $\pm$ SEM, $n=5$) which is not significantly different ($P < 0.05$) to the calculated log EC50 value of -14.43 ± 0.5468 (mean $\pm$ SEM, $n=12$) for the BRET² system. This demonstrates that the ODR-10 affinity for diacetyl is statistically similar for both detection systems inferring that diacetyl is specifically interacting with the ODR-10 receptor and not with the RET components themselves.

EXAMPLE 3 – DUAL-TAGGED Str112 (TGTR)

MATERIALS AND METHODS

The TGTR sequence flanked by *NcoI* and *NotI* restriction sites was synthesised by Genscript. The TGTR sequence was inserted into the *NcoI* and *NotI* sites of the pENTR11 vector. These were then recombined into pYES-DEST52 (Invitrogen) using Gateway[®] technology for subsequent expression in *S. cerevisiae*.

TGTR Expression

The same expression protocol was used for the expression of TGTR in InVSC1 as for OGOR expression. The TGTR coding sequence is provided as SEQ ID NO:51, whereas the amino acid sequence is provided as SEQ ID NO:52.

RET Analysis Protocol

The same RET analysis protocol as described for the OGOR selectivity studies. 1 μ M of the ligands pyruvic acid, citric acid, ethyl acetate, acetoin and diacetyl was assayed with 10 nM of TGTR

Preparation of Bacterial Extract Assays

The bacterial strain OP50 was grown in LB overnight at 37°C with shaking (200 rpm). *E.coli* strain OP50 is usually used as a food source for nematode growth in the laboratory (Brenner, 1974). Two assays were performed using the OP50 bacterial culture. The first assay was the addition of 10 μ L of the bacterial culture or 10 μ L of LB to 10 nM TGTR. The second assay required that 1 mL of the bacterial culture was placed in a microcentrifuge tube and centrifuged at 10000 $\times$ g for 1 minute. 10 μ L of the supernatant, following decanting into a fresh microcentrifuge tube, or 10 μ L of LB was added to 10 nM TGTR. Following bacterial sample addition the same protocol was followed as for the OGOR RET analysis.

RESULTS

The response of TGTR to the addition of 10 μ L of OP50 bacterial supernatant or bacterial culture in LB was significantly different ($P \leq 0.05$) to that of LB alone (Figure 9). Stimulating the Str112 receptor to a complex odorant mixture as released from OP50 during growth causes an increase in the BRET² ratio suggesting that the BRET² components move towards each other upon odorant binding.

A number of ligands were tested individually (Figure 10) and both pyruvic acid and acetoin produced significantly higher BRET² signals ($P \leq 0.05$) compared to the response of TGTR to water. This confirms that upon ligand binding the BRET² components move towards each other. An increase of 20.8% in the BRET² signal in response to acetoin is four times greater than the response of the FRET tagged α_{2A} adrenergic receptor to norepinephrine (Lohse et al., 2003). This is the first demonstration of odorant binding by the Str112 nematode receptor and also the first example of monitoring odorant binding by BRET² tagged Str112.

EXAMPLE 4 – RECEPTOR CHIMERAS

An ODR-10 transduction cassette is engineered so the ODR-10 N-terminus (aa1-32) and IC3 to C terminus (aa225 –aa339) with BRET tags flank a multiple cloning site (Figure 11). The chimeric receptors are expressed in yeast. Ligand binding regions of ORs are shuffled “in-frame” into this cassette and these chimeric receptors can be used to assess ligand binding by BRET.

EXAMPLE 5 – OLIGOMERIZATION OF OLFACTORY RECEPTORS

Odr10 labelled with Rluc at the C-terminus (SEQ ID NO:17) and Odr10 labelled with GFP2 at the C-terminus (SEQ ID NO:19) were co-expressed in yeast to show receptor oligomerization and BRET modulation.

MATERIALS AND METHODS

Transformant Culture and Tagged-Gene Induction Conditions

A colony of transformed yeast cells was grown in yeast synthetic drop-out medium (SCMM, Sigma) lacking appropriate nutrients (such as uracil (U), tryptophan (T)) to select for plasmid-containing cells with 2% glucose for overnight at 28°C with shaking 190 rpm; SCMM-U-T for Invsc1 co-expressed *pYesDest52-Odr10-Rluc* and *pDestpESC-Odr10-GFP2* (Invsc1/OR/OG), SCMM-U for Invsc1 expresses *pVV214-odr10-Rluc* (Invsc1/OR) alone and *pYesDest52 odr10-GFP2* (Invsc1/OG) alone respectively.

After overnight culture, OD600 was determined and the cells were suspended in induction medium (with 2% galactose and 1% raffinose instead of glucose as the culture medium) to give a final OD600 of 0.4. This induction culture was incubated for 24 hours with shaking at 15°C to induce fusion receptor expression. The culture was then pelleted and washed twice with cold phosphate-buffered saline (PBS). Cell pellet can be stored at -80°C and suspended in PBS buffer just before the BRET² assay.

Quantification of Yeast Cells for BRET² Assay

First Invsc1/OR/OG was quantified by GFP intensity (about 3000 RLU or c.p.s in 100 µl per well) by direct excitation of GFP² at Ex420nm in a white 96-well microplate (Perkin Elmer) using a SpectraMax M2 spectrofluorometer (Molecular Devices). The Renilla luciferase activity of the quantified Invsc1/OR/OG cells was determined by adding luciferin coelenterazine native (final concentration 5 µM) into a total volume of 100 µl cell in a 96-well white microplate and measuring luminescence signals immediately (Polarstar Optima, BMGLABTECH).

Invsc1/OG was normalised to have the similar GFP intensity as Invsc1/OR/OG. Invsc1/OR was normalised to have the similar Rluc activity as Invsc1/OR/OG. Negative control (host strain) Invsc1 was quantified to have same cell density as Invsc1/OR/OG.

Microplate BRET² Cell Based Assay

Assay procedure was modified from Issad and Jockers (2006) and conducted in a 96-well white microplate in a total volume of 100 µl and two repeat for each sample. Three biological independent assays will be conducted and data are pooled. Coelenterazine h DeepBlueC substrate (Biosynth AG) was added at a final concentration of 10 µM, and readings were performed with a dual wavelength lumino/fluorometer microplate reader (BMGLabtech). The following optimized filter settings were used to measure Rluc light emission (410±80 nm) and GFP² light emission (515±30 nm). The BRET ratio was defined as the difference of the emission at 515nm/410nm of co-expressed Rluc and GFP2 fusion proteins and the emission at the 515nm/410nm of the Rluc fusion protein alone. Results were expressed in milliBRET units, 1 milliBRET corresponding to the BRET ratio value multiplied by 1000 (Ayoub, 2002). Illumination of control cells (host strain Invsc1) that do not express a Rluc or GFP2 fusion at the same settings results in detection of cell autofluorescence. Subtraction of the autofluorescence from all tested samples was taken place before BRET ratio values were calculated.

Crude Membrane Preparation, Solubilisation, and Immunoprecipitation

Properly induced yeast cells (GFP² level was not more than 2 fold over background of host strain Invsc1 cells) were washed three times with ice-cold Buffer A (75 mM tris-HCl, pH7.4, 12 mM MgCl₂ and 2 mM EDTA) and the final pellet was suspended in Buffer B (Buffer A + protease inhibitor cocktail, complete Mini EDTA-

free (Roche Applied Science)). Then the yeast cells are disrupted using a French Press and centrifuged for 15 mins at 10,000 x g at 4°C. The supernatant was ultracentrifuged for 1 hour at 130000 x g and the pellet was suspended in cold Buffer B.

Membrane protein solubilisation and immunoprecipitation procedures were modified according to the method described by Ayoub et al. (2002). Crude membrane was solubilised with 0.5% digitonin (Sigma) and this mixture was stirred for 3h at 4°C. Non-solubilised membrane proteins were removed by centrifugation at 18,000 x g for 30 mins at 4°C. The digitonin concentration was adjusted to 0.2% using cold Buffer B and the polyclonal anti-GFP antibodies (Sapphire Bioscience, Cat No 120-29000) were added to a final dilution of 1:1000. Immune complex formation was allowed to proceed for 12h at 4°C with gentle agitation and Protein A-agarose suspension (Sigma) was added to a final volume of 1:10 (agarose suspension:sample) for incubation for another 6h with gentle agitation at 4°C. After centrifugation for 1 min at 5000 x g, supernatant was decanted and the agarose beads washed five times with 0.5 ml of cold Buffer C (Buffer B + 0.2% digitonin). Precipitates were analysed by assessing luciferase activity in a luminometer (BMGLabtech) using coelenterazine as substrate (Biosynth AG, Switzerland).

RESULTS

Visualisation of Nematode odr-10 in Yeast Cells – Confocal Microscopy

Confocal image of yeast strain Invsc1 co-expressed odr-10-Rluc and odr-10-GFP² after 24 hour induction reveals that GFP² fusion protein mostly only expressed on the membrane of the cells and no over-expression of tagged odr-10 observed. About 32% of the cells expressed tagged odr-10 protein. Increasing induction time up to 48 hours, confocal image shows GFP² over-expression in the cells and aggregated GFP² particles observed in the cytosol of the cells (Figure 12).

Detection of Constitutive odr-10 Oligomers in Living Yeast Cells by BRET²

BRET² experiment results shows that significant energy transfer was observed in yeast cells co-expressing odr-10-Rluc and odr-10-GFP² (Figure 13) indicating that odr-10 forms homo-oligomers. No significant energy transfer was obtained in the sample at which half yeast cells expressed odr-10-Rluc and another half cells expressed odr-10-GFP².

Detection of Constitutive Odr-10 Oligomers in Living Yeast Cells by Immunoprecipitation

Oligomerisation of Odr-10 in the living yeast cells was confirmed by the pairwise expression of receptor-Rluc and –GFP² fusion, followed by precipitation using anti-green fluorescence protein antibodies and measuring co-immunoprecipitated luciferase activity. The results represented in Figure 14 indicate that Odr-10 forms oligomers in the cells. To rule out the possibility that co-immunoprecipitation could result from receptor-independent, interaction between the GFP² and the Rluc, co-immunoprecipitation was also carried out using (1) odr-10-Rluc/GFP², (2) the mixture of solubilised membranes which either expressed receptor-GFP² or receptor-Rluc. Results from those samples in which less than 20% of luciferase activity was recovered comparing to co-expression of odr-10-GFP² and odr-10-Rluc in the same cells suggested the interaction between co-expressing odr-10-GFP² and odr-10-Rluc fusion proteins was specific.

EXAMPLE 6 – BRET² TAGGED MOUSE α_{2A} ADRENERGIC RECEPTOR

The mouse α_{2A} adrenergic receptor (SEQ ID NO:56) was engineered so that the GFP² was inserted into the 3rd intracellular loop with its N-terminus following Ala250 and its C-terminus adjacent to Arg372. RLuc was fused to Val461 in the C-terminus of the receptor (amino acid sequence provided as SEQ ID NO:54 and encoding open reading frame as SEQ ID NO:53).

The dual-tagged adrenergic receptors are expressed in yeast. These receptors can be used to monitor ligand binding by the mouse α_{2A} adrenergic receptor via changes in the BRET² ratio.

EXAMPLE 7 – WHOLE-CELL VS CELL-FREE BRET ASSAY

Comparison of the cell-free assay system of the invention to a whole cell assay system demonstrates that the intensity of the BRET² signal was more than forty time greater, in terms of light output when measured in the RLuc channel, for the cell-free assay compared to the whole-cell assay (Figure 15). Assaying 1 μ M of diacetyl resulted in no significant change ($P \leq 0.05$) in the BRET² signal using the whole cell assay, whereas a significant decrease ($P \leq 0.05$) of 52 % was observed using a cell-free assay system (Figure 16).

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The present application claims priority from US 61/148,271 filed 29 January 2009, the entire contents of which are incorporated herein by reference.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. A method of detecting a compound, the method comprising,
 - i) contacting a sample with a cell-free composition comprising
 - a) at least one G protein coupled receptor embedded in a lipid bilayer, and which is capable of binding the compound, and
 - b) optionally at least one accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor, wherein the G protein coupled receptor comprises one or more subunits that are the same or different, and wherein the G protein coupled receptor, and/or accessory molecule when present, in combination comprise a bioluminescent protein and an acceptor molecule,
 - ii) simultaneously or sequentially with step i) providing a substrate of the bioluminescent protein, and allowing the bioluminescent protein to modify the substrate,
 - iii) determining if step ii) modulates bioluminescent resonance energy transfer (BRET) between the bioluminescent protein and the acceptor molecule, wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the G protein coupled receptor, and wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.
2. The method of claim 1, wherein a subunit of the G protein coupled receptor which is capable of binding the compound comprises
 - i) the bioluminescent protein, and
 - ii) the acceptor molecule,and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the subunit.
3. The method of claim 1, wherein the G protein coupled receptor comprises
 - i) a first subunit comprising
 - a) the bioluminescent protein, and
 - b) the acceptor molecule, and
 - ii) a second subunit which is capable of binding the compound,

and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the second subunit.

4. The method of claim 2 or claim 3, wherein

i) the bioluminescent protein forms part of the third non-transmembrane loop of the subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop, or

ii) the acceptor molecule forms part of the third non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop, or

iii) the bioluminescent protein forms part of the first non-transmembrane loop of the subunit, and the acceptor molecule forms part of the third non-transmembrane loop, or

iv) the acceptor molecule forms part of the first non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the third non-transmembrane loop, or

v) the bioluminescent protein forms part of the fifth non-transmembrane loop of the subunit, and the acceptor molecule forms part of the C-terminus, or

vi) the acceptor molecule forms part of the fifth non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the C-terminus.

5. The method of claim 1, wherein the G protein coupled receptor comprises

i) a first subunit comprising the bioluminescent protein, and

ii) a second subunit comprising the acceptor molecule.

6. The method of claim 5, wherein

i) the bioluminescent protein forms part of the third non-transmembrane loop of the first subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop of the second subunit, or

ii) the acceptor molecule forms part of the third non-transmembrane loop of the first subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop of the second subunit, or

iii) the bioluminescent protein forms part of the first non-transmembrane loop of the first subunit, and the acceptor molecule forms part of the third non-transmembrane loop of the second subunit, or

iv) the acceptor molecule forms part of the first non-transmembrane loop of the first subunit, and the bioluminescent protein forms part of the third non-transmembrane loop of the second subunit, or

v) the bioluminescent protein forms part of the fifth non-transmembrane loop of the first subunit, and the acceptor molecule forms part of the C-terminus of the second subunit, or

vi) the acceptor molecule forms part of the fifth non-transmembrane loop of the first subunit, and the bioluminescent protein forms part of the C-terminus of the second subunit.

7. The method of claim 1, wherein

i) the G protein coupled receptor comprises a subunit comprising the bioluminescent protein, and the accessory molecule comprises the acceptor molecule, or

ii) the G protein coupled receptor comprises a subunit comprising the acceptor molecule, and the accessory molecule comprises the bioluminescent protein.

8. The method of claim 1, wherein the accessory molecule comprises

i) the bioluminescent protein, and

ii) the acceptor molecule.

9. The method of claim 1, wherein the composition comprises at least two accessory molecules, and wherein a first accessory molecule comprises the bioluminescent protein and a second accessory molecule comprises the acceptor molecule.

10. The method according to any one of claims 1 to 9, wherein the G protein coupled receptor is an odorant receptor.

11. The method of claim 10, wherein the odorant receptor is a chordate receptor, a nematode receptor, or a biologically active variant or fragment of any one thereof.

12. The method of claim 11, wherein the odorant receptor comprises:

i) an amino acid sequence as provided in any one of SEQ ID NO's 1 to 6, and

ii) an amino acid sequence which is at least 40% identical to any one or more of SEQ ID NO:1 to 6.

13. The method according to any one of claims 1 to 12, wherein the subunit is a chimera of a portion of two or more different G protein coupled receptor subunits.

14. The method of claim 13, wherein the subunit comprises the N-terminus and at least a majority of the first transmembrane domain of a first G protein coupled receptor subunit, at least a majority of the first non-transmembrane loop through to at least a majority of the fifth transmembrane domain of a second G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of the first G protein coupled receptor subunit.

15. The method of claim 13, wherein the subunit comprises the N-terminus through to at least a majority of the fifth transmembrane domain of a first G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of a second G protein coupled receptor subunit.

16. The method according to any one of claims 1 to 15, wherein the accessory molecule is a G protein or arrestin.

17. The method according to any one of claims 1 to 16, wherein the level of BRET is indicative of the relative amount of the compound in the sample.

18. The method according to any one of claims 1 to 17, wherein the bioluminescent protein is a luciferase, a β -galactosidase, a lactamase, a horseradish peroxidase, an alkaline phosphatase, a β -glucuronidase or a β -glucosidase.

19. The method of claim 18, wherein the luciferase is a *Renilla* luciferase, a Firefly luciferase, a Coelenterate luciferase, a North American glow worm luciferase, a click beetle luciferase, a railroad worm luciferase, a bacterial luciferase, a *Gaussia* luciferase, Aequorin, a *Arachnocampa* luciferase, or a biologically active variant or fragment of any one, or chimera of two or more, thereof.

20. The method according to any one of claims 1 to 19, wherein the substrate is luciferin, calcium, coelenterazine, or a derivative of coelenterazine.

21. The method according to any one of claims 1 to 20, wherein the acceptor molecule is a protein.

22. The method of claim 21, wherein the acceptor molecule is green fluorescent protein (GFP), blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, t-dimer2, t-dimer2(12), mRFP1, pocilloporin, Renilla GFP, Monster GFP, paGFP, Kaede protein or a Phycobiliprotein, or a biologically active variant or fragment of any one thereof.

23. The method according to any one of claims 1 to 20, wherein the acceptor molecule is a non-protein.

24. The method of claim 23, wherein the acceptor molecule is an Alexa Fluor dye, Bodipy dye, Cy dye, fluorescein, dansyl, umbelliferone, fluorescent microsphere, luminescent microsphere, fluorescent nanocrystal, Marina Blue, Cascade Blue, Cascade Yellow, Pacific Blue, Oregon Green, Tetramethylrhodamine, Rhodamine, Texas Red, rare earth element chelates, or any combination or derivatives thereof.

25. The method according to any one of claims 1 to 24, which further comprises simultaneously or sequentially with step i) or step ii) providing a co-factor of the bioluminescent protein.

26. The method of claim 25, wherein the co-factor is ATP, magnesium, oxygen, FMNH₂, calcium, or a combination of any two or more thereof.

27. The method according to any one of claims 1 to 26, wherein the cell-free composition was obtained by producing the G protein coupled receptor in a recombinant cell and disrupting the membrane of the cell.

28. The method of claim 27, wherein the recombinant cell does not produce any non-endogenous proteins which associate with the G protein coupled receptor.

29. The method of claim 27 or claim 28, wherein the recombinant cell is a yeast cell.
30. The method according to any one of claims 1 to 29, wherein the G protein coupled receptor is embedded in the lipid bilayer of a liposome.
31. The method according to any one of claims 1 to 30, wherein the method is preformed using microfluidics.
32. A purified and/or recombinant polypeptide for detecting a compound, the polypeptide comprising,
i) a subunit of a G protein coupled receptor, and
ii) a bioluminescent protein and/or an acceptor molecule,
wherein when expressed in a cell the N-terminus of the subunit is outside the cell and the C-terminus inside the cell.
33. The polypeptide of claim 32, wherein
i) the bioluminescent protein forms part of the third non-transmembrane loop of the subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop, or
ii) the acceptor molecule forms part of the third non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop, or
iii) the bioluminescent protein forms part of the first non-transmembrane loop of the subunit, and the acceptor molecule forms part of the third non-transmembrane loop, or
iv) the acceptor molecule forms part of the first non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the third non-transmembrane loop, or
v) the bioluminescent protein forms part of the fifth non-transmembrane loop of the subunit, and the acceptor molecule forms part of the C-terminus, or
vi) the acceptor molecule forms part of the fifth non-transmembrane loop of the subunit, and the bioluminescent protein forms part of the C-terminus, or
vii) the bioluminescent protein or the acceptor molecule forms part of the first non-transmembrane loop of the subunit, or

viii) the bioluminescent protein or the acceptor molecule forms part of the third non-transmembrane loop of the subunit, or

ix) the bioluminescent protein or the acceptor molecule forms part of the fifth non-transmembrane loop of the subunit, or

x) the bioluminescent protein or the acceptor molecule forms part of the C-terminus of the subunit.

34. The polypeptide of claim 32 which comprises the bioluminescent protein and the acceptor molecule, and wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the compound binds the polypeptide.

35. A purified and/or recombinant polypeptide for detecting a compound, the polypeptide comprising,

i) an accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor, and

ii) a bioluminescent protein and/or an acceptor molecule,
wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.

36. The polypeptide according to any one of claims 32 to 35, wherein the G protein coupled receptor is an odorant receptor.

37. The polypeptide of claim 36, wherein the odorant receptor is a chordate receptor, a nematode receptor, or a biologically active variant or fragment of any one thereof.

38. The polypeptide of claim 37, wherein the odorant receptor comprises:

i) an amino acid sequence as provided in any one of SEQ ID NO's 1 to 6, and

ii) an amino acid sequence which is at least 40% identical to any one or more of SEQ ID NO:1 to 6.

39. The polypeptide according to any one of claims 32 to 38, wherein the subunit is a chimera of a portion of two or more different G protein coupled receptor subunits.

40. The polypeptide of claim 39, wherein the subunit comprises the N-terminus and at least a majority of the first transmembrane domain of a first G protein coupled receptor subunit, at least a majority of the first non-transmembrane loop through to at least a majority of the fifth transmembrane domain of a second G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of the first G protein coupled receptor subunit.

41. The polypeptide of claim 39, wherein the subunit comprises the N-terminus through to at least a majority of the fifth transmembrane domain of a first G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of a second G protein coupled receptor subunit.

42. An isolated and/or exogenous polynucleotide encoding a polypeptide according to any one of claims 32 to 41.

43. A vector comprising the polynucleotide of claim 42.

44. The vector of claim 43, wherein the polynucleotide is operably linked to a promoter.

45. A host cell comprising the polynucleotide of claim 42 and/or the vector of claim 43 or claim 44.

46. The host cell of claim 45 further comprising the polypeptide according to any one of claims 32 to 41.

47. The host cell of claim 45 or claim 46 which is a yeast cell.

48. A composition comprising the polypeptide according to any one of claims 32 to 41, the polynucleotide of claim 42, the vector of claim 43 or claim 44, and/or the host cell according to any one of claims 45 to 47.

49. The composition of claim 48, wherein the polypeptide is embedded in a lipid bilayer.

50. A cell-free composition comprising;

i) the polypeptide according to any one of claims 32 to 34 or 36 to 41, wherein the polypeptide is embedded in a lipid bilayer, and/or

ii) the polypeptide of claim 35 directly or indirectly bound to an intracellular loop and/or the C-terminus of a G protein coupled receptor, wherein the G protein coupled receptor is embedded in a lipid bilayer, and wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.

51. The cell-free composition of claim 50, wherein the lipid bilayer is a yeast lipid bilayer.

52. The cell-free composition of claim 50 or claim 51, which further comprises a substrate of the bioluminescent protein and/or a co-factor of the bioluminescent protein.

53. A method of producing a cell-free composition according to any one of claims 50 to 52, the method comprising obtaining a cell according to any one of claims 45 to 47 and disrupting the membrane of the cells.

54. The method of claim 53, wherein the cells are permeabilized or lysed.

55. The method of claim 54, wherein the cells are lysed in a French press.

56. A biosensor comprising a polypeptide according to polypeptide according to any one of claims 32 to 41, a host cell according to any one of claims 45 to 47, a composition of claim 48 or claim 49 and/or a cell-free composition according to any one of claims 50 to 52.

57. A method for screening for a compound that binds a G protein coupled receptor, the method comprising,

i) contacting a candidate compound with a cell-free composition comprising

a) at least one G protein coupled receptor embedded in a lipid bilayer, and which is capable of binding the compound, and

b) optionally at least one accessory molecule that directly or indirectly binds an intracellular loop and/or the C-terminus of the G protein coupled receptor, wherein the G protein coupled receptor comprises one or more subunits that are the same or different, and wherein the G protein coupled receptor, and/or accessory

molecule when present, in combination comprise a bioluminescent protein and an acceptor molecule,

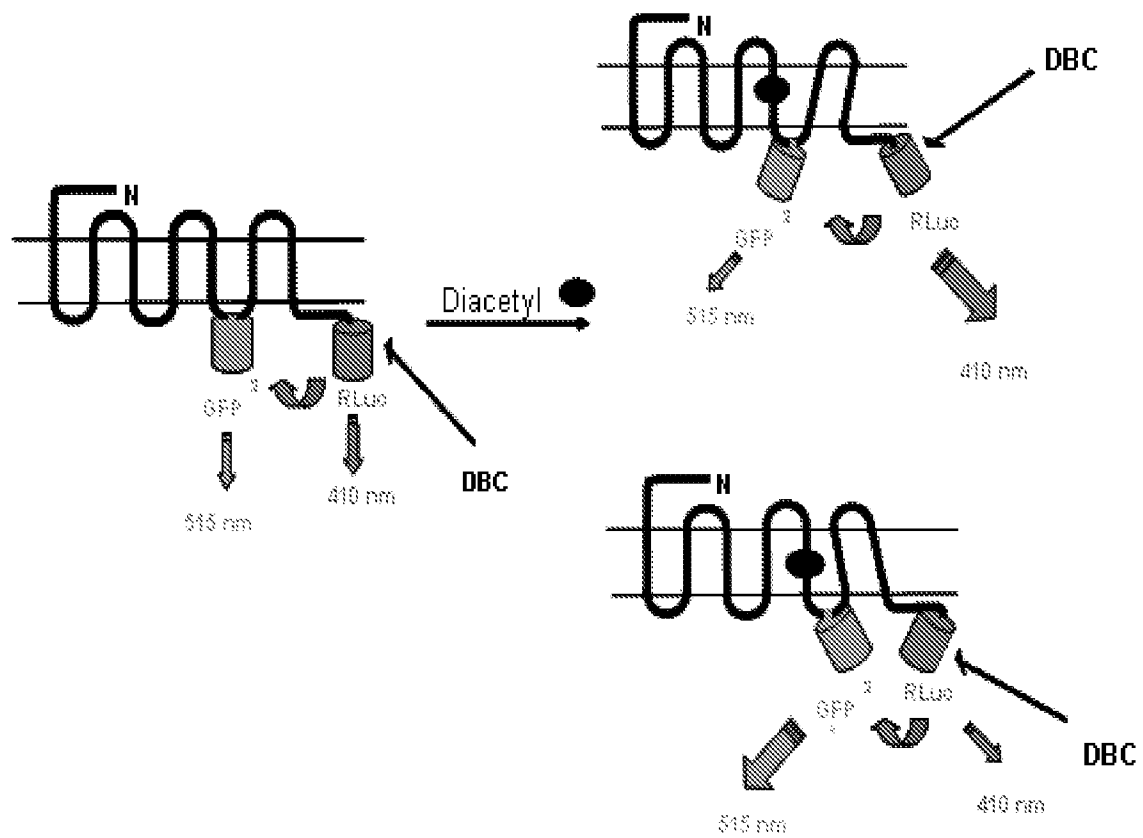
ii) simultaneously or sequentially with step i) providing a substrate of the bioluminescent protein, and allowing the bioluminescent protein to modify the substrate,

iii) determining if step ii) modulates bioluminescent resonance energy transfer (BRET) between the bioluminescent protein and the acceptor molecule,

wherein a modulation of BRET indicates that the compound binds the G protein coupled receptor, and wherein when expressed in a cell the N-terminus of the G protein coupled receptor, or subunits thereof, is outside the cell and the C-terminus inside the cell.

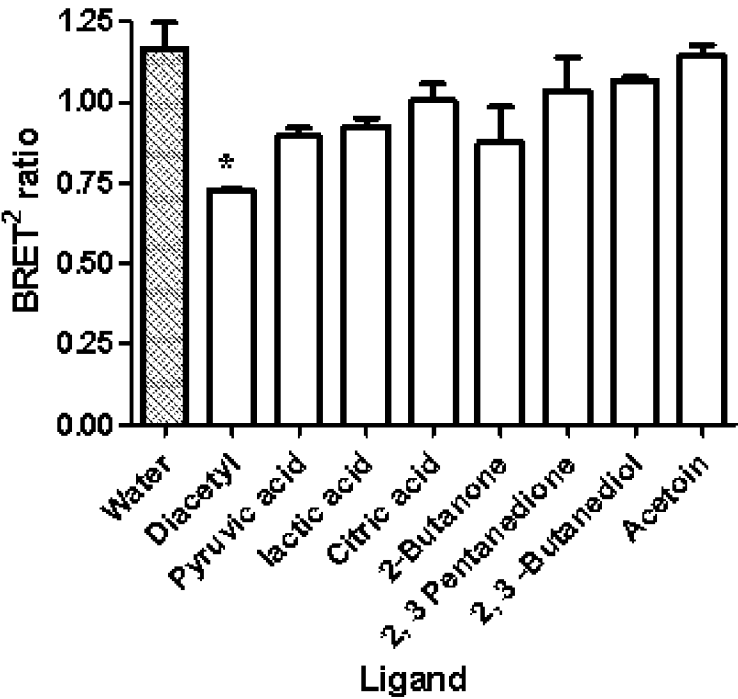
58. A kit comprising a polypeptide according to any one of claims 32 to 41, a polynucleotide of claim 42, a vector of claim 43 or claim 44, a host cell according to any one of claims 45 to 47, a composition of claim 48 or claim 49, a cell-free composition according to any one of claims 50 to 52, and/or a biosensor of claim 56.

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**Figure 1**

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A



B

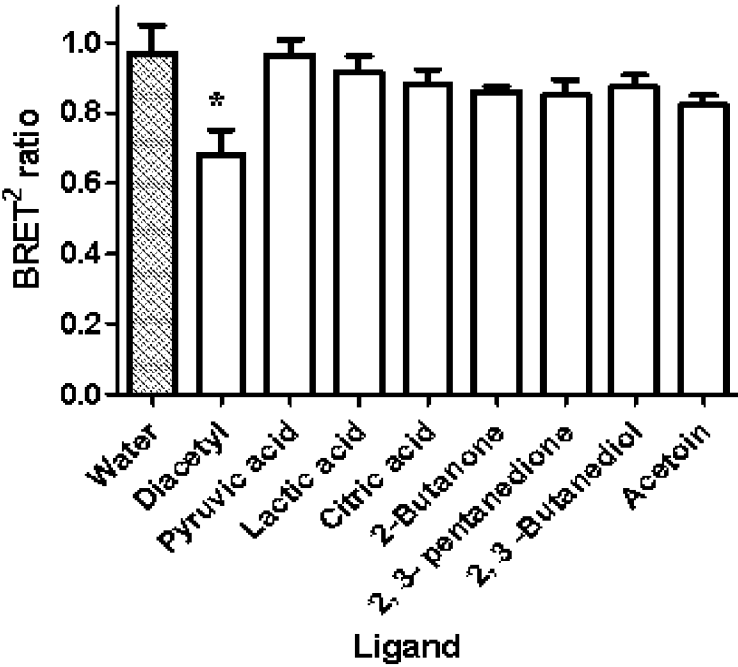


Figure 2

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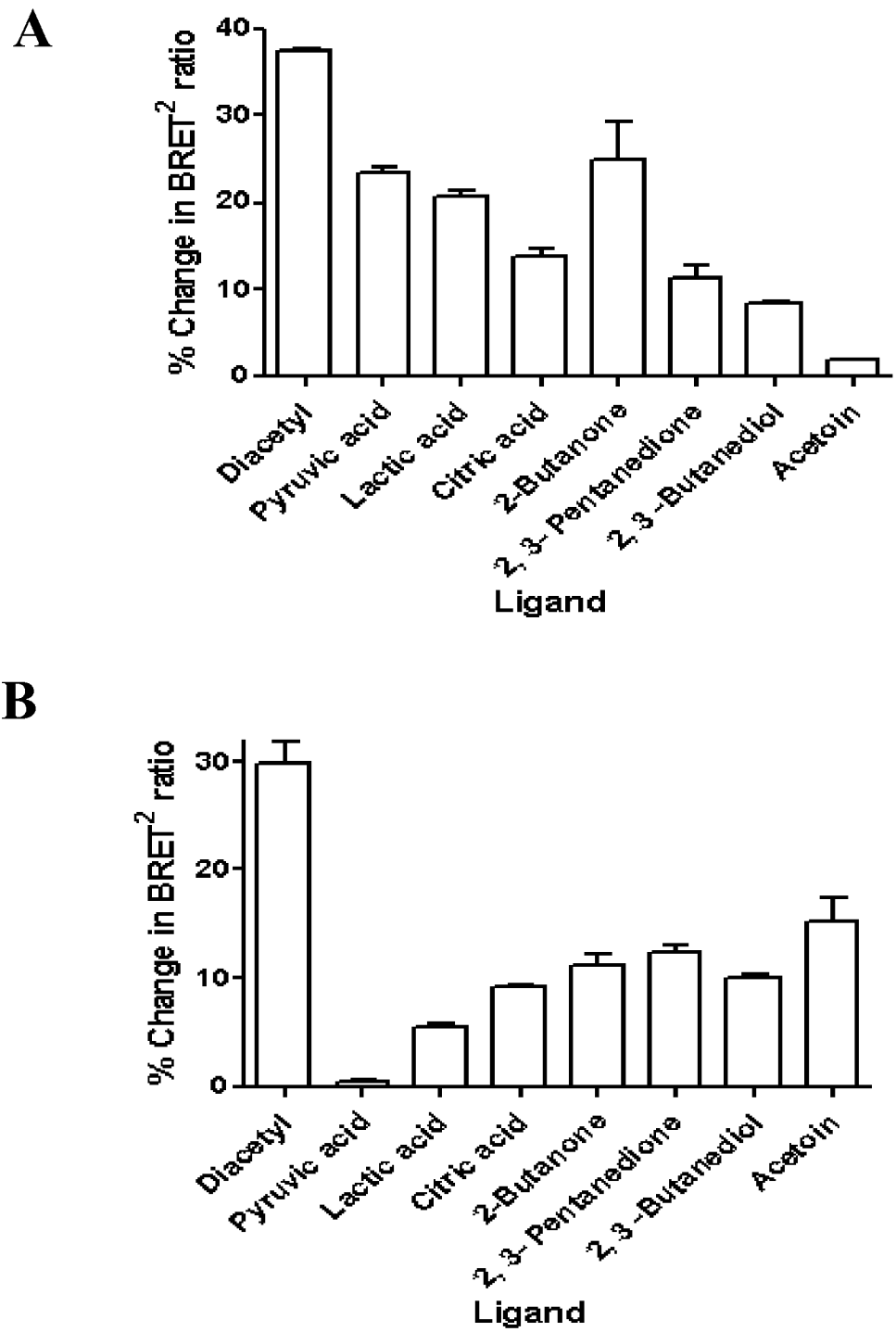
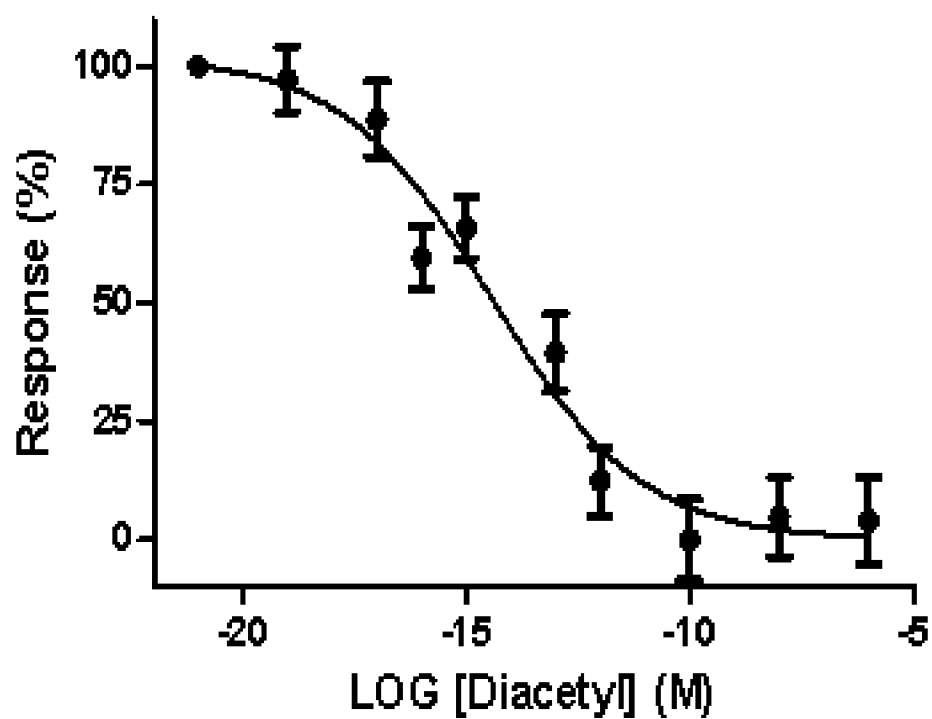
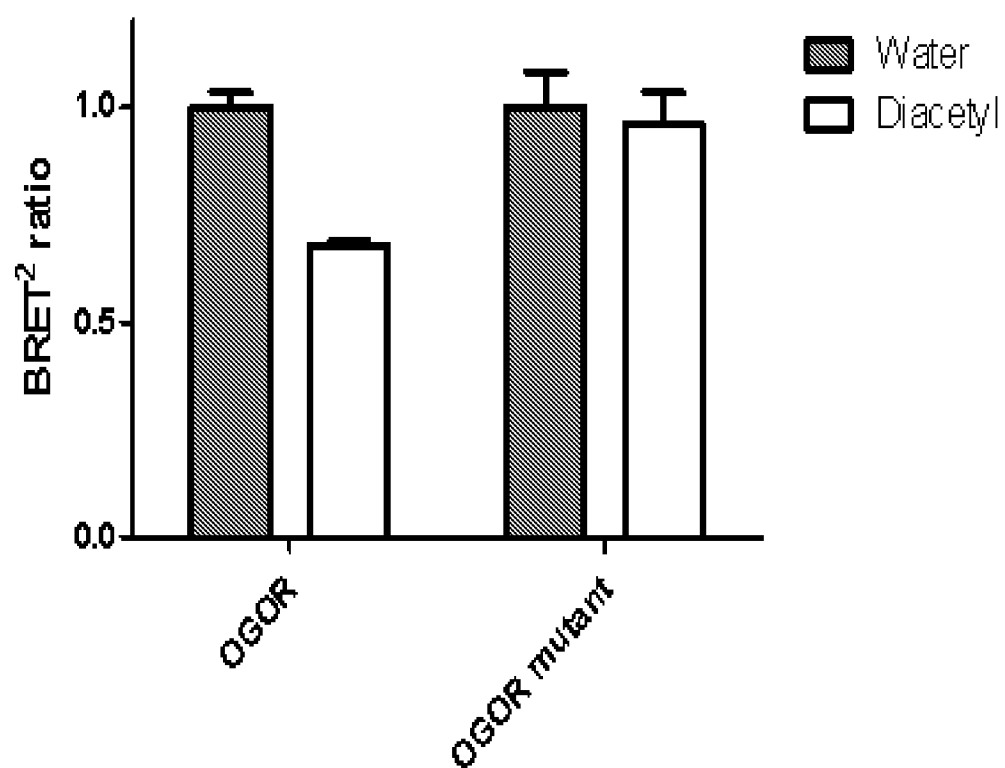


Figure 3

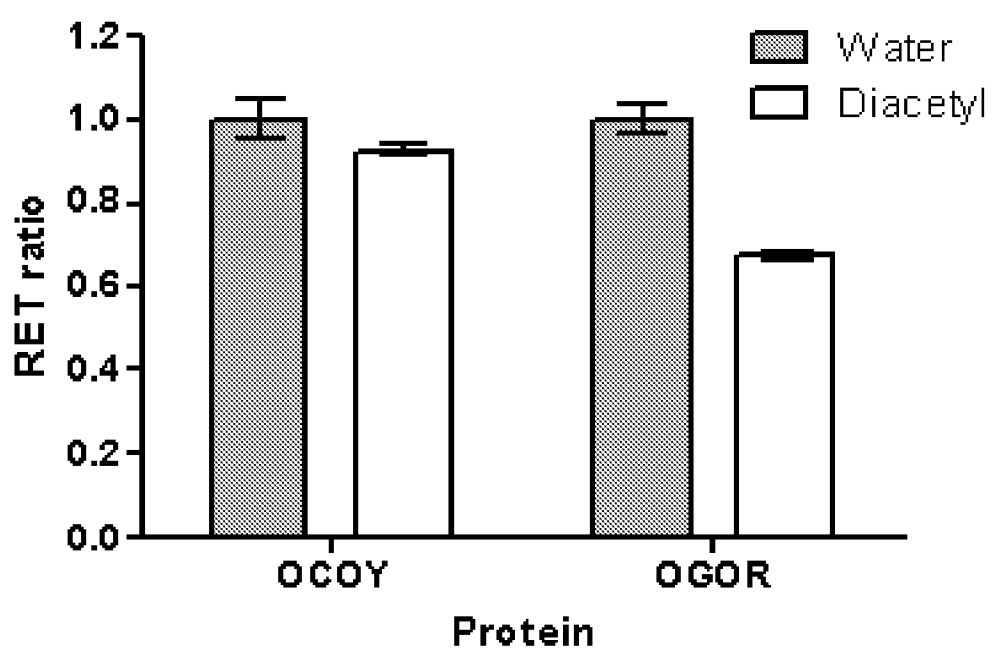
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**Figure 4**

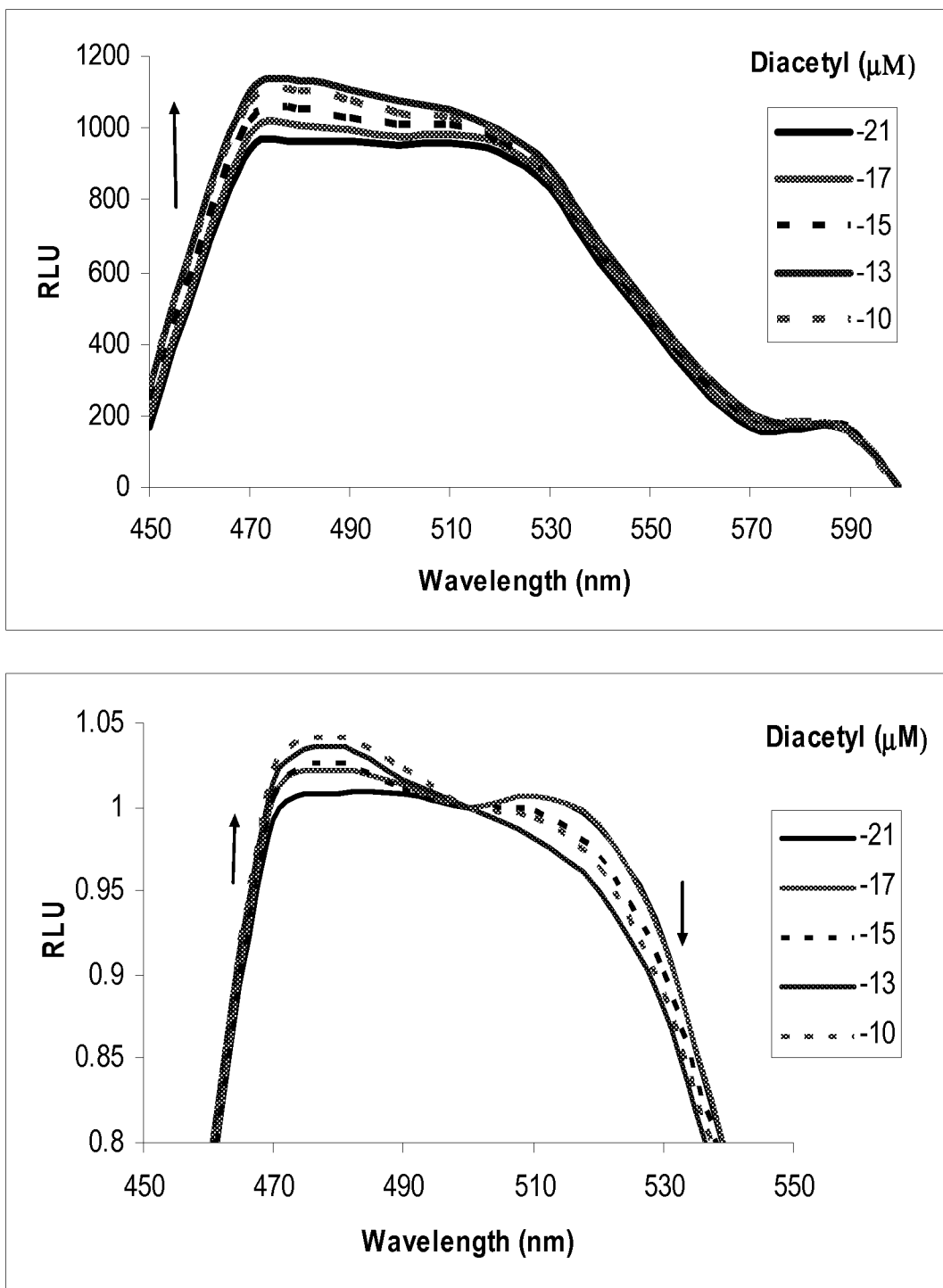
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**Figure 5**

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**Figure 6**

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**Figure 7**

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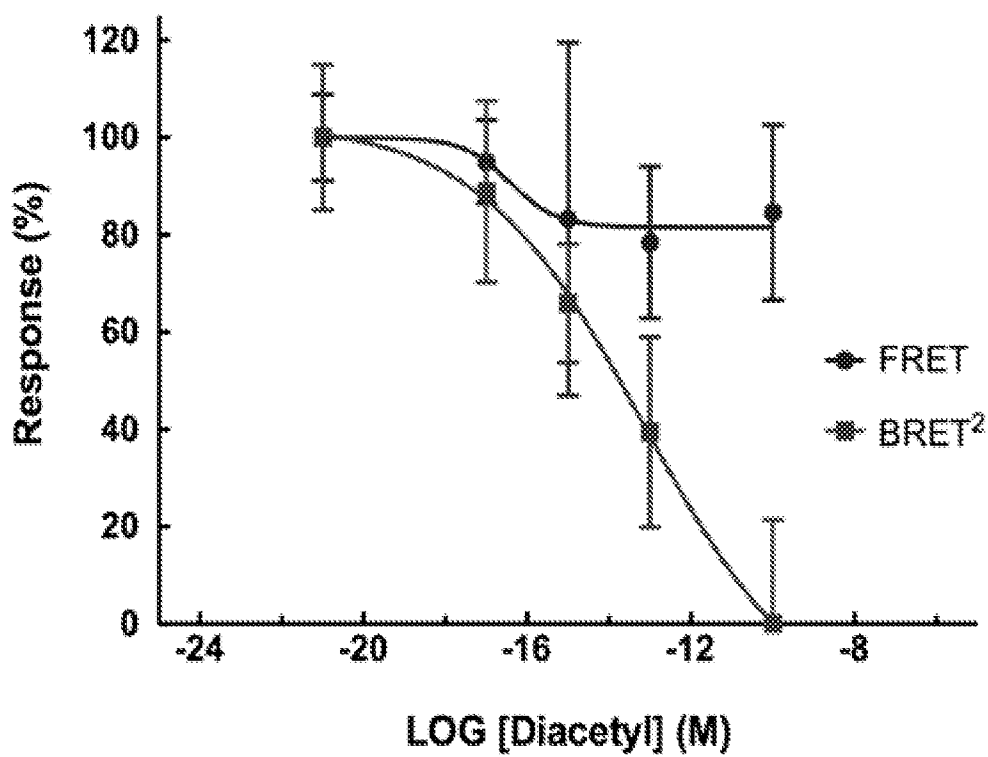
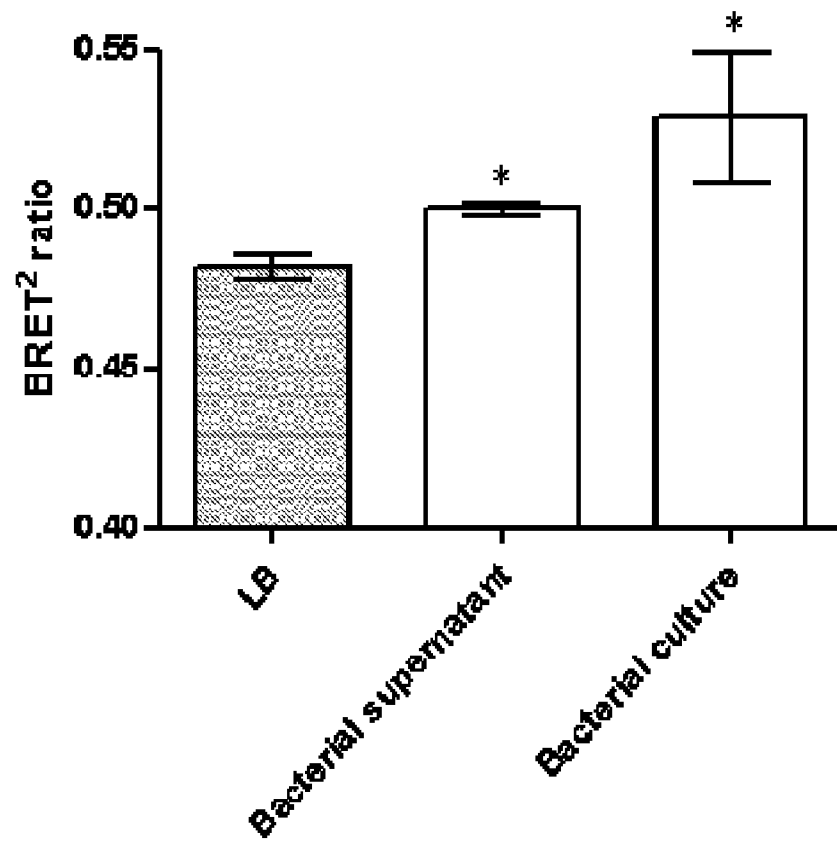
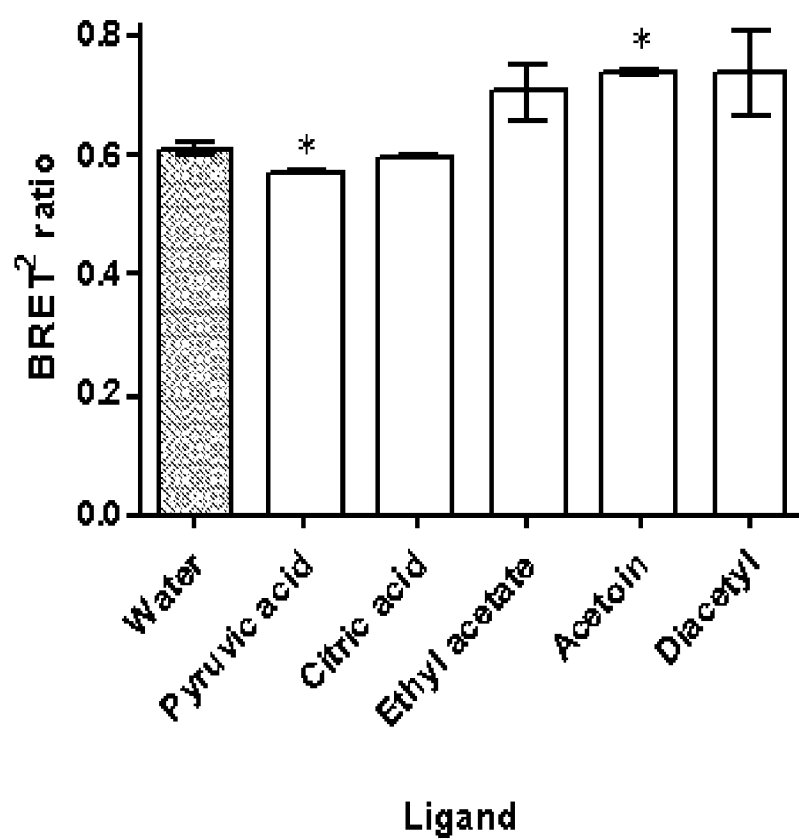


Figure 8

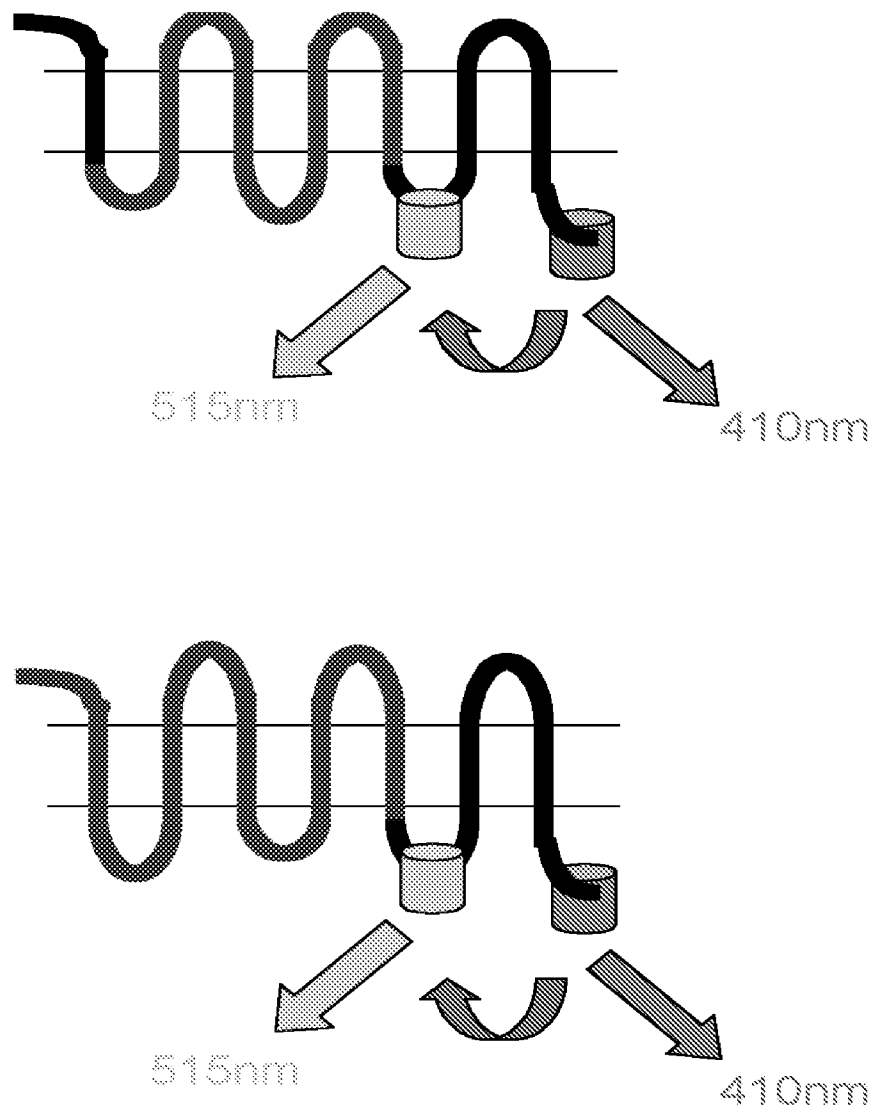
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**Figure 9**

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**Figure 10**

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**Figure 11**

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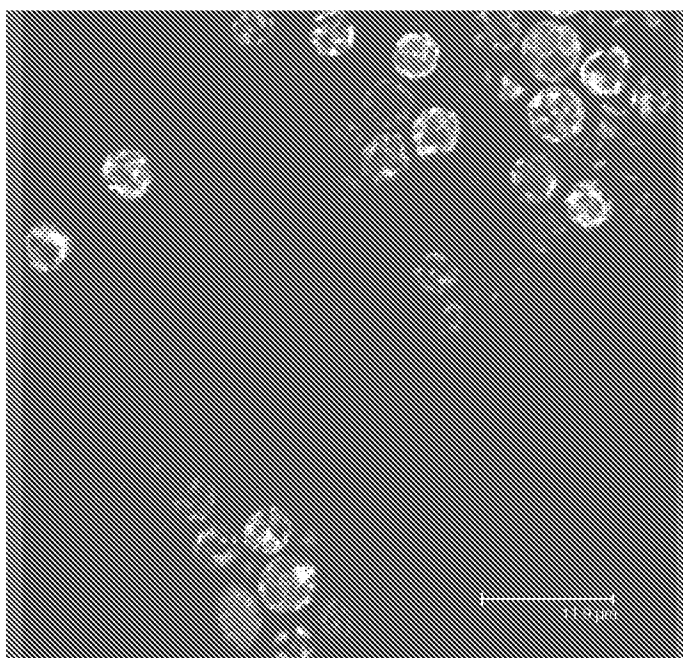
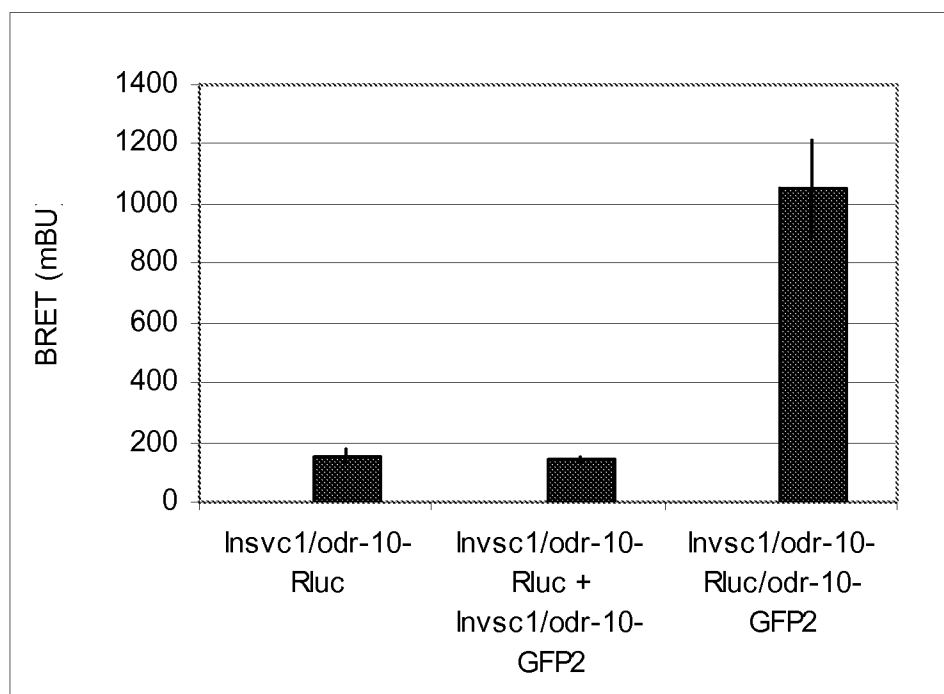
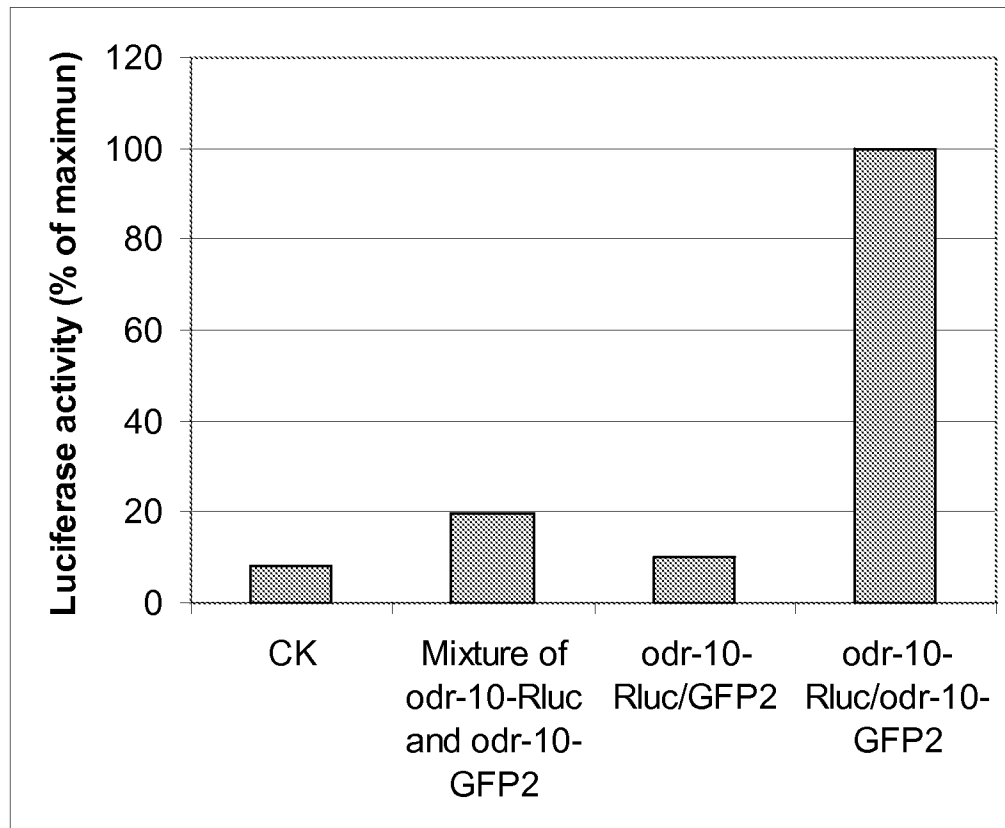


Figure 12

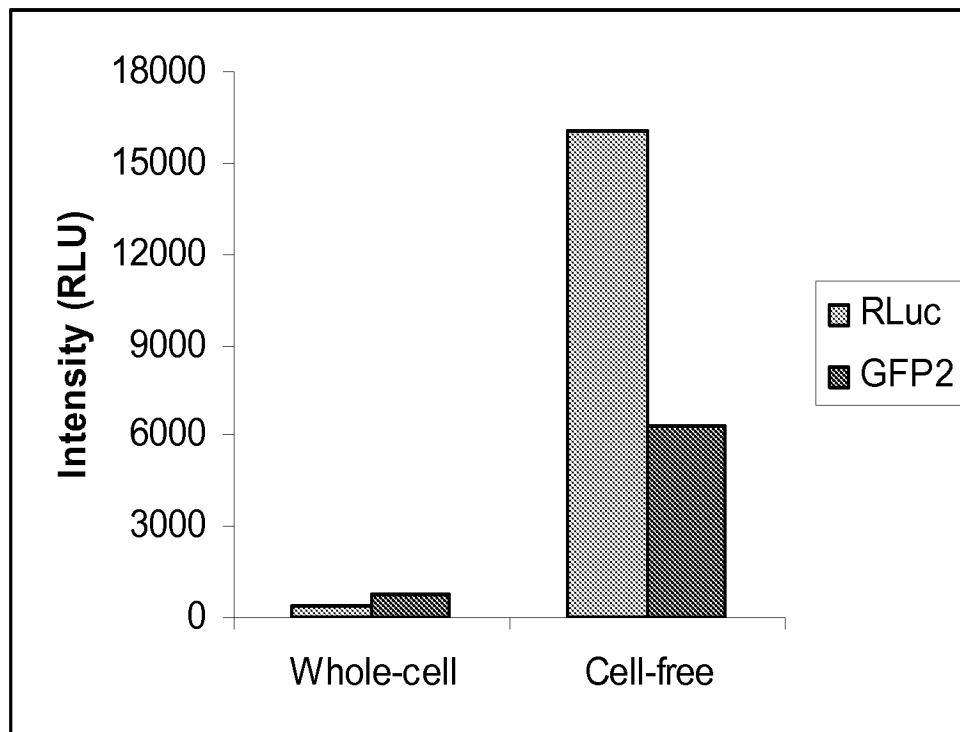
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**Figure 13**

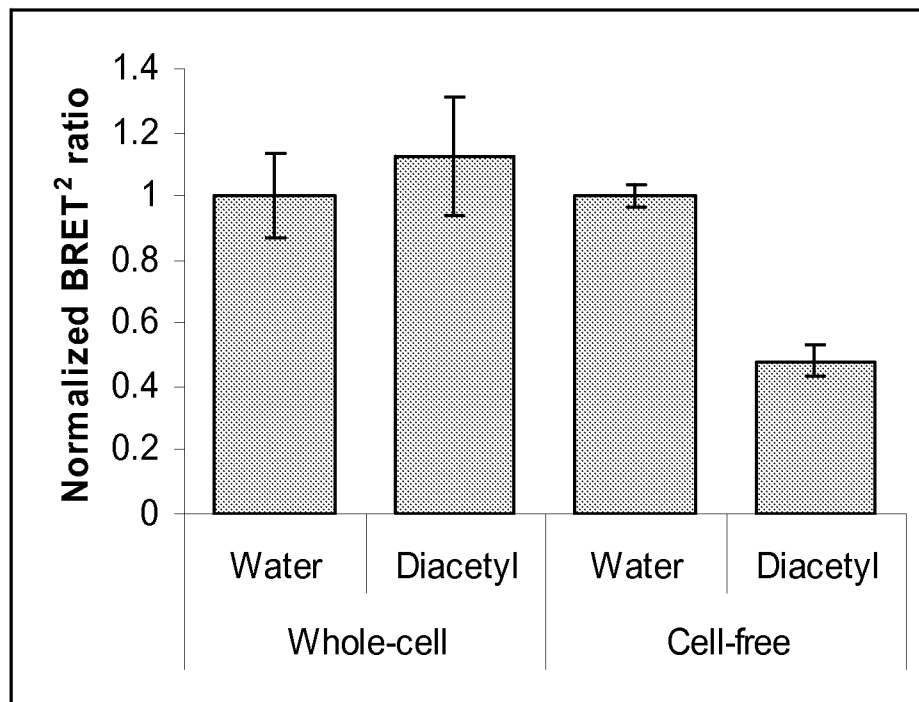
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**Figure 14**

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**Figure 15**

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**Figure 16**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2010/000080

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 33/53 (2006.01)

C12N 15/09 (2006.01)

G01N 21/76 (2006.01)

C07K 14/705 (2006.01)

G01N 21/64 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

MEDLINE, CAPLUS, BIOSIS, WPI, EPODOC; with GPCR, odorant receptor, BRET, cell-free, lipid bilayer, and the like terms.
GENOMEQUEST: SEQ ID NOs 1-6..

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/086883 A1 (UNIVERSITE DE MONTREAL), 24 August 2006 (p.1 l.17-20, p.6 l.20-22, p.9 p.23-29, p.17 l.13-p.19 l.8; Figures 1, 15, and 19; Examples 1-3, claims 17, 19, 21, 31, 33, 35, 45, 101)	1-58
X	WO 2004/057333 A1 (JULIUS-MAXIMILIANS-UNIVERSITAT WURZBURG), 8 July 2004 (Abstract, p.7 3rd paragraph-p.8 1st paragraph, p.11 2nd paragraph, p.12 2nd paragraph-p.14 1st paragraph, p.15 2nd paragraph-p.16 1st and 6th paragraphs, p.18 2nd paragraph, p.24 1st paragraph-p.29 2nd paragraph, p.32 2nd paragraph-p.33 1st paragraph, p.44 3rd paragraph; claims 3, 8, 11-15, 23)	1-58



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
22 March 2010Date of mailing of the international search report
29 MAR 2010

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2010/000080

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/0048811 A1 (DEVREOTES P.N. ET AL.), 25 April 2002 (Abstract, [0030]-[0035], [0038], [0041]-[0042]; claims 11, 36-39, 48, 53, 56, 60)	1, 9-31, 35-58
X	WO 2005/105850 A1 (UNIVERSITE DE MONTREAL), 10 November 2005 (p.1 2nd paragraph, p.12 1st paragraph-p.13 2nd paragraph, Figure 1, claim 40)	1, 8, 10-31, 35-58

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2010/000080

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

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

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

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

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

See Supplemental Box I

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

Remark on Protest

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

Supplemental Box I

(To be used when the space in any of Boxes I to IV is not sufficient)

Continuation of Box No: III

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

Note that Rule 13.2 states that where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only where there is a technical relationship among those inventions involving one or more of the same corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

This International Searching Authority has found that there are different inventions as follows:

Invention 1

Claims 1-34 (all completely), and 36-58 (all partially) directed to a method of detecting a compound and to a purified and/or recombinant polypeptide, comprising a G protein coupled receptor (GPCR) (subunit) and a bioluminescent protein and/or an acceptor molecule; wherein said method can optionally comprise an accessory molecule (e.g. a G protein) that binds to said GPCR, and that in combination with said GPCR comprise a bioluminescent protein and an acceptor molecule.

Invention 2

Claims 35 (completely) 36-58 (all partially) directed to a purified and/or recombinant polypeptide comprising an accessory molecule that is capable of binding to a GPCR and a bioluminescent protein and/or an acceptor molecule.

The only feature common to Inventions 1 and 2 is an accessory molecule that binds to a GPCR and that comprises a bioluminescent protein or an acceptor molecule. However, this concept is not novel in light of the following document:

US 2002/0048811 A1 (DEVREOTES P. N. ET AL.), 25 April 2002. This document discloses a G protein accessory molecule that binds to a GPCR and that comprises a bioluminescent protein or an acceptor molecule (see Abstract, [0030]).

This means that the common features can not constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since they make no contribution over the prior art.

Because the common features do not satisfy the requirement for being a special technical feature it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore the claims do not satisfy the requirement of unity of invention a posteriori.

However, despite the technical lack of unity of invention, all claims were searchable without effort justifying additional fees. Therefore this Authority did not invite payment of additional fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2010/000080

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	2006086883	US	2009298162
WO	2004057333	AU	2003293959
US	2002048811	AU	13234/02
WO	2005105850	CA	2607015
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			
END OF ANNEX			