

SYSTEMS AND METHODS FOR THE ASSESSMENT OF G-PROTEIN ACTIVATION

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. provisional application serial No. 62/573,853 filed on October 18, 2017, which is incorporated herein by reference in its entirety.

5 TECHNICAL FIELD

The present invention generally relates to the monitoring of G-protein activation in cells.

BACKGROUND ART

Heterotrimeric G proteins are the canonical signaling partners of G protein-coupled receptors (GPCRs), and also known to be involved in the signaling of other types of receptors, notably receptor tyrosine kinases (RTKs) through the G α -Interacting Vesicle-associated protein (GIV; also known as Girdin) (Ghosh P, 2016, *Pharmacol Res.* 105:99-107). Receptor activation triggers the exchange of a G α -bound GDP for GTP, resulting in a conformational rearrangement of the heterotrimeric G protein that promotes dissociation of G α and G β / γ subunits. GTP-bound G α and free G β / γ subunits are then available to engage specific effectors.

15 G proteins are grouped into families based on the signaling outcomes following activation of the G α subunit. The Gs family (G α s, G α olf) bolsters the production of cAMP through direct activation of adenylyl cyclases (AC). Conversely, the Gi family (G α i1, G α i2, G α i3, G α oA, G α oB and G α z) reduces cAMP levels by inhibiting specific ACs. The Gq family (G α q, G α 11, G α 14 and G α 15) activates Phospholipases C β (PLC β s) to produce the second messengers diacylglycerol (DAG) and inositol triphosphate (IP $_3$), which subsequently promote the activation of PKCs and Ca $^{2+}$ release from the endoplasmic reticulum, respectively. Finally, G12/13 family (G α 12 and G α 13) is known to control Rho-GEFs such as LARG, p115 and TRIO and thus influence processes linked to cytoskeletal remodeling (*e.g.*, chemotaxis). In view of the distinct outcomes of signaling following activation of G proteins belonging to different families, systems and assays that permit to monitor G protein activation in a G protein family-selective manner are needed, for example to better characterize cell surface receptor activation by various ligands (*e.g.*, signaling pathways activated by a given ligand) and identity more specific modulators of G proteins.

Although cell surface receptor activation occurs primarily at the plasma membrane (PM) in response to ligand binding, signaling of cell surface receptors (*e.g.*, GPCRs, RTKs) has been shown to occur in other cellular compartments, including the endosomes and the Golgi. Upon activation, many cell surface receptors enter the endosomes, and trafficking of a ligand-receptor complex within the endosomes provides a mechanism to either terminate signaling through degradation of the receptor (in lysosomes and proteasomes), or to sustain signaling through recycling of the receptor back to the cell surface. However, it has been demonstrated that receptor

signaling can also be initiated, sustained, and terminated in the endosomes (Murphy *et al.*, 2009, *PNAS*, vol. 106 no. 42, 17615-17622; Tsvetanova *et al.*, 2015, *J. Biol. Chem.* 290(11), pp. 6689-6696; Vilardaga *et al.*, 2014, *Nature Chemical Biology* 10, 700-706). Similarly, some G proteins have been found associated with, and activated at, the Golgi (Lo *et al.*, 2015, *Dev. Cell.* **33**: 189-203) and the endoplasmic reticulum (ER)-Golgi interface (Bastin *et al.*, *Front Bioeng Biotechnol.* 2015 Sep 1;3: 128). Different signals can arise from receptors at the PM and other compartments such as the endosomes and the Golgi, resulting in distinct physiological responses, and different mechanisms regulate signaling of receptors at these compartments. These distinct mechanisms of signaling and regulation raise the possibility of novel therapies based on targeting signaling at other cellular compartments (rather than PM), and thus systems and assays to monitor G protein activation at different cellular compartments are needed.

U.S. Patent No. 9,029,097 and WO/2016/058094 disclose BRET-based biosensors for monitoring G protein activation. However, these biosensors require the tagging of one or more of the G protein subunits ($G\alpha$, $G\beta$, and/or $G\gamma$), and/or of the GPCR, which may influence the activity of these proteins. Also, these biosensors detect global G protein activation in the cells, but do not allow the monitoring of G protein activation at different cellular compartments and in a G protein family-selective manner.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY

The present disclosure provides the following items 1 to 67:

1. A system for measuring modulation of G protein activation in a $G\alpha$ protein subunit family-selective manner, said system comprising a cell expressing:

(i) a first component comprising a $G\alpha$ subunit interacting polypeptide (GASIP) tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein: if said $G\alpha$ protein subunit family is G_i , said GASIP comprises a domain of a protein that specifically binds to G_i ; if said $G\alpha$ protein subunit family is G_q , said GASIP comprises a domain of a protein that specifically binds to G_q ; and if said $G\alpha$ protein subunit family is $G_{12/13}$, said GASIP comprises a domain of a protein that specifically binds to $G_{12/13}$; and

(ii) a second component comprising a plasma membrane (PM)-targeting moiety, an endosomal-targeting moiety or a Golgi-targeting moiety tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein if said GASIP is tagged with said fluorescent acceptor molecule, said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said bioluminescent donor molecule, and if said GASIP is tagged with said bioluminescent donor molecule, said PM-

targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said fluorescent acceptor molecule.

2. The system of item 1, wherein said domain of a protein that specifically binds to Gi is the G protein-binding domain of Rap1GAP or of a Regulator of G-protein signaling (RGS) protein.

5 3. The system of item 2, wherein said GASIP comprises the G protein-binding domain of Rap1GAP.

4. The system of item 3, wherein said G protein-binding domain of Rap1GAP comprises residues 1 to 442 of Rap1GAP (SEQ ID NO:8), or a variant thereof in which one or more of the serine residues at positions 437, 439 and 441 are mutated or absent.

10 5. The system of item 4, wherein said G protein-binding domain of Rap1GAP comprises residues 1 to 420 or 1 to 436 of Rap1GAP.

6. The system of item 4, wherein all three serine residues at positions 437, 439 and 441 are mutated.

7. The system of item 4 or 6, wherein said serine residues are substituted for alanine.

15 8. The system of item 2, wherein said GASIP comprises the G protein-binding domain of an RGS protein.

9. The system of item 8, wherein said RGS protein is RGS17, RGS19 or RGS20.

10 10. The system of item 9, wherein said G protein-binding domain comprises residues 64 to 210 of RGS17 (SEQ ID NO:17), residues 70-217 of RGS19 (SEQ ID NO:18), or residues 242-388 of RGS20 (SEQ ID NO:19).

11. The system of item 1, wherein said domain of a protein that specifically binds to Gq is the G protein-binding domain of P63RhoGEF or GRK2.

12. The system of item 11, wherein said GASIP comprises the G protein-binding domain of P63RhoGEF.

25 13. The system of item 12, wherein said G protein-binding domain of P63RhoGEF comprises residues 295 to 502 of P63RhoGEF (SEQ ID NO:25).

14. The system of item 11, wherein said GASIP comprises the G protein-binding domain of GRK2.

15 15. The system of item 14, wherein said G protein-binding domain of GRK2 comprises residues 30 to 203 of GRK2 (SEQ ID NO:27).

16. The system of item 1, wherein said domain of a protein that specifically binds to G12/13 is the G protein-binding domain of PDZRhoGEF or P115RhoGEF.

17. The system of item 16, wherein said GASIP comprises the G protein-binding domain of PDZRhoGEF.

35 18. The system of item 17, wherein said G protein-binding domain of PDZRhoGEF comprises residues 281 to 483 of PDZRhoGEF (SEQ ID NO:21).

19. The system of item 16, wherein said GASIP comprises the G protein-binding domain of P115RhoGEF.
20. The system of item 19, wherein said G protein-binding domain of P115RhoGEF comprises residues 1 to 244 of P115RhoGEF (SEQ ID NO:23).
- 5 21. The system of any one of items 1 to 20, wherein said GASIP is tagged with said bioluminescent donor molecule and said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said fluorescent acceptor molecule.
22. The system of any one of items 1 to 21, wherein said PM targeting moiety is a PM protein or a fragment thereof that localizes to the PM.
- 10 23. The system of item 22, wherein said PM protein or fragment thereof comprises (a) a palmitoylation, myristoylation, and/or prenylation signal sequence and/or (b) a polybasic sequence.
24. The system of item 22 or 23, wherein said PM targeting moiety comprises the amino acid sequence GCMSCCKVLS (SEQ ID NO:62), GCMGLPCVVM (SEQ ID NO:63), CVKIKKCIIM (SEQ ID NO:64), KKKKKKSKTKCVIM (SEQ ID NO:65), KNGKKKRKSLAKRIRERCCIL (SEQ ID NO: 45), CMSCKCCIL (SEQ ID NO:46), or SPKKGLLQRLFKRQHQNNSSKS (SEQ ID NO:47).
- 15 25. The system of item 24, wherein said PM targeting moiety comprises the amino acid sequence GKKKKKSKTKCVIM (SEQ ID NO:1).
26. The system of any one of items 1 to 21, wherein said endosomal targeting moiety is an endosomal protein or a fragment thereof that localizes to the endosomes.
- 20 27. The system of item 26, wherein said endosomal protein or fragment thereof comprises a FYVE domain.
28. The system of item 27, wherein said endosomal targeting moiety comprises the FYVE domain of human endofin.
- 25 29. The system of item 28, wherein said endosomal targeting moiety comprises residues 739 to 806 of human endofin (SEQ ID NO:39).
30. The system of any one of items 1 to 21, wherein said Golgi targeting moiety is a Golgi protein or a fragment thereof that localizes to the Golgi.
31. The system of item 30, wherein said Golgi targeting moiety is eNOS1 or a fragment thereof that localizes to the Golgi.
- 30 32. The system of item 31, wherein said Golgi targeting moiety comprises residues 1 to 73 of human eNOS1 (SEQ ID NO:48).
33. The system of any one of items 1 to 32, wherein said first component further comprises a linker between (i) said GASIP and (ii) said bioluminescent donor molecule or fluorescent acceptor molecule.
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34. The system of any one of items 1 to 33, wherein said second component further comprises a linker between (i) said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety and (ii) said bioluminescent donor molecule or fluorescent acceptor molecule.
35. The system of item 33 or 34, wherein said linker is a peptide linked of 5 to 25 amino acids.
- 5 36. The system of any one of items 1 to 35, further comprising a third component that is a cell surface receptor that signals through said G protein.
37. The system of item 36, where said cell surface receptor is a GPCR, an RTK or an integrin receptor.
38. The system of any one of items 1 to 37, wherein said G protein activation is G protein-coupled receptor (GPCR)-mediated G protein activation.
- 10 39. The system of any one of items 1 to 38, further comprising a fourth component that is a recombinant G α subunit polypeptide.
40. The system of item 39, wherein said G protein activation is non-receptor guanine nucleotide exchange factor (GEF)-mediated G protein activation, wherein said recombinant G α subunit polypeptide comprises at least one mutation in the carboxy (C)-terminal domain of said
- 15 G α subunit polypeptide, and wherein said C-terminal domain corresponds to the last seven residues of said G α subunit polypeptide.
41. The system of item 40, wherein said mutation is a deletion or substitution of at least the last two C-terminal residues.
- 20 42. The system of item 41, wherein said mutation is a deletion or substitution of at least the last five C-terminal residues.
43. The system of item 40, wherein said mutation is a deletion or substitution of at least one of the conserved leucine residues in said C-terminal domain.
44. The system of item 43, wherein said mutation is a substitution of the last conserved leucine
- 25 residue in said C-terminal domain.
45. The system of item 44, wherein said substitution is a leucine to aspartate, leucine to proline, or leucine to arginine substitution.
46. The system of any one of items 40 to 45, wherein said GEF is GIV/Girdin, and wherein said GASIP comprises the G protein-binding domain of Rap1GAP as defined in any one of items
- 30 1 to 7.
47. The system of item 46, wherein said GEF is activated by a receptor tyrosine kinase (RTK).
48. The system of any one of items 1 to 47, wherein said bioluminescent donor molecule is a luciferase, preferably a *Renilla* luciferase protein (*rLuc*).
49. The system of any one of items 1 to 48, wherein said fluorescent acceptor molecule is a
- 35 green fluorescent protein (GFP), preferably a *Renilla* GFP (*rGFP*).
50. One or more nucleic acids encoding the first and/or second components of the system of any one of items 1 to 49.

51. The one or more nucleic acids of item 50, comprising a first nucleic acid encoding the first component of the system of any one of items 1 to 49 and a second nucleic acid encoding the second component of the system of any one of items 1 to 49.
52. One or more vectors comprising the one or more nucleic acids of item 50 or 51.
- 5 53. A host cell expressing the components of the system defined any one of items 1 to 49.
54. A method for determining whether an agent modulates the activation of a G protein of interest, said method comprising: (a) contacting the system of any one of items 1 to 49 with a substrate for said bioluminescent donor molecule; and (b) measuring the BRET signal in the system in the presence and absence of said agent; wherein a difference in said BRET signal in
10 the presence of said agent relative to the absence thereof is indicative that said agent modulates the activation of said G protein of interest.
55. The method of item 54, wherein said G protein of interest is of the Gi protein subunit family, and wherein said GASIP comprises the G protein-binding domain of Rap1GAP as defined in any one of items 1 to 7.
- 15 56. The method of item 49, wherein the system comprises a recombinant G α subunit polypeptide of the Gi protein subunit family.
57. The method of item 56, wherein the method is performed using a plurality of systems, and wherein each of said systems comprises a different recombinant G α subunit polypeptide of the Gi protein subunit family.
- 20 58. The method of item 54, wherein said G protein of interest is of the Gq protein subunit family, wherein said GASIP comprises the G protein-binding domain of P63RhoGEF or GRK2 as defined in any one of items 1 and 11 to 15.
59. The method of item 58, wherein the system comprises a recombinant G α subunit polypeptide of the Gq protein subunit family.
- 25 60. The method of item 59, wherein the method is performed using a plurality of systems, and wherein each of said systems comprises a different recombinant G α subunit polypeptide of the Gq protein subunit family.
61. The method of item 54, wherein said G protein of interest is of the G12/13 protein subunit family, wherein said GASIP comprises the G protein-binding domain of PDZRhoGEF or
30 P115RhoGEF as defined in any one of items 1 and 16 to 20.
62. The method of item 61, wherein the system comprises a recombinant G α subunit polypeptide of the G12/13 protein subunit family.
63. The method of item 62, wherein the method is performed using a plurality of systems, and wherein each of said systems comprises a different recombinant G α subunit polypeptide of the
35 G12/13 protein subunit family.
64. A method for determining whether an agent modulates non-receptor guanine nucleotide exchange factor (GEF)-mediated G protein activation, said method comprising (a) contacting the

system of any one of items 40 to 47 with a substrate for said bioluminescent donor molecule; and
 (b) measuring the BRET signal in the system in the presence and absence of said agent; wherein
 a difference in said BRET signal in the presence of said agent relative to the absence thereof is
 indicative that said agent modulates non-receptor GEF-mediated G protein activation.

5 65. The method of any one of items 54 to 64, wherein the BRET signal is measured using a
 plate reader or by microscopy.

66. The method of any one of items 54 to 65, wherein the substrate is a coelenterazine
 substrate.

67. The method of item 66, wherein the coelenterazine substrate is methoxy e-coelenterazine.

10 Other objects, advantages and features of the present disclosure will become more
 apparent upon reading of the following non-restrictive description of specific examples thereof,
 given by way of example only with reference to the accompanying drawings.

15 In one aspect, the present disclosure refers to a system for measuring modulation of G
 protein activation in a G α protein subunit family-selective manner, said system comprising a cell
 expressing:

(i) a first component comprising a G α subunit interacting polypeptide (GASIP) tagged with a
 bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein:

20 if said G α protein subunit family is Gi, said GASIP comprises the G protein-binding domain
 of RAP1 GTPase Activating Protein 1 (Rap1GAP) or of a Regulator of G-protein signaling (RGS)
 protein;

if said G α protein subunit family is Gq, said GASIP comprises the G protein-binding
 domain of Rho guanine nucleotide exchange factor 25 (P63RhoGEF); and

25 if said G α protein subunit family is G12/13, said GASIP comprises the G protein-binding
 domain of Rho guanine nucleotide exchange factor 11 (PDZRhoGEF) or Rho guanine nucleotide
 exchange factor 1 (P115RhoGEF); and

(ii) a second component comprising a plasma membrane (PM)-targeting moiety, an endosomal-
 targeting moiety or a Golgi-targeting moiety tagged with a bioluminescent donor molecule or a
 fluorescent acceptor molecule;

30 wherein if said GASIP is tagged with said fluorescent acceptor molecule, said PM-
 targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said
 bioluminescent donor molecule, and if said GASIP is tagged with said bioluminescent donor
 molecule, said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is
 35 tagged with said fluorescent acceptor molecule and wherein there is no protein-protein interaction
 between (i) the PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety and
 (ii) the GASIP.

40 In another aspect, the present disclosure refers to a host cell expressing the components
 of the system as disclosed herein.

In another aspect, the present disclosure refers to a method for determining whether an
 agent modulates the activation of a G protein of interest, said method comprising:

(a) contacting the system as disclosed herein with a substrate for said bioluminescent donor molecule; and

(b) measuring the Bioluminescence Resonance Energy Transfer (BRET) signal in the system in the presence and absence of said agent;

5 wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates the activation of said G protein of interest.

In another aspect, the present disclosure refers to a method for determining whether an agent modulates non-receptor guanine nucleotide exchange factor (GEF)-mediated G protein
10 activation, said method comprising

(a) contacting the system as disclosed herein with a substrate for said bioluminescent donor molecule; and

(b) measuring the BRET signal in the system in the presence and absence of said agent;

15 wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates non-receptor GEF-mediated G protein activation.

BRIEF DESCRIPTION OF DRAWINGS

In the appended drawings:

20 **FIG. 1A** depicts the principle of an effector-based sensor to monitor GPCR-mediated direct G protein activation (upper panel **i**) or guanine-nucleotide exchange factor (GEF)-mediated G protein activation (lower panel **ii**). Cells expressing a receptor, a cellular compartment (or subcellular localization) marker, such as a plasma-membrane (PM) or early endosomes (EE) marker, tagged with a suitable Bioluminescent Resonance Energy Transfer (BRET) donor or acceptor (e.g., rGFP), the G α -interaction domain of a specific G protein effector tagged with
25 suitable BRET donor or acceptor (e.g., RlucII) are exposed to an agonist to activate the coexpressed G protein. In panel **i**), the agonist-induced GPCR stimulation activates directly G proteins, which recruits a tagged effector from the cytoplasm to the labeled membrane. In panel **ii**) G protein activation is mediated by the recruitment of a GEF such as GIV/Girdin following the activation of an RTK (e.g., EGFR) or an integrin $\alpha\beta$ complex.

30 **FIGs 1B-1E** show examples of specificity obtained with three different G protein effectors. Receptors coupled to members of Gq, Gi and G12/13 families were co-expressed with these G proteins, with an rGFP fused to a PM marker (rGFP-CAAX, GKKKKKKSKTKCVIM, SEQ ID NO: 1) and a G protein effector tagged with RlucII. **FIG. 1B** shows dose response curves (DRCs) obtained with platelet-activating factor (PAF)/PAFR-mediated G protein (Gq, G11, G14,
35 G12, GoA, GoB, Gz, Gi1, Gi2, Gi3) activation, using Rap1GAP (SSS-AAA)-RlucII and rGFP-CAAX (PM marker). Mock response represent activation of endogenously expressed Gi1, i2 and i3 proteins. **FIG. 1C** shows DRCs obtained with U46619/TPaR-mediated G protein (Gq, G11, G14, G15, G12, G13, Gz) activation, using PDZRG-RlucII and rGFP-CAAX. Mock response represent activation of endogenously expressed G12 and G13 proteins. **FIGs. 1D and 1E** show
40 DRCs obtained with PAF/PAFR-mediated G protein (Gq, G11, G14, G12, GoA, GoB, Gz, Gi1,

Gi2, Gi3) activation (**FIG. 1D**) and U46619/TP α R-mediated G protein (Gq, G11, G14, G15, G12, G13, Gz) activation (**FIG. 1E**), using P63RG-RlucII and rGFP-CAAX. Mock response represent activation of endogenously expressed Gq and G11 proteins.

FIG. 2A depicts various constructs tested of a Rap1GAP-based system for monitoring activation of G proteins of the Gi family. The first construct consists of a portion (residues 1-442) of Rap1GAP tagged in C-terminal with the BRET donor, Rluc8. From this construct, a second construct was derived using a different linker and using RlucII instead of Rluc8. The results of the experiments are depicted in **FIGs. 2B-2Z**. LinkerA = GSGGGSGGGA (SEQ ID NO: 6) and LinkerB = GSAGTGGRAIDIKLPAT (SEQ ID NO: 7). Rap1GAP 1-442 = SEQ ID NO: 8; Rap1GAP Δ cterm (1-420) = SEQ ID NO: 10; Rap1GAP Δ SSS = SEQ ID NO: 9; Rap1GAP SS-AA = SEQ ID NO: 13; Rap1GAP SS-DA = SEQ ID NO: 14; Rap1GAP SS-AD = SEQ ID NO: 15; Rap1GAP SS-DD = SEQ ID NO: 16; Rap1GAP SSS-AAA = SEQ ID NO: 11; Rap1GAP SSS-TTT = SEQ ID NO: 12.

FIGs. 2B-2E show DRCs using the human Mu opioid receptor (hMOR1)-mediated activation of Gi1 (**FIG. 2B**), Gi2 (**FIG. 2C**), GoA (**FIG. 2D**) and Gz (**FIG. 2E**) upon AR-M100390 (ARM) stimulation using three different Rap1GAP-Rluc constructs: Rap1GAP (1-442)-Rluc8 (circles), Rap1GAP (1-442)-RlucII (triangles) and Rap1GAP (Δ CT)-RlucII (diamonds).

FIGs. 2F-2H show DRCs using hMOR1/ARM-promoted activation of Gz at the PM in the presence of forskolin (triangles) or vehicle (DMSO/Tyrode, circles), which promotes an increase cAMP production and activation of protein kinase A leading to phosphorylation of different proteins, using Rap1GAP (1-442)-Rluc8 (**FIG. 2F**), Rap1GAP (1-442)-RlucII (**FIG. 2G**) or RAP1GAP (Δ CT)-RlucII (**FIG. 2H**).

FIGs. 2I-2N show DRCs using hMOR1/ARM-promoted activation of Gi2 at the PM in the presence of forskolin (squares) or vehicle (circles) using Rap1GAP (1-442)-RlucII (**FIG. 2I**), the truncated (residues 1-436) version Rap1GAP (Δ SSS)-RlucII (**FIG. 2J**), Rap1GAP (SS-AD)-RlucII (**FIG. 2K**), Rap1GAP (SS-DA)-RlucII (**FIG. 2L**), Rap1GAP (SS-AA)-RlucII (**FIG. 2M**), or Rap1GAP (SS-DD)-RlucII (**FIG. 2N**).

FIGs. 2O-2Q show DRCs using hMOR1/ARM-promoted activation of GoB at the PM in the presence of forskolin (triangles) or vehicle (circles) using Rap1GAP (1-442)-RlucII (**FIG. 2O**), Rap1GAP (SSS-TTT)-RlucII (**FIG. 2P**) and Rap1GAP (SSS-AAA)-RlucII (**FIG. 2Q**).

FIG. 2R shows DRCs of G protein activation following dopamine-promoted stimulation of the Dopamine D4 receptor (D4R) using Rap1GAP (SSS-AAA)-RlucII translocation to the plasma membrane, with co-expression of Gi1, Gi2, Gi3, GoA or GoB.

FIGs. 2S-2W show DRCs of Gz activation following stimulation of the dopamine receptors D4R (**FIG. 2S**), D1R (**FIG. 2T**), D2R (**FIG. 2U**), D3R (**FIG. 2U**) or D1R (**FIG. 2W**) with the ligands A412,997, Dopamine, L741,742 and Way-100635.

FIGs. 2X-2Z are graphs depicting the Z' factors for the assays, which is an indication of the robustness of the assays. HEK293 were co-transfected with D4R, Rap1GAP (SSS-AAA)-

RlucII, rGFP-CAAX, together with WT Gi2 (**FIG. 2X**), with WT GoA (**FIG. 2Y**), or WT Gz (**FIG. 2Z**), plated in a 96-well plate and stimulated with 100nM dopamine (triangles) or vehicle (DMSO/Tyrode; squares) at 37°C, for 6-8 min. Recruitment of Rap1GAP (SSS-AAA)-RlucII to the PM was evaluated in BRET2. BRET values are expressed per well in the presented graphs.

5 **FIG. 3A** depicts other constructs for monitoring activation of G proteins of the Gi family based on the RGS domain of members of the Regulator of G-protein signaling (RGS) proteins used in the studies described herein. A fragment comprising the RGS (Gi-binding) domain of RGS17 (residues 64-210 of SEQ ID NO: 17), RGS19 (residues 70-217 of SEQ ID NO: 18) and RGS20 (residues 242-388 of SEQ ID NO: 19) is tagged in C-terminal with the BRET donor RlucII.
10 LinkerB' = GSAGTGGRAIDIKLASAT (SEQ ID NO: 20).

FIGs. 3B-3D show DRCs of G protein activation following dopamine-promoted stimulation of D4) using RGS(RGS17)-RlucII (**FIG. 3B**), RGS(RGS19)-RlucII (**FIG. 3C**) or RGS(RGS20)-RlucII (**FIG. 3D**) translocation to the PM, with co-expression of GoA, GoB, Gz, Gi1, Gi2 or Gi3.

15 **FIG. 4A** depicts the PDZRhGEF (PDZRG)-RlucII construct for monitoring activation of G protein of the G12/13 family used in the studies described herein. A fragment comprising the G12/13 binding domain of PDZRhGEF (residues 281-483 of SEQ ID NO: 21) is tagged in C-terminal with the BRET donor RlucII. LinkerD = GIRLREALKLPAT (SEQ ID NO: 22).

FIGs. 4B and 4C show DRCs of G12 (**FIG. 4B**) and G13 (**FIG. 4C**) activation in HEK293
20 cells cotransfected with thromboxane receptor (TPaR), PDZRG-RlucII, rGFP-CAAX and with either no Ga (Mock), 5 ng of Ga, 20 ng of Ga or 100 ng of Ga, following stimulation with the TPaR agonist I-BOP (CAS Number: 128719-90-4).

FIGs. 4D and 4E show DRCs of TPaR ligands on G12 (**FIG. 4D**) and 13 (**FIG. 4E**) activation at the PM using PDZRG-RlucII. HEK293 cells cotransfected with thromboxane receptor
25 (TPaR), PDZRG-RlucII, rGFP-CAAX were stimulated with known full agonists (U46619, I-BOP, CTA2), with a partial agonist (U51605) and the antagonists I-SAP and SQ 29,558. Results were normalized and presented as % of I-BOP response (n=4) +/- SEM.

FIGs. 4F and 4G are graphs depicting the Z' factors for the assays using the PDZRG-RlucII construct. HEK293 were co-transfected with TPaR, PDZRG-RlucII, rGFP-CAAX and with
30 WT G12 (**FIG. 4F**) or WT G13 (**FIG. 4G**), plated in a 96-well plate and stimulated with 100 nM of the TPaR agonist U46619 (triangles) or vehicle (methyl acetate/Tyrode; squares) at 37°C, for 6-8 min. Recruitment of PDZRG-RlucII to the PM was evaluated in BRET2. BRET values are expressed per well in the presented graphs.

FIG. 5A depicts the P115RhGEF(P115RG)-RlucII construct for monitoring activation of
35 G protein of the G12/13 family used in the studies described herein. A fragment comprising the G12/13 binding domain of P115RhGEF (residues 1-244 of SEQ ID NO:23) is tagged in C-terminal with the BRET donor RlucII. LinkerC = RLKLPAT (SEQ ID NO: 24).

FIGs. 5B and 5C show DRCs of TP α R ligands on G12 (**FIG. 5B**) and G13 (**FIG. 5C**) activation at the PM using P115RG-RlucII. HEK293 cells cotransfected with thromboxane receptor (TP α R), P115RG-RlucII, rGFP-CAAX were stimulated with U46619, I-BOP, CTA2, U51605, I-SAP and SQ 29,558. Results were normalized and presented as % of I-BOP response (n=4) +/- SEM.

FIGs. 5D and 5E are graphs depicting the Z' factors for the assays using the PDZRG-RlucII construct. HEK293 were co-transfected with TP α R, P115RG-RlucII, rGFP-CAAX and with WT G12 (**FIG. 5D**) or WT G13 (**FIG. 5E**), plated in a 96-well plate and stimulated with 100 nM of the TP α R agonist U46619 (triangles) or vehicle (methyl acetate/Tyrode; squares) at 37°C, for 6-8 min. Recruitment of P115RG-RlucII to the PM was evaluated in BRET2. BRET values are expressed per well in the presented graphs.

FIG. 6A depicts the P63RhoGEF (P63RG) construct for monitoring activation of G proteins of the Gq family (Gq, G11, G14 & G15) used in the studies described herein. A fragment comprising the Gq binding domain of P63RhoGEF (residues 295-502 of SEQ ID NO: 25) is tagged in C-terminal with the BRET donor RlucII. LinkerE = ASGSAGTGGRADIKLPAT (SEQ ID NO: 26).

FIGs. 6B-6E show DRCs of Gq (**FIG. 6B**), G11 (**FIG. 6C**), G14 (**FIG. 6D**) and G15 (**FIG. 6E**) activation at the PM in HEK293 cells cotransfected with TP α R, P63RG-RlucII, rGFP-CAAX, and either no G α (Mock, responses obtained from endogenous G proteins) or different quantities of G α subunit, following stimulation with the TP α R agonist U46619.

FIGs. 6F-6I show DRCs of Gq (**FIG. 6F**), G11 (**FIG. 6G**), G14 (**FIG. 6H**) and G15 (**FIG. 6I**) activation at the early endosomes (EE) in HEK293 cells cotransfected with TP α R, P63RG-RlucII, rGFP-FYVE, and either no G α (Mock, responses obtained from endogenous G proteins) or different quantities of G α subunit, following stimulation with the TP α R agonist U46619.

FIGs. 6J-6M show DRCs of TP α R ligands on Gq (**FIG. 6J**), G11 (**FIG. 6K**), G14 (**FIG. 6L**) and G15 (**FIG. 6M**) activation at the PM using P63RG-RlucII under the optimal conditions determined in **FIGs. 6B-6E**. HEK293 cells cotransfected with thromboxane receptor (TP α R), P63RG-RlucII, rGFP-CAAX were stimulated with U46619, I-BOP, CTA2, U51605, I-SAP and SQ 29,558. Results were normalized and presented as % of I-BOP response (between n=3 and n=5) +/- SEM.

FIGs. 6N-6Q show DRCs of TP α R ligands on Gq (**FIG. 6N**), G11 (**FIG. 6O**), G14 (**FIG. 6P**) and G15 (**FIG. 6Q**) activation at the EE using P63RG-RlucII under the optimal conditions determined in **FIGs. 6F-6I**. HEK293 cells cotransfected with thromboxane receptor (TP α R), P63RG-RlucII, rGFP-FYVE were stimulated with U46619, I-BOP, CTA2, U51605, I-SAP and SQ 29,558. Results were normalized and presented as % of I-BOP response (between n=3 and n=5) +/- SEM.

FIGs. 6R and **6S** are graphs depicting the Z' factors for the assays using the P63RG-RlucII construct. HEK293 were co-transfected with TPαR, P63RG-RlucII, rGFP-CAAX and with WT Gq (**FIG. 6R**) or WT G11 (**FIG. 6S**), plated in a 96-well plate and stimulated with 100 nM of the TPαR agonist U46619 (triangles) or vehicle (methyl acetate/Tyrode; squares) at 37°C, for 6-8 min. Recruitment of P63RG-RlucII to the PM was evaluated in BRET2. BRET values are expressed per well in the presented graphs.

FIG. 7A depicts two RGS(GRK2) constructs for monitoring activation of G proteins of the Gq family (Gq, G11, G14 & G15) used in the studies described herein. A fragment comprising the Gq binding domain (RGS domain) of GRK2 (residues 30-203 of SEQ ID NO: 27) is tagged at the N-terminal RlucII-RGS(GRK2) or C-terminal (RGS(GRK2)-RlucII) with the BRET donor RlucII. LinkerB' = GSAGTGGRAIDIKLASAT (SEQ ID NO: 20).

FIGs. 7B and **7C** show DRCs of the activation of Gq, G11, G14 and G15 at the PM in two ligand/receptor systems, namely At1AR/ANGII (**FIG. 7B**) and TPαR/U46619 (**FIG. 7C**), using RlucII-RGS(GRK2).

FIGs. 7D and **7E** show DRCs of the activation of Gq, G11, G14 and G15 at the PM in two ligand/receptor systems, namely At1AR/ANGII (**FIG. 7D**) and TPαR/U46619 (**FIG. 7E**), using RGS(GRK2)-RlucII.

FIG. 7F is a graph depicting the Z' factors for the assays using the RlucII-RGS(GRK2) construct. HEK293 were co-transfected with TPαR, RlucII-RGS(GRK2), rGFP-CAAX and with WT Gq, plated in a 96-well plate and stimulated with 100 nM of the TPαR agonist U46619 (triangles) or vehicle (methyl acetate/Tyrode; squares) at 37°C, for 6-8 min. Recruitment of RlucII-RGS(GRK2) to the PM was evaluated in BRET2. BRET values are expressed per well in the presented graphs.

FIG. 8A depicts mutated Gi2 and GoB proteins tested in the studies described herein for their ability to monitor GEF-mediated activation of G proteins, i.e. G protein activation not mediated by GPCRs. Gai2 Δ5 = SEQ ID NO: 28; Gai2 Δ2 = SEQ ID NO: 29; Gai2 L-2G = SEQ ID NO: 31; Gai2 L-2P = SEQ ID NO: 33; Gai2 L-2R = SEQ ID NO: 34; Gai2 L-2D = SEQ ID NO: 32; Gai2 L-7G = SEQ ID NO: 30; GaoB Δ5 = SEQ ID NO: 36; GaoB L-2G = SEQ ID NO: 35.

FIGs. 8B-8G show DRCs of the activation of deletion mutants of Gi2 and GoB, namely Gi2 Δ2 (**FIGs. 8B** and **8C**), Gi2 Δ5 (**FIGs. 8D** and **8E**) and GoB Δ5 (**FIGs. 8F** and **8G**). HEK293 were co-transfected with constructs encoding either the EGF receptor (EGFR, **FIGs. 8B, 8D, 8F**) or the bradykinin receptor (BKB2R, **FIGs. 8C, 8E, 8G**), Rap1GAP (SSS-AAA)-RlucII, rGFP-CAAX and the WT or mutated Gi2/GoB subunits.

FIGs. 8H-8M show DRCs of the activation of Leu to Gly mutants of Gi2 and GoB, namely Gi2 L-7G (**FIGs. 8H** and **8I**), Gi2 L-2G (**FIGs. 8J** and **8K**) and GoB L-2G (**FIGs. 8L** and **8M**). HEK293 were co-transfected with constructs encoding either EGFR (**FIGs. 8H, 8J, 8L**) or BKB2R

(**FIGs. 8I, 8K, 8M**), Rap1GAP (SSS-AAA)-RlucII, rGFP-CAAX and the WT or mutated Gi2/GoB subunits.

FIGs. 8N-8S show DRCs of the activation of position -2 mutants of Gi2, namely Gi2 L-2D (**FIGs. 8N** and **8O**), Gi2 L-2P (**FIGs. 8P** and **8Q**) and Gi2 L-2R (**FIGs. 8R** and **8S**). HEK293 were co-transfected with constructs encoding either EGFR (**FIGs. 8N, 8P, 8R**) or BKB2R (**FIGs. 8O, 8Q, 8S**), Rap1GAP (SSS-AAA)-RlucII, rGFP-CAAX and the WT or mutated Gi2 subunits.

FIGs. 8T-8U are graphs depicting the Z' factors for the assay monitoring GEF-mediated activation of G proteins using the Rap1GAP (SSS-AAA)-RlucII construct. HEK293 were co-transfected with EGFR, Rap1GAP (SSS-AAA)-RlucII, rGFP-CAAX and WT Gi2 (**FIG. 8T**) or mutant Gi2 L-2P (**FIG. 8U**), plated in a 96-well plate and stimulated with 10 ng/ml of EGF (triangles) or vehicle (Tyrode; squares) at RT, for 2 min. Recruitment of Rap1GAP (SSS-AAA)-RlucII to the PM was evaluated in BRET2. BRET values are expressed per well in the presented graphs.

FIGs. 8V-8X show DRCs of Gi2 activation by two GPCRs, delta-opioid receptor (DOR) in **FIG. 8V** and D2R in **FIG. 8W**, and an RTK (EGFR; **FIG. 8X**), monitored using Rap1GAP (SSS-AAA)-RlucII construct (circles) and RGS(RGS17)-RlucII (triangles). HEK293 were co-transfected with constructs encoding a receptor (DOR, D2R or EGFR), Rap1GAP (SSS-AAA)-RlucII or RGS(RGS17)-RlucII, rGFP-CAAX and WT Gi2, plated in a 96-well plate and stimulated with the indicated doses at RT, for 8 min with a GPCR agonist (**FIGs. 8V, 8W**) or 7 min with EGF (**FIG. 8X**). Recruitment of Rap1GAP (SSS-AAA)-RlucII and RGS(RGS17)-RlucII to the PM was evaluated in BRET2. DRCs presented are representative of three independent experiments.

FIGs. 9A-9D show the amino acid sequences of polypeptides used in the studies described herein.

DISCLOSURE OF INVENTION

Terms and symbols of genetics, molecular biology, biochemistry and nucleic acid used herein follow those of standard treatises and texts in the field, *e.g.* Kornberg and Baker, *DNA Replication*, Second Edition (W University Science Books, 2005); Lehninger, *Biochemistry*, 6th Edition (W H Freeman & Co (Sd), New York, 2012); Strachan and Read, *Human Molecular Genetics*, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, *Oligonucleotides and Analogs: A Practical Approach* (Oxford University Press, New York, 1991); Gait, editor, *Oligonucleotide Synthesis: A Practical Approach* (IRL Press, Oxford, 1984); and the like. All terms are to be understood with their typical meanings established in the relevant art.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element. Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the

inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

The information, including the nucleotide and amino acid sequences, corresponding to the Genbank, RefSeq, UniProt, NCBI and/or Ensembl accession numbers (or any other database) referred to in the present specification is incorporated herein by reference.

All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context.

The use of any and all examples, or exemplary language ("*e.g.*", "such as") provided herein, is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed.

Herein, the term "about" has its ordinary meaning. The term "about" is used to indicate that a value includes an inherent variation of error for the device or the method being employed to determine the value, or encompass values close to the recited values, for example within 10% or 5% of the recited values (or range of values).

Any and all combinations and subcombinations of the embodiments and features disclosed herein are encompassed by the present invention. For example, the expression of any combination of 2, 3, 4, 5 or more of the genes identified herein may be used in the methods described herein.

The present disclosure relates to systems and assays allowing the monitoring of G protein activation in a G protein family-selective manner, and at different cellular compartments. These systems and assays are based on the use of specific effectors of G proteins and cellular compartment markers, tagged with suitable energy (i.e. BRET) donors and acceptors. These systems and assays advantageously do not require the modification (*e.g.*, tagging/fusion with a BRET donor or acceptor or other detectable labels) of any of the G protein subunits (G α , G β or G γ) as G protein activation at a particular cellular compartment is indirectly detected in a G protein family-selective manner by assessing the translocation of specific G protein effectors, thus minimizing the risk that the function/activity of the G protein be altered. The dynamic window obtained with the system described herein is higher than that obtained with other systems for assessing G protein activation (*e.g.*, U.S. Patent No. 9,029,097 and WO/2016/058094), which may be important for high throughput screening (HTS) applications (including the identification of partial agonists), and also permits the detection of the signal mediated by endogenous G proteins (at the G protein family level). Specificity is achieved by using G protein-binding domains of G protein effectors specific to the G protein subunit(s) of interest, such as the G protein-binding domain of Rap1GAP for the Gi family (Gi1, Gi2, Gi3, GoA, GoB, Gz), the G protein-binding domain of P63RhoGEF (P63RG) for the Gq family (Gq, G11, G14 & G15) and the G protein-binding domain of PDZRhoGEF or P115RhoGEF for the G12/13 family. When needed, specificity at the

G protein subunit level may be achieved by co-expressing the G protein subunit(s) of interest with the studied receptor.

Accordingly, in an aspect, the present disclosure provides a system for measuring modulation of G protein activation in a G α protein subunit family-selective manner, said system comprising:

a cell expressing:

(i) a first component comprising a G α subunit interacting polypeptide (GASIP) tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein:

if said G α protein subunit family is Gi, said GASIP comprises a domain of a protein that binds to Gi;

if said G α protein subunit family is Gq, said GASIP comprises a domain of a protein that binds to Gq;

if said G α protein subunit family is G12/13, said GASIP comprises a domain of a protein that binds to G12/13; and

if said G α protein subunit family is Gs, said GASIP comprises a domain of a protein that binds to Gs; and

(ii) a second component comprising a plasma membrane (PM)-targeting moiety, an endosomal-targeting moiety or a Golgi-targeting moiety tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein if said GASIP is tagged with said fluorescent acceptor molecule, said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said bioluminescent donor molecule, and if said GASIP is tagged with said bioluminescent donor molecule, said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said fluorescent acceptor molecule.

In another aspect, the present disclosure provides a system for measuring modulation of G protein activation in a G α protein subunit family-selective manner, said system comprising:

a cell expressing:

(i) a first component comprising a G α subunit interacting polypeptide (GASIP) tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein:

if said G α protein subunit family is Gi, said GASIP comprises a G protein-binding domain of: Rap1GAP, a Regulator of G-protein signaling (RGS) protein or TNFAIP8, preferably a G protein-binding domain of Rap1GAP or an RGS protein;

if said G α protein subunit family is Gq, said GASIP comprises a G protein-binding domain of: a RhoGEF protein (*e.g.*, P63RhoGEF or TRIO), GRK2, GRK3, TPR1 (tetratricopeptide repeat

domain 1) or an RGS protein (*e.g.*, RGS2), preferably a G protein-binding domain of P63RhoGEF or GRK2/GRK3;

if said G α protein subunit family is G12/13, said GASIP comprises a G protein-binding domain of a RhoGEF protein (PDZRhoGEF, P115RhoGEF or LARG), JNK-Interacting Leucine
5 Zipper Protein (JLP), cadherin, Axin1, PP2A, SNAP-alpha, polycystin1, RGS16, AKAP110 or HAX1 (HS1-associated protein X1), preferably a G protein-binding domain of a PDZRhoGEF or P115RhoGEF;

if said G α protein subunit family is Gs (G α_s , XLG α_s , G α_{olf}), said GASIP comprises a G protein-binding domain of SNX13 (RGS-PX1), AXIN1 or TPR1 (tetratricopeptide repeat domain
10 1);

(ii) a second component comprising a plasma membrane (PM)-targeting moiety, an endosomal-targeting moiety or a Golgi-targeting moiety tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein if said GASIP is tagged with said fluorescent acceptor molecule, said PM-
15 targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said bioluminescent donor molecule, and if said GASIP is tagged with said bioluminescent donor molecule, said cellular M-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said fluorescent acceptor molecule.

The bioluminescent donor molecule (or BRET donor) and the fluorescent acceptor
20 molecule (or BRET acceptor) are selected so that the emission spectrum of the bioluminescent donor molecule overlaps with the absorbance spectrum of the fluorescent acceptor molecule. Under such conditions, the light energy delivered by the bioluminescent donor molecule is at a wavelength that is able to excite the fluorescent acceptor molecule, i.e. bioluminescence resonance energy transfer (BRET). Resonance energy transfer (abbreviated RET) is a
25 mechanism describing energy transfer between two chromophores, having overlapping emission/absorption spectra. When the two chromophores (the "donor" and the "acceptor"), are within a short distance (*e.g.*, 10-100 Angstroms) of one another and their transition dipoles are appropriately oriented, the donor chromophore is able to transfer its excited-state energy to the acceptor chromophore through non-radiative dipole-dipole coupling. Bioluminescence
30 Resonance Energy Transfer (BRET) is based on the non-radiative transfer of energy between a donor bioluminescent molecule (bioluminescent enzyme such as *renilla* luciferase) and an acceptor fluorescent molecule (*e.g.*, *renilla* GFP).

As used herein, the term bioluminescent donor molecule refers to any molecule able to
generate luminescence following either action on a suitable substrate, or its own excitation by an
35 external source. There are a number of different bioluminescent donor molecules that can be employed in the present disclosure. 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 PCT publications No. WO 00/024878 and WO 99/049019. In an embodiment, the bioluminescent donor molecule is a luciferase. Examples of
 5 bioluminescent proteins with luciferase activity are disclosed in US Patents Nos. 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* luciferases and (ii) *Firefly* luciferases.

In an embodiment, the bioluminescent donor molecule is a *Renilla* luciferase (*rLuc*). The term *Renilla* luciferase as used herein refers to an oxidative enzyme used in bioluminescence and that is derived from an organism of the genus *Renilla*, such as *Renilla reniformis* or *Renilla mulleri*. It includes the native luciferase from a *Renilla* organism, or variants thereof, for example the native form (in terms of amino acid sequence) of *Renilla reniformis* luciferase (Rluc) or variants thereof such as RlucII, Rluc3, Green *Renilla* Luciferase or Rluc8. The term "RlucII" refers to a mutant form of *Renilla reniformis* luciferase that comprises the following amino acid substitutions:
 15 A55T, C124A and M185V relative to a native *Renilla* luciferase. In an embodiment, the RlucII comprises the sequence depicted in **FIG. 9C**. The term "Rluc8" refers to a mutant form of *Renilla reniformis* luciferase that comprises the following amino acid substitutions: A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L relative to a native *Renilla reniformis* luciferase. The amino acid sequence of native *Renilla mulleri* luciferase is disclosed in GenBank accession No.
 20 AAG54094.1.

Natural and synthetic luminescent substrates for these enzymes are well known in the art and are commercially available. Examples of luciferase substrates include luciferin (*e.g.*, D-luciferin and salts thereof, Latia luciferin, bacterial luciferin, Dinoflagellate luciferin, etc.), coelenterazine, coelenterazine h, coelenterazine e, coelenterazine f, coelenterazine fcp,
 25 coelenterazine cp, coelenterazine hcp, coelenterazine i, coelenterazine ip, coelenterazine n, coelenterazine 400a (DeepBlueC™), methoxy e-Coelenterazine (Prolume® Purple I from NanoLight Technology®), Methoxy-Coelenterazine-Methoxy (Prolume® Purple II from NanoLight Technology®), Methoxy-Coelenterazine-F (Prolume® Purple III from NanoLight Technology®), Methoxy-Coelenterazine-Iodine (Prolume® Purple IV from NanoLight Technology®), Methoxy-v-
 30 Coelenterazine- Methoxy (Prolume® Purple V from NanoLight Technology®), ViviRen™ (from Promega®). The suitable substrate of the bioluminescent donor molecule may be selected by the skilled person based on the desired wavelength and/or intensity of the light emitted by the bioluminescent protein. In an embodiment, the luciferase substrate is coelenterazine-h, methoxy e-Coelenterazine or coelenterazine 400A.

35 As used herein, the term fluorescent acceptor molecule refers to any compound which can accept energy emitted as a result of the activity of a bioluminescent donor molecule, and re-emit it as light energy. Representative fluorescent acceptor proteins can include, but are not

limited to, green fluorescent protein (GFP), variant of green fluorescent protein (such as GFP10), blue fluorescent protein (BFP), cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, mAmetrine, LSS-mOrange, LSS-mKate, Emerald, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, mRFPI, pocilloporin, *Renilla* GFP (*r*GFP), Monster GFP, paGFP, Kaede protein or a Phycobiliprotein, or a biologically active variant or fragment of any one thereof. The most frequently used bioluminescent or fluorophore is the GFP from the jellyfish *Aequorea victoria* and numerous other variants (GFPs) obtained for example mutagenesis and chimeric protein technologies. 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.

Examples of non-proteinaceous fluorescent acceptor molecules are Alexa™ (Molecular Probes), fluor dye, Bodipy dye™ (Life technologies), Cy dye™ (Life technologies), fluorescein, dansyl, umbelliferone (7-hydroxycoumarin), fluorescent microsphere, luminescent nanocrystal, Marina blue™ (Life technologies), Cascade blue™ (Life technologies), Cascade yellow™ (Life technologies), Pacific blue™ (Life technologies), Oregon green™ (Life technologies), Tetramethylrhodamine, Rhodamine, Texas red™ (Life technologies), rare earth element chelates, or any combination or derivatives thereof.

Other representative fluorescent acceptor molecules can include, but are not limited to sgGFP, sgBFP, BFP blue-shifted GFP (Y66H), Cyan GFP, DsRed, monomeric RFP, EBFP, ECFP, GFP (S65T), GFP red-shifted (rsGFP), non-UV excitation (wtGFP), UV excitation (wtGFP), GFPuv, HcRed, rsGFP, Sapphire GFP, sgBFP™, sgBFP™ (super glow BFP), sgGFP™, sgGFP™ (super glow GFP), Yellow GFP, semiconductor nanoparticles (e.g., raman nanoparticles), 1,5 IAEDANS; 1,8-ANS; 4-Methylumbelliferone; 5-carboxy-2,7-dichlorofluorescein; 5-Carboxyfluorescein (5-FAM); 5-Carboxynaphthofluorescein; 5 -Carboxy tetramethylrhodamine (5-TAMRA); 5-FAM (5-Carboxyfluorescein); 5-HAT (Hydroxy Tryptamine); 5-Hydroxy Tryptamine (HAT); 5-ROX (carboxy-X-rhodamine); 5-TAMRA (5-Carboxytetramethylrhodamine); 6-Carboxyrhodamine 6G; 6-CR 6G; 6-JOE; 7-Amino-4-methylcoumarin; 7-Aminoactinomycin D (7-AAD); 7-Hydroxy-4-methylcoumarin; 9-Amino-6-chloro-2-methoxyacridine; ABQ; Acid Fuchsin; ACMA (9-Amino-6-chloro-2-methoxyacridine); Acridine Orange; Acridine Red; Acridine Yellow; Acriflavin; Acriflavin Feulgen SITS A; Aequorin (Photoprotein); AFPs (AutoFluorescent Protein; Quantum Biotechnologies); Alexa Fluor 350™; Alexa Fluor 430™; Alexa Fluor 488™; Alexa Fluor 532™; Alexa Fluor 546™; Alexa Fluor 568™; Alexa Fluor 594™; Alexa Fluor 633™; Alexa Fluor 647™; Alexa Fluor 660™; Alexa Fluor 680™; Alizarin Complexon; Alizarin Red; Allophycocyanin (APC); AMC, AMCA-S; AMCA

(Aminomethylcoumarin); AMCA-X; Aminoactinomycin D; Aminocoumarin; Aminomethylcoumarin (AMCA); Anilin Blue; Anthrocyll stearate; APC (Allophycocyanin); APC-Cy7; APTRA-BTC; APTS; Astrazon Brilliant Red 4G; Astrazon Orange R; Astrazon Red 6B; Astrazon Yellow 7 GLL; Atabrine; ATTO-TAG™ CBQCA; ATTO-TAG™ FQ; Auramine; Aurophosphine G;

5 Aurophosphine; BAO 9 (Bisaminophenyloxadiazole); BCECF (high pH); BCECF (low pH); Berberine Sulphate; Beta Lactamase; Bimane; Bisbenzamide; Bisbenzimidazole (Hoechst); bis-BTC; Blancophor FFG; BlancophorSV; BOBO™-1 ; BOBO™-3; Bodipy 492/515; Bodipy 493/503; Bodipy 500/510; Bodipy 505/515; Bodipy 530/550; Bodipy 542/563; Bodipy 558/568; Bodipy 564/570; Bodipy 576/589; Bodipy 581/591; Bodipy 630/650-X; Bodipy 650/665-X; Bodipy

10 665/676; Bodipy FI; Bodipy FL ATP; Bodipy FI-Ceramide; Bodipy R6G SE; Bodipy TMR; Bodipy TMR-X conjugate; Bodipy TMR-X, SE; Bodipy TR; Bodipy TR ATP; Bodipy TR-X SE; B0-PRO™-1 ; B0-PRO™-3; Brilliant Sulphoflavin FF; BTC; BTC-5N; Calcein; Calcein Blue; Calcium Crimson™; Calcium Green; Calcium Green-1 Ca²⁺ Dye; Calcium Green-2 Ca²⁺; Calcium Green-5N Ca²⁺; Calcium Green-C18 Ca²⁺; Calcium Orange; Calcofluor White; Carboxy-X-rhodamine (5-

15 ROX); Cascade Blue™; Cascade Yellow; Catecholamine; CCF2 (GeneBlazer); CFDA; Chlorophyll; Chromomycin A; Chromomycin A; CL-NERF; CMFDA; Coumarin Phalloidin; C-phycocyanine; CPM Methylcoumarin; CTC; CTC Formazan; Cy2™; Cy3.18; Cy3.5™; Cy3™; Cy5.18; Cy5.5™; Cy5™; Cy7™; cyclic AMP Fluorosensor (FiCRhR); Dabcyl; Dansyl; Dansyl Amine; Dansyl Cadaverine; Dansyl Chloride; Dansyl DHPE; Dansyl fluoride; DAPI; Dapoxyl;

20 Dapoxyl 2; Dapoxyl 3'DCFDA; DCFH (Dichlorodihydrofluorescein Diacetate); DDAO; DHR (Dihydrohodamine 123); Di-4-ANEPPS; Di-8-ANEPPS (non-ratio); DiA (4-Di-16-ASP); Dichlorodihydrofluorescein Diacetate (DCFH); DiD-Lipophilic Tracer; DiD (DiI18(5)); DIDS; Dihydrohodamine 123 (DHR); DiI (DiI18(3)); Dinitrophenol; DiO (DiOC18(3)); DiR; DiR (DiI18(7)); DM-NERF (high pH); DNP; Dopamine; DTAF; DY-630-NHS; DY-635-NHS; ELF 97;

25 Eosin; Erythrosin; Erythrosin ITC; Ethidium Bromide; Ethidium homodimer-1 (EthD-1); Euchrysin; EukoLight; Europium (III) chloride; EYFP; Fast Blue; FDA; Feulgen (Pararosaniline); FIF (Formaldehyd Induced Fluorescence); FITC; Flazo Orange; Fluo-3; Fluo-4; Fluorescein (FITC); Fluorescein Diacetate; Fluoro-Emerald; Fluoro-Gold (Hydroxystilbamidine); Fluor-Ruby; FluorX; FM 1-43™; FM 4-46; Fura Red™ (high pH); Fura Red™/Fluo-3; Fura-2; Fura-2/BCECF; Genacryl

30 Brilliant Red B; Genacryl Brilliant Yellow 10GF; Genacryl Pink 3G; Genacryl Yellow 5GF; GeneBlazer (CCF2); Gloxalic Acid; Granular blue; Haematoporphyrin; Hoechst 33258; Hoechst 33342; Hoechst 34580; HPTS; Hydroxycoumarin; Hydroxystilbamidine (FluoroGold); Hydroxytryptamine; Indo-1, high calcium; Indo-1, low calcium; Indodicarbocyanine (DiD); Indotricarbocyanine (DiR); Intrawhite Cf; JC-1 ; JO-JO-1 ; JO-PRO-1 ; LaserPro; Laurodan; LDS

35 751 (DNA); LDS 751 (RNA); Leucophor PAF; Leucophor SF; Leucophor WS; Lissamine Rhodamine; Lissamine Rhodamine B; Calcein/Ethidium homodimer; LOLO-1; LO-PRO-1; Lucifer Yellow; Lyso Tracker Blue; Lyso Tracker Blue-White; Lyso Tracker Green; Lyso Tracker Red;

Lyso Tracker Yellow; LysoSensor Blue; LysoSensor Green; LysoSensor Yellow/Blue; Mag Green; Magdala Red (Phloxin B); Mag-Fura Red; Mag-Fura-2; Mag-Fura-5; Mag-Indo-1; Magnesium Green; Magnesium Orange; Malachite Green; Marina Blue; Maxilon Brilliant Flavin 10 GFF; Maxilon Brilliant Flavin 8 GFF; Merocyanin; Methoxycoumarin; Mitotracker Green FM; Mitotracker Orange; Mitotracker Red; Mitramycin; Monobromobimane; Monobromobimane (mBBBr-GSH); Monochlorobimane; MPS (Methyl Green Pyronine Stilbene); NBD; NBD Amine; Nile Red; Nitrobenzoxadidole; Noradrenaline; Nuclear Fast Red; Nuclear Yellow; Nylosan Brilliant Iavin E8G; Oregon Green; Oregon Green 488-X; Oregon Green™; Oregon Green™ 488; Oregon Green™ 500; Oregon Green™ 514; Pacific Blue; Pararosanine (Feulgen); PBFI; PE-Cy5; PE-Cy7; PerCP; PerCP-Cy5.5; PE-TexasRed [Red 613] ; Phloxin B (Magdala Red); Phorwite AR; Phorwite BKL; Phorwite Rev; Phorwite RPA; Phosphine 3R; PhotoResist; Phycoerythrin B [PE]; Phycoerythrin R [PE]; PKH26 (Sigma); PKH67; PMIA; Pontochrome Blue Black; POPO-1; POPO-3; PO-PRO-1 ; PO-PRO-3; Primuline; Procion Yellow; Propidium Iodid (PI); PyMPO; Pyrene; Pyronine; Pyronine B; Pyrozal Brilliant Flavin 7GF; QSY 7; Quinacrine Mustard; Red 613 [PE-TexasRed]; Resorufin; RH 414; Rhod-2; Rhodamine; Rhodamine 110; Rhodamine 123; Rhodamine 5 GLD; Rhodamine 6G; Rhodamine B; Rhodamine B 200; Rhodamine B extra; Rhodamine BB; Rhodamine BG; Rhodamine Green; Rhodamine Phallicidine; Rhodamine Phalloidine; Rhodamine Red; Rhodamine WT; Rose Bengal; R-phycoerythrin (PE); S65A; S65C; S65L; S65T; SBFI; Serotonin; Sevron Brilliant Red 2B; Sevron Brilliant Red 4G; Sevron Brilliant Red B; Sevron Orange; Sevron Yellow L; SITS; SITS (Primuline); SITS (Stilbene Isothiosulphonic Acid); SNAFL calcein; SNAFL-1; SNAFL-2; SNARF calcein; SNARF1; Sodium Green; SpectrumAqua; SpectrumGreen; SpectrumOrange; Spectrum Red; SPQ (6-methoxy-N-(3-sulfopropyl)quinolinium); Stilbene; Sulphorhodamine B can C; Sulphorhodamine Extra; SYTO 11 ; SYTO 12; SYTO 13; SYTO 14; SYTO 15; SYTO 16; SYTO 17; SYTO 18; SYTO 20; SYTO 21; SYTO 22; SYTO 23; SYTO 24; SYTO 25; SYTO 40; SYTO 41 ; SYTO 42; SYTO 43; SYTO 44; SYTO 45; SYTO 59; SYTO 60; SYTO 61 ; SYTO 62; SYTO 63; SYTO 64; SYTO 80; SYTO 81; SYTO 82; SYTO 83; SYTO 84; SYTO 85; SYTOX Blue; SYTOX Green; SYTOX Orange; Tetracycline; Tetramethylrhodamine (TRITC); Texas Red™; Texas Red-X™ conjugate; Thiadibocyanine (DiSC3); Thiazine Red R; Thiazole Orange; Thioflavin 5; Thioflavin S; Thioflavin TCN; Thiolyte; Thiozole Orange; Tinopol CBS (Calcofluor White); TMR; TO-PRO-1 ; TO-PRO-3; TO-PRO-5; TOTO-1; TOTO-3; TriColor (PE-Cy5); TRITC TetramethylRhodamineIsoThioCyanate; True Blue; TruRed; Ultralite; Uranine B; Uvitex SFC; WW 781; X-Rhodamine; XRITC; Xylene Orange; Y66F; Y66H; Y66W; YO-PRO-1 ; YO-PRO-3; YOYO-1; YOYO-3, SYBR Green, and Thiazole orange (interchelating dyes).

In an embodiment, the fluorescent acceptor molecule is a *Renilla* GFP (*rGFP*). The term “*Renilla* GFP” refers to a green fluorescent protein that is derived from organisms of the genus *Renilla*, such as *Renilla reniformis* or *Renilla mulleri*. It includes the native GFP from a *Renilla*

organism, or variants thereof. In an embodiment, the *Renilla* GFP is a *Renilla reniformis* GFP, in a further embodiment, the native form (in terms of amino acid sequence) of *Renilla reniformis* GFP. In an embodiment, the rGFP comprises the sequence depicted in **FIG. 9D**. The amino acid sequence of native *Renilla mulleri* GFP is disclosed in GenBank accession No. AAG54098.1. The

5 nucleic acid sequence of the *Renilla* luciferase and/or *Renilla* GFP may be codon-optimized for expression in human cells (i.e. "humanized", see, *e.g.*, WO 2002/057451 for a humanized version of *Renilla mulleri* GFP).

Representative combinations of bioluminescent donor and fluorescent acceptor molecule suitable for BRET (referred to as BRET pairs) include luciferase (Luc)/GFP, Luc/Venus,

10 Luc/Topaz, Luc/GFP-10, Luc/GFP-2, Luc/YFP, Luc/rGFP, and the like. In another embodiment, one of the following BRET configurations is used in the biosensors and methods described herein: RlucII/coel-400a/enhanced blue (EB) FP2, RlucII/coel-400a/super cyan fluorescent protein (SCFP3A), RlucII/coel-400a/mAmetrine, RlucII/coel-400a/rGFP, RlucII/coel-400a/mAmetrine.

In an embodiment, the bioluminescent donor molecule is a *Renilla* luciferase and the

15 fluorescent acceptor molecule is *Renilla* GFP.

In an embodiment, the second component comprises a PM-targeting moiety. The term "plasma membrane (PM) targeting moiety" as used herein refers to any moiety capable of recruiting or sequestering the bioluminescent donor or fluorescent acceptor molecule (*e.g.*, *Renilla* GFP or *Renilla* Luc) to the PM. The bioluminescent donor or fluorescent acceptor molecule

20 may thus be fused to any protein found at the plasma membrane (*e.g.*, receptors or any other protein found at the PM), or fragments thereof. An example of such proteins is Caveolin-1, which the main component of the caveolae (a type of lipid raft that correspond to small (50-100 nm) invaginations of the plasma membrane) found in many cell types. Two isoforms of Caveolin-1, generated by alternative splicing of the CAV1 gene, have been identified: Caveolin-1 α (comprising residues 2-178) and Caveolin-1 β (corresponding to the 32-178 sequence). Other

25 examples of such moiety include peptides/polypeptides comprising a signal sequence for protein lipidation/fatty acid acylation, such as myristoylation, palmitoylation and prenylation, as well as polybasic domains. Several proteins are known to be myristoylated, palmitoylated and/or prenylated (*e.g.*, protein kinases and phosphatases such as Yes, Fyn, Lyn, Lck, Hck, Fgr, G α proteins, nitric oxide synthase, ADP-ribosylation factors (ARFs), calcium binding proteins and

30 membrane or cytoskeleton-associated structural proteins such as MARCKS (see, *e.g.*, Wright et al., *J Chem Biol.* Mar 2010; 3(1): 19-35; Alcart-Ramos et al., *Biochimica et Biophysica Acta (BBA) – Biomembranes*, Volume 1808, Issue 12, December 2011, Pages 2981-2994), and thus the myristoylation, palmitoylation and prenylation (*e.g.*, geranylgeranylation) signal sequences from

35 any of these proteins may be used in the biosensor. In an embodiment, the myristoylation and/or palmitoylation sequence is from the Lyn kinase.

In an embodiment, the PM membrane targeting moiety comprises a CAAX motif (C is cysteine residue, AA are two aliphatic residues, and X represents any amino acid. CAAX motifs are found in "CAAX proteins" that are defined as a group of proteins with a specific amino acid sequence at C-terminal that directs their post translational modification. CAAX proteins encompass a wide variety of molecules that include nuclear lamins (intermediate filaments) such as prelamin A, lamin B1 and lamin B2, Ras and a multitude of GTP-binding proteins (G proteins) such as Ras, Rho, Rac, and Cdc42, several protein kinases and phosphatases, etc. (see, *e.g.*, Gao *et al.*, *Am J Transl Res.* 2009; 1(3): 312-325). The proteins that have a CAAX motif or box at the end of the C-terminus typically need a prenylation process before the proteins migrate to the plasma membrane or nuclear membrane and exert different functions. In an embodiment, the CAAX box is derived from a human RAS family protein, for example HRAS, NRAS, Ral-A, KRAS4A or KRAS4b. The last C-terminal residues of RAS, NRAS, KRAS4A or KRAS4b (referred to as the hypervariable region or HVR) are depicted below, with the putative minimal plasma membrane targeting region in italics and the CAAX box underlined (see, *e.g.*, Ahearn *et al.*, *Nature Reviews Molecular Cell Biology* **13**: 39-51, January 2012): HRAS: KLNPPDESGP GCMSCKCVLS (SEQ ID NO: 41); NRAS: KLNSSDDGTQ GCMGLPCVVM (SEQ ID NO: 42); KRAS4A: KISKEEKTPG CVKIKKCIIM (SEQ ID NO: 43); KRAS4B: KMSKDG KKKKKKSKTKCVIM (SEQ ID NO: 44); Ral-A/Ral1: KNGKKKRKSLAKRIRERCCIL (SEQ ID NO: 45). In an embodiment, the PM targeting moiety comprises the amino acid sequence GCMSCKCVLS (SEQ ID NO:60), GCMGLPCVVM (SEQ ID NO:61), CVKIKKCIIM (SEQ ID NO:62), KKKKKKSKTKCVIM (SEQ ID NO:63), or KNGKKKRKSLAKRIRERCCIL (SEQ ID NO: 45), preferably the PM targeting moiety comprises the sequence GKKKKKSKTKCVIM (SEQ ID NO:1) from KRAS4B. In another embodiment, the PM targeting moiety comprises the the plasma-membrane targeting palmitoylation sequence from HRAS and prenylation signal sequence from Ral-A/Ral1 (sequence: CMSCKCCIL, SEQ ID NO: 4).

Several proteins also contain a non-lipid, polybasic domain that targets the PM such as Ras small GTPases, phosphatase PTEN, nonreceptor tyrosine kinase Src, actin regulators WASP and MARCKS, and G protein-coupled receptor kinases (GRKs) such as GRK5. In an embodiment, the polybasic domain is from GRK5, and comprises the sequence SPKKGLLQRLFKRQHQNNSSKS (SEQ ID NO: 5).

In an embodiment, the second component comprises an endosomal targeting moiety. The term "endosomal targeting moiety" as used herein refers to any moiety capable of recruiting or sequestering the bioluminescent donor or fluorescent acceptor molecule to the endosomes, *e.g.*, the early endosomes. Several endosomal targeting moieties/markers are known in the art and include the Rab family of proteins (RAB4, RAB5, RAB7, RAB9 and RAB11), mannose 6-phosphate receptor (M6PR), caveolin-1 and -2, transferrin and its receptor, clathrin, as well as proteins comprising a FYVE domain such as early endosome autoantigen 1 (EEA1), Rabenosyn-

5, Smad anchor for receptor activation (SARA), Vps27p and Endofin. Some markers are more specific to early endosomes (*e.g.*, RAB4, Transferrin and its receptor, and proteins comprising a FYVE domain), others are more specific to late endosomes (*e.g.*, RAB7, RAB9, and M6PR) and others are more specific to recycling endosomes (*e.g.*, RAB11, RAB4). Thus, these proteins or suitable fragments thereof may be fused to the bioluminescent donor molecule (*e.g.*, *Renilla* Luc) or fluorescent acceptor molecule (*e.g.*, *Renilla* GFP) to link/target them to an endosomal localization.

In an embodiment, the endosomal targeting moiety comprises a FYVE domain. The FYVE domain is defined by the three conserved elements: the N-terminal WxxD, the central RR/KHHCR, and the C-terminal RVC motifs. Examples of human proteins containing a FYVE domain include ANKFY1, EEA1 FGD1, FGD2, FGD3, FGD4, FGD5, FGD6, FYCO1, HGS MTMR3, MTMR4, PIKFYVE, PLEKHF1, PLEKHF2, RUFY1, RUFY2, WDF3, WDFY1, WDFY2, WDFY3, ZFYVE1, ZFYVE16, ZFYVE19, ZFYVE20, ZFYVE21, ZFYVE26, ZFYVE27, ZFYVE28 and ZFYVE9 (EMBL-EBI, family FYVE (PF01363)). In an embodiment, the endosomal targeting moiety comprises the FYVE domain of human ZFYVE16/Endofin (UniProtKB - Q7Z3T8, SEQ ID NO: 39), for example about residues 747 to 805 or about residues 739 to 806 human Endofin.

In an embodiment, the second component comprises a Golgi targeting moiety. The term "Golgi targeting moiety" as used herein refers to any moiety capable of recruiting or sequestering the bioluminescent donor or fluorescent acceptor molecule to the Golgi apparatus. Several Golgi targeting moieties/markers are known in the art and include eNOS (*e.g.*, the N-terminal portion thereof, J. Liu *et al.*, Biochemistry, 35 (1996), pp. 13277-13281), GM130, Golgin-97, the 58K protein, Trans-Golgi network membrane protein 2 (TGOLN2), TGN46, TGN38, Mannosidase 2, Syntaxin 6, GM130 (GOLGA2), Golgin-160, Membrin (GS27), GS28, Coatamer proteins, Rbet1 and RCAS1. Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to a Golgi apparatus localization. In an embodiment, the Golgi targeting moiety the N-terminal portion of a human eNOS protein (SEQ ID NO:46), for example residues 1 to 73 of human eNOS1.

In an embodiment, there is no direct protein-protein interaction between (i) the PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety and (ii) the GASIP.

The bioluminescent donor or fluorescent acceptor molecule may be fused N-terminal, within or C-terminal relative to the targeting moiety. In an embodiment, the PM targeting moiety is fused to the C-terminal end of said bioluminescent donor or fluorescent acceptor molecule. In an embodiment, the PM targeting moiety is fused to the fluorescent acceptor molecule, preferably to the C-terminal end of the fluorescent acceptor molecule. In an embodiment, the endosomal targeting moiety is fused to the C-terminal end of said bioluminescent donor or fluorescent acceptor molecule, in a further embodiment to the C-terminal end of said fluorescent acceptor molecule.

The bioluminescent donor or fluorescent acceptor molecule may be fused N-terminal, within, or C-terminal relative to the GASIP. In an embodiment, the bioluminescent donor or fluorescent acceptor molecule is fused to the N-terminal end of the GASIP. In another embodiment, the bioluminescent donor or fluorescent acceptor molecule is fused to the C-terminal end of the GASIP. In a further embodiment, the bioluminescent donor molecule is fused to the GASIP, preferably to the C-terminal end of the GASIP.

The term "G protein-binding domain of Rap1GAP" refers to a polypeptide comprising the domain of the Rap1 GTPase-activating protein 1 (Rap1GAP) protein (UniProt accession No. P47736, SEQ ID NO:47), or a variant thereof, that has the ability to bind to G α subunit protein(s) of the Gi family. The G protein-binding domain of Rap1GAP is located in the N-terminal portion of Rap1GAP, and for example comprises at least 50, 100, 150, 200, 250, 300, 350 or 400 residues from native Rap1GAP. The G protein-binding domain of Rap1GAP may comprise one or more mutations that do not abrogate the binding to the G α subunit protein. In an embodiment, the G protein-binding domain of Rap1GAP comprises residues 1 to 420 or 1 to 436 of native Rap1GAP, or a variant thereof that retains the ability to bind to G α subunit protein(s) of the Gi family. In another embodiment, the G protein-binding domain of Rap1GAP comprises residues 1 to 442, or a variant thereof that retains the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of Rap1GAP is fused to the N-terminal end of the bioluminescent donor molecule.

In another embodiment, the G protein-binding domain of Rap1GAP comprises a mutation that reduces its sensitivity to downstream signalling events such as kinase activation (*e.g.*, protein kinase A). Such reduction in sensitivity to downstream signalling events such as kinase activation may be achieved, for example, by introduction of mutation(s) (*e.g.*, deletion, substitution) of one or more of the putative phosphorylation sites, for example the serine residues at position 431, 437, 439 and/or 441 of native Rap1GAP. In a further embodiment, one or more of the serine residues at positions 437, 439 and/or 441 are mutated in the G protein-binding domain of Rap1GAP, preferably at least two of the serine residues at positions 437, 439 and/or 441 are mutated in the G protein-binding domain of Rap1GAP, and more preferably all three serine residues at positions 437, 439 and 441 are mutated. In a further embodiment, the mutation is a substitution, for example by an amino acid that cannot be phosphorylated, for example an alanine residue. In an embodiment, all three serine residues at positions 437, 439 and 441 are substituted by an alanine in the G protein-binding domain of Rap1GAP. In an embodiment, the G protein-binding domain of Rap1GAP comprises one of the sequences depicted in **FIG. 9A** and **FIG. 9B**.

The term "G protein-binding domain of a Regulator of G-protein signaling (RGS) protein" as used herein refers to a polypeptide comprising the domain of an RGS protein, or a variant thereof, that has the ability to bind to G α subunit protein(s) of the Gi family. RGS proteins are a family of 22 proteins (RGS1-RGS22) comprising an RGS-box or RGS domain (PROSITE entry

PS50132). The G protein-binding domain of the RGS protein may comprise at least 50, 100, 150, 200, 250, 300, 350 or 400 residues from an RGS protein, or a variant thereof retaining the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the GASIP comprises the G protein-binding domain of an RGS protein of the RZ/A subfamily, preferably RGS17 (RGSZ2, UniProt accession No. Q9UGC6, SEQ ID NO:17), RGS19 (GAIP, UniProt accession No. P49795, SEQ ID NO:18) or RGS20 (RGSZ1, UniProt accession No. O76081, SEQ ID NO:19), or a variant thereof that has the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of RGS17 comprises residues 84-200 of native RGS17 or a variant thereof that has the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of RGS17 comprises residues 64-210 of native RGS17 or a variant thereof that has the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of RGS19 comprises residues 90-206 of native RGS19 or a variant thereof that has the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of RGS19 comprises residues 70-217 of native RGS19 or a variant thereof that has the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of RGS20 comprises residues 262-378 of native RGS20 or a variant thereof that has the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of RGS20 comprises residues 242-388 of native RGS20 or a variant thereof that has the ability to bind to G α subunit protein(s) of the Gi family. In an embodiment, the G protein-binding domain of a RGS protein is fused to the N-terminal end of the bioluminescent donor molecule

Another protein comprising a domain that binds G α subunit protein(s) of the Gi family that may be used in the systems/methods described herein is tumor necrosis factor- α (TNF α)-induced protein 8 (TNFAIP8, UniProtKB accession No. O95379, SEQ ID NO: 48).

The term "G protein-binding domain of P63RhoGEF" refers to a polypeptide comprising the domain of the P63RhoGEF (Rho guanine nucleotide exchange factor 25) protein (UniProt accession No. Q86VW2, SEQ ID NO:25), or a variant thereof, that has the ability to bind to G α subunit protein(s) of the Gq family. The G protein-binding domain of P63RhoGEF is located in the C-terminal portion of P63RhoGEF, and for example comprises at least 50, 100, 150, 200, 250, 300, 350 or 400 residues from native P63RhoGEF, or a variant thereof that retains the ability to bind to G α subunit protein(s) of the Gq family. The G protein-binding domain of P63RhoGEF may comprise one or more mutations that do not abrogate the binding to the G α subunit protein. In an embodiment, the G protein-binding domain of P63RhoGEF comprises residues 348 to 466 of native P63RhoGEF, or a variant thereof that retains the ability to bind to G α subunit protein(s) of the Gq family. In another embodiment, the G protein-binding domain of P63RhoGEF comprises residues 295 to 502 of native P63RhoGEF, or a variant thereof that retains the ability to bind to

Gα subunit protein(s) of the Gq family. In an embodiment, the G protein-binding domain of P63RhoGEF is fused to the N-terminal end of the bioluminescent donor molecule.

The term "G protein-binding domain of GRK2" refers to a polypeptide comprising the domain of the GRK2 (Beta-adrenergic receptor kinase 1) protein (UniProt accession No. P25098, SEQ ID NO: 27), or a variant thereof that has the ability to bind to Gα subunit protein(s) of the Gq family. The G protein-binding domain of GRK2 is located in the N-terminal portion of GRK2, and for example comprises at least 50, 100, 150, 200, 250, 300, 350 or 400 residues from native GRK2, or a variant thereof that retains the ability to bind to Gα subunit protein(s) of the Gq family. The G protein-binding domain of GRK2 may comprise one or more mutations that do not abrogate the binding to the Gα subunit protein. In an embodiment, the G protein-binding domain of GRK2 comprises residues 54 to 175 of native GRK2, or a variant thereof that retains the ability to bind to Gα subunit protein(s) of the Gq family. In another embodiment, the G protein-binding domain of GRK2 comprises residues 30 to 203 of native GRK2, or a variant thereof that retains the ability to bind to Gα subunit protein(s) of the Gq family. In an embodiment, the G protein-binding domain of GRK2 is fused to the C-terminal end of the bioluminescent donor molecule.

Other proteins comprising a domain that binds Gα subunit protein(s) of the Gq family that may be used in the systems/methods described herein include GRK3 (*e.g.*, residues ~54-175), PLCβ proteins, RGS proteins (*e.g.*, RGS2, residues ~83-199), TRP1 and other RhoGEF proteins (*e.g.*, Triple functional domain protein, TRIO). Polypeptides comprising these domains or variants thereof that retains the ability to bind to Gα subunit protein(s) of the Gq family as defined above may be used in the systems/methods described herein.

The term "G protein-binding domain of PDZRhoGEF" refers to a polypeptide comprising the domain of the PDZRhoGEF (Rho guanine nucleotide exchange factor 11) protein (UniProt accession No. O15085, SEQ ID NO:21), or a variant thereof that has the ability to bind to Gα subunit protein(s) of the G12/13 family. The G protein-binding domain of PDZRhoGEF is located in the central portion of PDZRhoGEF, and for example comprises at least 50, 100, 150, 200, 250, 300, 350 or 400 residues from native PDZRhoGEF, or a variant thereof that retains the ability to bind to Gα subunit protein(s) of the G12/13 family. The G protein-binding domain of PDZRhoGEF may comprise one or more mutations that do not abrogate the binding to the Gα subunit protein. In an embodiment, the G protein-binding domain of PDZRhoGEF comprises residues 306 to 486 of native PDZRhoGEF, or a variant thereof that retains the ability to bind to Gα subunit protein(s) of the G12/13 family. In another embodiment, the G protein-binding domain of PDZRhoGEF comprises residues 281 to 483 of native PDZRhoGEF, or a variant thereof that retains the ability to bind to Gα subunit protein(s) of the G12/13 family. In an embodiment, the G protein-binding domain of PDZRhoGEF is fused to the N-terminal end of the bioluminescent donor molecule.

The term "G protein-binding domain of P115RhoGEF" refers to a polypeptide comprising the domain of the P115RhoGEF (Rho guanine nucleotide exchange factor 1) protein (UniProt

accession No. Q92888, SEQ ID NO:23), or a variant thereof that has the ability to bind to G α subunit protein(s) of the G12/13 family. The G protein-binding domain of P115RhoGEF is located in the N-terminal portion of P115RhoGEF, and for example comprises at least 50, 100, 150, 200, 250, 300, 350 or 400 residues from native P115RhoGEF, or a variant thereof that retains the ability to bind to G α subunit protein(s) of the G12/13 family. The G protein-binding domain of P115RhoGEF may comprise one or more mutations that do not abrogate the binding to the G α subunit protein. In an embodiment, the G protein-binding domain of P115RhoGEF comprises residues 41 to 232 of native P115RhoGEF, or a variant thereof that retains the ability to bind to G α subunit protein(s) of the G12/13 family. In another embodiment, the G protein-binding domain of P115RhoGEF comprises residues 1 to 244 of native P115RhoGEF, or a variant thereof that retains the ability to bind to G α subunit protein(s) of the G12/13 family. In an embodiment, the G protein-binding domain of P115RhoGEF is fused to the N-terminal end of the bioluminescent donor molecule.

Other proteins comprising a domain that binds G α subunit protein(s) of the G12/13 family that may be used in the systems/methods described herein include RhoGEFs such as Rho guanine nucleotide exchange factor 12 (LARG, UniProtKB accession No. Q9NZN5, SEQ ID NO:49) (*e.g.*, residues ~367-558), JNK-Interacting Leucine Zipper Protein (JLP, the C-terminal portion) cadherin proteins, AXIN1 (UniProtKB accession No. O15169, SEQ ID NO: 50, residues ~88-212, more selective for G12), PP2A (more selective for G12), Alpha-soluble NSF attachment protein (alphaSNAP, UniProtKB accession No. P54920, SEQ ID NO: 51, N-terminal portion, more selective for G12), polycystin-1 (UniProtKB accession No. P98161, SEQ ID NO: 52, more selective for G12), RGS16 (UniProtKB accession No. O15492, SEQ ID NO: 53, N-terminal portion, more selective for G13), AKAP110 (UniProtKB accession No. O75969, SEQ ID NO: 54, C-terminal portion, more selective for G13), HS1-associated protein X1 (HAX1, UniProtKB accession No. O00165, SEQ ID NO: 55, residues ~176-247, more selective for G13). Polypeptides comprising these domains or variants thereof that retains the ability to bind to G α subunit protein(s) of the G12/13 family as defined above may be used in the systems/methods described herein. Using domains more selective for one of the subunits (G12 or G13) may be useful for assessing the selective activation of the endogenous G12 or G13 proteins.

Domain that binds G α subunit protein(s) of the Gs family includes SNX13 (RGS-PX1, UniProtKB accession No. Q9Y5W8, SEQ ID NO: 56, residues ~373-496), AXIN1 (UniProtKB accession No. O15169, SEQ ID NO: 50, residues ~88-211) or TPR1 (tetratricopeptide repeat domain 1, UniProtKB accession No. Q99614, SEQ ID NO: 57, C-terminal portion). In an embodiment, the G α subunit protein of the Gs family is the membrane-anchored XL(alpha)s subunit.

In an embodiment, the length of the GASIP is about 50, 75 or 100 amino acids to about 500, 600, 700 or 800 amino acids, for example about 100 or 150 amino acids to about 200, 250, 300, 350, 400 or 500 amino acids.

In an embodiment, the GASIP does not comprise the full-length sequences of Rap1GAP, the RGS protein (*e.g.*, RGS17, 19 or 20), P63RhoGEF, GRK2, PDZRhoGEF or P115RhoGEF, *i.e.*, it comprises one or more mutations (substitutions, deletions, etc.) relative to the full-length proteins. In an embodiment, the GASIP lacks the residues or domain involved in the recruitment or anchoring to the PM, *e.g.*, myristoylation, palmitoylation and/or prenylation signal sequences. Using truncated and/or mutated version of these proteins advantageously permits to minimize the effects of downstream signalling events (*e.g.*, kinase activation) and/or to ensure proper localization (cytosolic) and translocation of the GASIP to different compartments upon G protein activation, which improves the reliability and/or sensitivity of the assay.

In an embodiment, the system described herein further comprises a recombinant G α protein subunit. G α protein subunit as defined herein includes, but is not limited to, the 17 different known isoforms, their splice variants, and any mutated G α proteins, for example those leading to non-selective/promiscuous G α . In one non-limiting embodiment, the herein described G α protein is selected amongst any of the natural mammalian G α proteins, which includes Gq, Gs, Gi1, Gi2, Gi3, Gt-cone, Gt-rod, Gt-gust, Gz, GoA, GoB, Golf, G11, G12, G13, G14, and G15/G16 (also designated GNA15), the splice variants of these isoforms, as well as functional variants thereof. In an embodiment, the recombinant G α protein subunit is of the Gi family, *e.g.*, Gi1, Gi2, Gi3, GoA, GoB, Gt-cone, Gt-rod, Ggus, and/or Gz. In an embodiment, the G α protein subunit is of the G $_{\alpha}$ family, *e.g.*, Gq, G11, G14 and/or G15/16). In an embodiment, the G α protein subunit is of the G12/13 family.

In an embodiment, the recombinant G α subunit polypeptide comprises at least one mutation that decreases or abrogates the activation by GPCRs. Such mutated G α subunit polypeptide may be useful for assessing non-receptor guanine nucleotide exchange factor (GEF)-mediated G protein activation, *i.e.* G protein activation not induced through GPCR engagement. In an embodiment, the mutation is in the carboxy (C)-terminal domain, and preferably a mutation in one or more of the last seven residues at the C-terminal of said G α subunit polypeptide. In an embodiment, the mutation is a truncation of the last one, 2, 3, 4, 5, 6 or 7 residues at the C-terminal. In another embodiment, the mutation is a substitution of at least one, 2, 3, 4, 5, 6 or all residues at the C-terminal. In an embodiment, the mutation is a deletion or substitution of at least one of the conserved leucine residues in said C-terminal domain, preferably a deletion or substitution of the last conserved leucine residue (penultimate residue of the native protein) in said C-terminal domain. In a further embodiment, the mutation is a substitution of the last conserved leucine residue, preferably a substitution for an aspartic acid (D), a proline (P) or an arginine (R) residue. In an embodiment, the mutated recombinant G α subunit polypeptide is of

the Gi family, for example Gi2 or GoB. In a further embodiment, the mutated recombinant Ga subunit polypeptide comprises one of the sequences depicted in **FIGs. 9B** and **9C**, *e.g.*, Gai2 Δ 5 (SEQ ID NO: 28); Gai2 Δ 2 (SEQ ID NO: 29); Gai2 L-2G (SEQ ID NO: 31); Gai2 L-2P (SEQ ID NO: 33); Gai2 L-2R (SEQ ID NO: 34); Gai2 L-2D (SEQ ID NO: 32); Gai2 L-7G (SEQ ID NO: 30);

5 GaoB Δ 5 (SEQ ID NO: 36); or GaoB L-2G (SEQ ID NO: 35).

The term "recombinant" as used herein refers to a protein molecule which is expressed from a recombinant nucleic acid molecule, i.e. a nucleic acid prepared by means of molecular biology/genetic engineering techniques, for example a protein that is expressed following transfection/transduction of a cell (or its progeny) with a nucleic acid (*e.g.*, present in a vector)

10 encoding the protein (as opposed to a protein that is naturally expressed by a cell).

In an embodiment, the system described herein further comprises a cell surface receptor. The cell of the biosensor may naturally express the cell surface receptor, or the cell surface receptor may be a recombinant cell surface receptor (*e.g.*, the cell has been transfected or transformed with a nucleic acid encoding the cell surface receptor). The term "cell surface

15 receptor" as used herein refers to a protein attached to or embedded with the plasma membrane and that induces G protein activation upon binding of a ligand. Examples of cell surface receptors that induces G protein activation include G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), integrins. Whereas G protein activation typically occurs through GPCRs engagement by a ligand, G protein activation may also be achieved via non-receptor guanine

20 nucleotide exchange factors (GEF) such as GIV (G α -interacting vesicle-associated protein, also known as Girdin), NUCB1 (nucleobindin1, also known as calnuc), NUCB2 and DAPLE (Dishevelled-associating protein). For example, GIV activity is associated with RTKs (*e.g.*, EGFR) and integrin α - β complex modulation of Gi activity. In an embodiment, the GASIP comprises the G protein-binding domain of Rap1GAP, and the cell surface receptor is a GPCR or a RTK. In

25 another embodiment, the GASIP comprises the G protein-binding domain of a RGS protein, preferably RGS17, and the cell surface receptor is a GPCR.

"GPCR" refers to full-length native GPCR molecules as well as mutant GPCR molecules. A list of GPCRs is given in Foord *et al.* (2005) Pharmacol Rev. 57, 279-288, which is incorporated herein by reference, and an updated list of GPCRs is available in the IUPHAR-DB database

30 (Harmar AJ, et al. (2009) IUPHAR-DB: the IUPHAR database of G protein-coupled receptors and ion channels. *Nucl. Acids Res.* **37** (Database issue): D680-D685; Sharman JL, *et al.*, (2013) IUPHAR-DB: updated database content and new features. *Nucl. Acids Res.* **41** (Database Issue): D1083-8).

"RTK" refers to full-length native RTK proteins as well as mutant RTK proteins. RTKs

35 (EC 2.7.10.1 according to the IUBMB Enzyme Nomenclature) are cell surface receptors for many polypeptide growth factors, cytokines, and hormones, characterized by an intracellular region comprising catalytic domains responsible for the kinase activity of these receptors, which

catalyses receptor autophosphorylation and tyrosine phosphorylation of RTK substrates. There are 58 known RTK in humans, distributed into 20 subfamilies (Robinson et al., *Oncogene* 2000, 19(49):5548-57).

"Integrin" refers to full-length native integrin proteins as well as mutant integrin proteins.

- 5 Integrins are composed of two noncovalently associated transmembrane glycoprotein subunits called α and β . A variety of human integrin heterodimers are formed from 9 types of β subunits and 24 types of α subunits. Representative integrins found in vertebrates include $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_{IIb}\beta_3$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, and $\alpha_6\beta_4$.

- The term "variant" (or "mutant") as used herein refers to a protein/polypeptide having has
 10 a sequence identity of at least 60% with a reference (*e.g.*, native) sequence and retains a desired activity thereof, for example the capacity to bind to G α subunit or to act as a BRET donor or acceptor. In further embodiments, the variant has a similarity or identity of at least 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% with a reference (*e.g.*, native) sequence and retains a desired activity thereof. "Similarity" and "identity" refers to sequence similarity/identity between
 15 two polypeptide molecules. The similarity or identity can be determined by comparing each position in the aligned sequences. A degree of similarity or identity between amino acid sequences is a function of the number of matching or identical amino acids at positions shared by the sequences. Optimal alignment of sequences for comparisons of similarity or identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and
 20 Waterman, 1981, *Adv. Appl. Math* 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443, the search for similarity method of Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85: 2444, and the computerized implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, Wis., U.S.A.). Sequence similarity or identity may
 25 also be determined using the BLAST algorithm, described in Altschul *et al.*, 1990, *J. Mol. Biol.* 215: 403-10 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information web site.

- In embodiments, the domains of the components (fusion molecules) described herein may be covalently linked either directly (*e.g.*, through a peptide bond) or "indirectly" via a suitable
 30 linker moiety, *e.g.*, a linker of one or more amino acids or another type of chemical linker (*e.g.*, a carbohydrate linker, a lipid linker, a fatty acid linker, a polyether linker, PEG, etc. In an embodiment, one or more additional domain(s) may be inserted before (N-terminal), between or after (C-terminal) the domains defined above. In an embodiment, the domains of the fusion molecules are covalently linked through a peptide bond. In another embodiment, one or more of
 35 the components of the fusion molecules are linked through a peptide linker. In one embodiment, the linkers are peptide linkers, typically ranging from 2 to 30 amino acids in length, for example about 5 to about 20-25 amino acids or about 10 to about 15-20 amino acids. The composition and

length of each of the linkers may be chosen depending on various properties desired such as flexibility and aqueous solubility. For instance, the peptide linker may comprise relatively small amino acid residues, including, but not limited to, glycine; small amino acid residues may reduce the steric bulk and increase the flexibility of the peptide linker. The peptide linker may also
5 comprise polar amino acids, including, but not limited to, serine. Polar amino acid residues may increase the aqueous solubility of the peptide linker. Furthermore, programs such as Globplot 2.3 (Linding *et al.*, GlobPlot: exploring protein sequences for globularity and disorder, *Nucleic Acid Res* 2003 - Vol. 31, No.13, 3701-8), may be used to help determine the degree of disorder and globularity, thus also their degree of flexibility. In an embodiment, the peptide linker comprises
10 one or more of the amino acid sequences disclosed in the Examples below and/or the figures (SEQ ID NOs:6, 7, 20, 22, 24 and 26).

In an embodiment, the system further comprises a cell expressing the various components defined herein, i.e. the first and second components, and optionally the recombinant Ga protein subunit and/or cell surface receptor.

15 In another aspect, the present disclosure provides a nucleic acid or a plurality of nucleic acids encoding the above-defined first and/or second component(s) defined herein. In an embodiment, the nucleic acid(s) is/are present in a vector/plasmid (or a plurality of vectors/plasmids), in a further embodiment expression vector(s)/plasmid(s). Such vectors comprise nucleic acid(s) encoding the above-defined first and/or second component(s) operably
20 linked to one or more transcriptional regulatory sequence(s), such as promoters, enhancers and/or other regulatory sequences. In an embodiment, the nucleic acid encodes the first and second components (polycistronic construct).

The term "vector" refers to a nucleic acid molecule, which is capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e.,
25 a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". A recombinant expression vector of the present invention can be constructed by standard techniques known to one of ordinary skill in the art and found, for
30 example, in Sambrook *et al.*, *supra*. A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and can be readily determined by persons skilled in the art. The vectors of the present invention may also contain other sequence elements to facilitate vector propagation and selection in bacteria and host cells. In addition, the vectors of the present invention may comprise a sequence of
35 nucleotides for one or more restriction endonuclease sites. Coding sequences such as for selectable markers and reporter genes are well known to persons skilled in the art.

A recombinant expression vector comprising one or more of the nucleic acids defined herein may be introduced into a cell (a host cell), which may include a living cell capable of expressing the protein coding region from the defined recombinant expression vector. The living cell may include both a cultured cell and a cell within a living organism. Accordingly, the invention
5 also provides host cells containing the recombinant expression vectors of the invention. The terms "cell", "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are
10 still included within the scope of the term as used herein.

Vector DNA can be introduced into cells via conventional transformation or transfection techniques. The terms "transformation" and "transfection" refer to techniques for introducing foreign nucleic acid into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection
15 and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can for example be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals. "Transcriptional regulatory sequence/element" is a generic term that refers to DNA sequences, such as initiation and termination signals, enhancers, and promoters, splicing signals, polyadenylation signals
20 which induce or control transcription of protein coding sequences with which they are operably linked. A first nucleic acid sequence is "operably-linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably-linked to a coding sequence if the promoter affects the transcription or expression of the coding sequences. Generally, operably-linked DNA
25 sequences are contiguous and, where necessary to join two protein coding regions, in reading frame. However, since for example, enhancers generally function when separated from the promoters by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably-linked but not contiguous.

In another aspect, the present disclosure provides a kit comprising a first nucleic acid
30 encoding the first component and a second nucleic acid encoding the second component, or a nucleic acid encoding the first and second components. In an embodiment, the kit further comprises a nucleic acid encoding a Gα protein subunit. In another embodiment, the kit further comprises a nucleic acid encoding a cell surface receptor.

In another aspect, the present disclosure provides a cell comprising or expressing the
35 above-defined first and/or second component(s). In an embodiment, the cell has been transfected or transformed with a nucleic acid encoding the above-defined first and/or second component(s). In an embodiment, the cell further comprises or expresses a recombinant Gα protein subunit. In

an embodiment, the cell further comprises or expresses a recombinant cell surface receptor, *e.g.*, a GPCR, an RTK or an integrin.

The present disclosure further provides a recombinant expression system, vectors and cells, such as those described above, for the expression of the first and/or second component(s) of the invention, using for example culture media and reagents well known in the art. The cell may be any cell capable of expressing the first and second component(s) defined above. Suitable host cells and methods for expression of proteins are well known in the art. Any cell capable of expressing the component(s) defined above may be used. For example, eukaryotic host cells such as mammalian cells may be used (*e.g.*, rodent cells such as mouse, rat and hamster cell lines, human cells/cell lines). In another embodiment, the above-mentioned cell is a human cell line, for example an embryonic kidney cell line (*e.g.*, HEK293 or HEK293T cells).

In another aspect, the present disclosure provides a method for determining whether an agent modulates the activation of a G protein of interest, said method comprising:

contacting the system defined herein with a substrate for the bioluminescent donor molecule;

measuring the BRET signal in the system in the presence and absence of said agent;

wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates the activation of said G protein of interest.

In an embodiment, the agent is an agonist and the difference in said BRET signal is an increase.

In an embodiment, the G protein of interest is of the Gi protein subunit family, and wherein the GASIP comprises the G protein-binding domain of Rap1GAP or of an RGS protein as defined herein. In an embodiment, the system used in the above-mentioned method comprises a recombinant G α subunit polypeptide of the Gi protein subunit family, i.e. a recombinant Gi1, Gi2, Gi3, GoA, GoB, or Gz subunit polypeptide. To identify the profile or signature of a given test agent (i.e. to identify the specific Gi protein subunit(s) activated by the test agent), the above-mentioned method may be performed using a plurality (i.e. two or more) of systems, each system comprising a different Gi protein subunit, *e.g.*, a first system comprising a recombinant Gi1, a second system comprising a recombinant Gi2, a third system comprising a recombinant Gi3, etc.

In another embodiment, the G protein of interest is of the Gq protein subunit family, and wherein the GASIP comprises the G protein-binding domain of P63RhoGEF or GRK2 as defined herein. In an embodiment, the system used in the above-mentioned method comprises a recombinant G α subunit polypeptide of the Gq protein subunit family, i.e. a recombinant Gq, G11, G14 or G15 subunit polypeptide. To identify the profile or signature of a given test agent (i.e. to identify the specific Gq protein subunit(s) activated by the test agent), the above-mentioned method may be performed using a plurality (i.e. two or more) of systems, each system comprising

a different Gq protein subunit, *e.g.*, a first system comprising a recombinant Gq, a second system comprising a recombinant G11, a third system comprising a recombinant G14, etc.

In another embodiment, the G protein of interest is of the G12/13 protein subunit family, and wherein the GASIP comprises the G protein-binding domain of PDZRhoGEF or P115RhoGEF as defined herein. In an embodiment, the system used in the above-mentioned method comprises a recombinant G α subunit polypeptide of the G12/13 protein subunit family, i.e. a recombinant Gq, G12 or G13 subunit polypeptide. To identify the profile or signature of a given test agent (i.e. to identify the specific G12/13 protein subunit(s) activated by the test agent), the above-mentioned method may be performed using a plurality (i.e. two or more) of systems, each system comprising a different G12/13 protein subunit, *e.g.*, a first system comprising a recombinant G12 and a second system comprising a recombinant G13.

In another aspect, the present disclosure provides a method for determining whether an agent modulates non-receptor guanine nucleotide exchange factor (GEF)-mediated G protein activation, said method comprising:

contacting the system defined herein with a substrate for the bioluminescent donor molecule;

measuring the BRET signal in the system in the presence and absence of said agent, wherein said system comprises a recombinant G α subunit polypeptide comprising at least one mutation that decreases or abrogates the activation by GPCRs;

wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates non-receptor GEF-mediated G protein activation.

In an embodiment, the agent is an agonist and the difference in said BRET signal is an increase (or higher BRET signal).

In another embodiment, the agent is an antagonist and the difference in said BRET signal is a decrease (or lower BRET signal). In an embodiment, to identify whether an agent is an antagonist, the system is contacted with a known agonist, and a decrease in the BRET signal induced by the known agonist in the presence of the agent is indicative that the agent is an antagonist.

In another aspect, the present disclosure provides a method for determining whether an agent induces GPCR-mediated Gprotein activation or RTK/GEF-mediated Gprotein activation, the method comprising:

measuring the BRET signal in the presence of the agent using a first biosensor in which the GASIP comprises the G protein-binding domain of Rap1GAP, preferably a G protein-binding domain of Rap1GAP that comprises a mutation that reduces its sensitivity to downstream signalling events (*e.g.*, kinase activation), more preferably comprising a mutation at one or more

of the serine residues at positions 437, 439 and/or 441 (*e.g.*, Rap1GAP(SSS-AAA)), as defined above;

measuring the BRET signal in the presence of the agent using a second biosensor in which the GASIP comprises the G protein-binding domain of an RGS protein, preferably RGS17,
5 as defined above;

wherein an increase (*e.g.*, dose-dependent increase) in the BRET signal in the presence of the agent in the first biosensor without an increase in the BRET signal in the presence of the agent in the second biosensor is indicative that the agent induces RTK/GEF-mediated Gprotein activation; and wherein an increase (*e.g.*, dose-dependent increase) in the BRET signal in the
10 presence of the agent in the first and second biosensors is indicative that the agent induces GPCR-mediated Gprotein activation.

The term "compound", "agent", "test compound" or "test agent" refers to any molecule (*e.g.*, drug candidates) that may be screened by the system/assay described herein may be obtained from any number of sources including libraries of synthetic or natural compounds. For
15 example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical
20 means.

In an embodiment, the "increased signal" or "higher signal" as used herein refers to signal that is at least 10, 20, 30, 40, 45 or 50% higher relative to the reference signal measured in the absence of the test agent. In another embodiment, the "higher signal" is determined by showing a statistically significant difference (determined using a suitable statistical analysis) in the signal
25 measured in the presence relative to the absence of the test agent, for example by combining the results obtained in a plurality of samples. Statistical analysis (ANOVA, Student *t*-test, Chi square, etc.) to determine significant differences between different sets of data are known in the art, and such analysis may be performed using suitable computer programs.

Positive controls and negative controls may be used in the methods/assays described
30 herein. Control and test samples may be performed multiple times to obtain statistically significant results.

In an embodiment, the above-mentioned methods are high-throughput methods (high-throughput screening, HTS). The term "high-throughput screening" (HTS) as used herein refers to a method that allow screening rapidly and in parallel large numbers of compounds (hundreds,
35 thousands) for binding activity or biological activity against target molecules. Such HTS methods are typically performed in microtiter plates having several wells, for example 384, 1536, or 3456 wells. For HTS, it is important that the readout signal be detected with high sensitivity, accuracy

and reproducibility. One way to determine whether a method is suitable or compatible with HTS is by measurement of the Z-factor, as described in the examples below. Ideally, the Z-factor should be at least 0.5 (i.e. between 0.5 and 1), and preferably at least 0.55, 0.6, 0.65, 0.7, 0.75 or 0.8.

5 Methods and devices to measure the BRET signal are well known in the art. The BRET signal may be measured, for example, by determining the intensity of the BRET acceptor signal (light intensity), and/or by calculating the ratio of the signal or light intensity emitted by the BRET acceptor over the signal or light intensity emitted by the BRET donor (BRET ratio). The BRET signal may be measured using a microplate reader or microscope with a suitable filter set for
10 detecting the BRET donor and/or BRET acceptor light emissions.

 The systems and methods described herein may be used in HTS to identify, for example, compounds that are active in modulating G protein activation (through GPCRs or other receptors signaling through G proteins) and are thus potential drug candidates or for use in treating disorders in which G protein signaling is involved, *e.g.*, for which G protein inhibition or stimulation
15 would be beneficial. The systems and methods described herein may also be used to perform ligand profiling, *e.g.*, to determine the pathways modulated by ligands of GPCRs or other receptors signaling through G proteins.

 The systems and methods described herein may be used for the screening of compound libraries to identify potential candidates for drug development. The change in BRET
20 signal ratio when the system described herein is contacted with a compound may identify drugs or pharmaceutically active compounds and may identify their effect on a cellular pathway. Also, the systems and methods described herein may also be used for the determination of the dosage dependence of drug candidates.

 Electromechanical plate readers can be used to detect signal ratio changes. Such plate
25 readers can be employed for HTS, drug candidate screening, and drug dosage dependence studies using the system of the present invention. Examples of plate readers that can be used in practicing the present invention include the Fusion™ family of plate readers offered by PerkinElmer (Boston, MA), including the PerkinElmer Fusion™ Universal Microplate Analyzer devices. The PerkinElmer EnVision™ HTS model can also be employed in practicing the methods
30 described herein.

MODE(S) FOR CARRYING OUT THE INVENTION

 The present invention is illustrated in further details by the following non-limiting examples.

35

Example 1: Materials and Methods

Materials. Angiotensin II (AngII; [Asp-Arg-Val-Tyr-Ile-His-Pro-Phe], SEQ ID NO: 58), Forkolin, Dopamine and poly-ornithine were from Sigma-Aldrich. u46619, I-BOP, CTA2, U51605, I-SAP, SQ 29558, were from Cayman Chemical® (Ann Arbor, MI). Human EGF was from Cedarlane laboratories. Kallidin ([Lys0]-Bradykinin) was from Eurogentec. WAY-100635, A 412997, L 741742, SNC-80 and AR-M100390 were from Tocris Bioscience (Bio-Techne). Platelet activating factor (PAF-C16) was from Enzo Life Sciences, Inc. Salmon sperm DNA, Dulbecco's modified Eagles medium (DMEM), fetal bovine serum, calf serum, OPTI-MEM®, and other cell culture reagents were purchased from Life technologies (Thermo Fisher Scientific) and from Wisent Inc. Polyethylenimine (PEI) 25 kDa linear was from Polysciences Inc. Prolume Purple I was purchased from Nanolight® Technology. Phusion DNA polymerase was from Thermo Fisher Scientific. Restriction enzymes, T4 DNA ligase and Gibson assembly mix were obtained from New England Biolabs Ltd. White 96-well Polystyrene Cell Culture Microplates, solid bottom (CellStar 655 083) were from Greiner.

Plasmids and constructions. The plasma-membrane marker rGFP-CAAX and the early endosome marker rGFP-FYVE constructs were already described (Namkung Y. *et al.* 2016; Nat Commun. 7:12178). Plasmids encoding TPα receptor construct, all Gα subunits, Gβ1 subunit and Gy1 subunits are from cdna.org. The coding sequence (cds) of the human receptors D4R, At1AR and Mu opiod receptor (hMOR1) with the signal peptide: MDSKGSSQKGSRLLLLLVSNLLLCQGVVS (SEQ ID NO: 59), was sequence-optimized, synthesized at GeneART (Thermo Fisher Scientific) and subcloned by Gibson assembly in pCDNA3.1 (+) from Invitrogen (Thermo Fisher Scientific); hMOR1 was subcloned in pLVX IRES puro (Clontech, CA). The construct encoding the human EGFR was provided by Dr Yosef Yarden (Tzahar E. *et al.* 1996; Molecular and Cellular Biology 16(10): 5276–5287). The Bradykinin B2 receptor (BKB2R) cds and RAPGAP(1-442)-Rluc8 construct (see **FIG. 9A** and **9B**) were sequence-optimized, synthesized and subcloned at Topgenetech in pCDNA3.1 (+); a peptidic linker GSGGGSGGGA (SEQ ID NO: 6) is present between the RapGAP(1-442) and Rluc8 (see **FIG. 2A**). The following construct encoding RlucII tagged version of RapGAP (1-442), Rap1GAP ΔCT(1-420), Rap1GAP ΔSSS(1-436), Rap1GAP SSS-AAA(1-442;S437A/S439A/S441A), Rap1GAP SSS-TTT(1-442;S437T/S439T/S441T), Rap1GAP SS-AA(1-442;S437A/S441A), Rap1GAP SS-DA(1-442;S437A/S441A), Rap1GAP SS-AD(1-442;S437A/S441D), Rap1GAP SS-DD(1-442;S437A/S441A) were done by PCR amplification from pCDNA3.1 (+) RapGAP(1-442)-Rluc8 and subcloned by Gibson assembly in pCDNA3.1 Hygro(+) GFP10-RlucII, replacing GFP10 with the coding sequences of the different variants of Rap1GAP (See **FIGs. 2A, 9A and 9B**). A peptidic linker: GSAGTGGRAIDIKLPAT (SEQ ID NO: 7) is present between RAPGAP and RlucII (See **FIG. 2A**). The construct encoding the RGS binding domain of the human RGS17 (residues: 64-210) tagged with RlucII was done by PCR amplification from pCDNA 3.1 HA-RGS17 (from cDNA.org) and subcloned by Gibson assembly

in pCDNA3.1 Hygro(+) GFP10-RlucII, replacing GFP10 with the cds of RGS(RGS17 64-210) (See **FIGs. 3A and 9D**). The construct encoding the RGS binding domain of the human RGS19 (residues: 70-217) tagged with RlucII was done by PCR amplification from pCDNA 3.1 HA-RGS19 (from cDNA.org) and subcloned by Gibson assembly in pCDNA3.1 Hygro(+) GFP10-RlucII, replacing GFP10 with the coding sequence of RGS19(70-217) (See **FIGs. 3A and 9D**). The construct encoding the RGS binding domain of the human RGS20 var1 (residues: 242-388) tagged with RlucII was done by PCR amplification from pCDNA 3.1 HA-RGS20 var1 (from cDNA.org) and subcloned by Gibson assembly in pCDNA3.1 Hygro(+) GFP10-RlucII, replacing GFP10 with the coding sequence of RGS(RGS20 242-388) (See **FIGs 3A and 9D**). A peptidic linker: GSAGTGGRAIDIKLASAT (SEQ ID NO: 20) is present between Rap1GAP and RlucII (See **FIG 3A**). The constructs encoding the G12/13 binding domain of the human PDZRhoGEF (residues: 281-483) and P115RhoGEF (residues: 1-244) tagged with RlucII were done by PCR amplification from IMAGE clones (OpenBiosystems) and subcloned by Gibson assembly in pCDNA3.1 Hygro(+) GFP10-RlucII, replacing GFP10 (See **FIGs. 4A, 5A and 9D**). Peptidic linkers: GILREALKLPAT (SEQ ID NO: 22) and RLKLPAT (SEQ ID NO: 24) are present between RlucII and the G12/13 binding domain of PDZRhoGEF and P115RhoGEF, respectively (See **FIGs. 4A and 5A**). The construct encoding the Gq binding domain of the human P63RhoGEF (residues: 295-502) tagged with RlucII was done from IMAGE clones (OpenBiosystems) and subcloned by Gibson assembly in pCDNA3.1 Hygro(+) GFP10-RlucII, replacing GFP10 (See **FIGs. 6A and 9B**). A peptidic linker: ASGSAGTGGRAIDIKLPAT (SEQ ID NO: 26) is present between the Gq binding domain and RlucII (See **FIG. 6A**). The constructs encoding the RGS domain of the human GRK2 (residues: 30-203) tagged with RlucII were done by PCR amplification from pCDNA3.1Z-GRK2-GFP10 and subcloned by Gibson assembly in pCDNA3.1 Hygro(+) GFP10-RlucII and pCDNA3.1 (+) RlucII-GFP10st2, replacing GFP10 and creating RGS(GRK2)-RlucII and RlucII-RGS(GRK2), respectively (See **FIGs. 7A and 9B**). A peptidic linker: GSAGTGGRAIDIKLASAT (SEQ ID NO: 20) is present between the RGS(GRK2) domain and RlucII, in both constructs (See **FIG. 7A**). Constructs encoding Gai2 and GaoB mutants: Gai2 $\Delta 5$ (=1-350), Gai2 $\Delta 2$ (=1-353), Gai2 L-7G(=L354G), Gai2 L-2G(=L354G), Gai2 L-2D(=L354D), Gai2 L-2P(=L354P), Gai2 L-2R(=L354R), GaoB L-2G(=L353G), GaoB $\Delta 5$ (=1-349), were created by PCR-amplification from pCDNA3.1 (+) GNAi2 and pCDNA3.1 (+) GNAoB (from cdna.org) and subcloning by Gibson assembly in pCDNA3.1 Zeo(+).

Cell culture and Transient Transfection. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂. HEK293SL cells were cultured in DMEM supplemented with 5% fetal bovine serum and 20 µg/ml gentamycin. Cells were grown at 37 °C in 5% CO₂ and 90% humidity.

Transfection using Poly(ethylenimine) (PEI): Two days before the experiments, HEK293 cells were washed with PBS containing no calcium or magnesium, detached and transfected with the indicated plasmids using PEI as a transfecting agent (at a ratio of 3 to 1, PEI/DNA). Cells were then directly seeded in 96-well plates pre-treated or not with poly-L-ornithine hydrobromide at a density of 35 000 cells per well. All experiments read in the Spark 10M reader were performed using non-treated plates.

BRET measurements. 48-hours post transfection, cells were washed twice with pre-warmed Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM D-glucose, 0.5 mM MgCl₂, 0.37 mM NaH₂PO₄, 25 mM HEPES, pH 7.4) before being stimulated with various concentrations of ligands at either room temperature (RT) or 37°C, as indicated. The cell-permeable substrate, coelenterazine Purple I was added at a final concentration of 1 µM in Tyrode's buffer for at least 6 min (at 37°C) to 10min (at RT) before BRET measurements. Measurements were either taken on a Spark 10M reader (Tecan Life Sciences; donor: 360nm/380nm & acceptor: 505nm/570nm), a LB 941 multimode plate reader (Berthold Technologies; donor: 400/470-nm and acceptor: 515/520-nm filters) or a Synergy Neo (Biotek; donor: 400/480 nm, acceptor: 515/530 nm). The BRET signal was determined by calculating the ratio of the light intensity emitted by the rGFP (acceptor) over the light intensity emitted by the RlucII (donor). All the BRET measurements were performed at 37 °C in the Tristar reader (**FIGs. 1-2M & FIGs. 2R-7F**), at RT in a Spark 10M reader (**FIGs. 2N-2Q & FIGs 8B-8S**) or at RT in a Synergy Neo (**FIGs. 8T-8X**).

Z'-factors determination. Z'-factor values were calculated as described by Zhang *et al.* (*J Biomol Screen.* 1999;4(2):67-73). A Z'-factor over 0.4 is considered a robust assay.

Data Analysis. Estimation of the EC₅₀ values were calculated using the GraphPad® Prism curve fitting program. The curves presented throughout this study, representing the best fits, and were also generated using this GraphPad® Prism program.

Example 2: Generation and specificity of systems and assays for monitoring G protein activation in a G protein family-selective manner

FIGs. 1A to 1E show the generation and specificity of systems and assays for monitoring G protein activation in a G protein family-selective manner, and at different cellular compartments, based on the translocation of Gα subunit interacting polypeptides (GASIP) from G protein effectors. **FIG. 1A** depicts the principle of an effector-based sensor to monitor, in **i**) GPCR-mediated direct Gprotein activation and, in **ii**) guanine-nucleotide exchange factor (GEF)-mediated Gprotein activation. Cells expressing a receptor, a subcellular localization domain (for example for the plasma-membrane (PM) or for early endosomes (EE)) tagged with rGFP, the Gα-interaction domain of a specific effector tagged with a BRET donor (*e.g.*, RlucII) are exposed to an agonist to activate the co-expressed G protein. In **i**), the agonist-induced GPCR stimulation

activates directly G proteins, which recruits a tagged-effector from the cytoplasm to the α GFP-labeled membrane. In ii), G protein activation is mediated from the recruitment of a GEF such as GIV/Girdin following the activation of an RTK (*e.g.*, EGFR) or an integrin α - β complex. The G protein subunits do not need to be modified to monitor their activation, and specificity is achieved by coexpressing the G α protein subunits with the studied receptor and by using a GASIP specific to each family of G proteins such as the G protein-binding domain of Rap1GAP for the Gi family (Gi1, Gi2, Gi3, GoA, GoB, Gz) (**FIG. 1B**), of P63RhoGEF (P63RG) for the Gq family (Gq, G11, G14 & G15) (**FIG. 1C**) and of PDZRhGEF (PDZRG) for the G12/13 family (**FIGs. 1D and 1E**). Only members of the Gi family showed a response greater than Mock condition (activation of endogenously expressed Gi1, i2 & i3 proteins) when the G protein-binding domain of Rap1GAP was used (**FIG. 1B**), only members of the G12/13 family showed a response greater than Mock condition (activation of endogenously expressed G12 and G13 proteins) when PDZRG was used (**FIG. 1C**), and only members of the Gq family show a response greater than Mock condition activation of endogenously expressed Gq and G11 proteins) when P63RG was used (**FIGs. 1D and 1E**), thus confirming the specificity of the assay.

Example 3: Systems and assays for monitoring activation of the G proteins of the Gi family

FIGs. 2A to 2Z show the optimization and use of a Rap1GAP-based BRET sensor for monitoring activation of the G proteins of the Gi family (Gi1, i2, i3, oA, oB, z). The various constructs tested are shown in **FIG. 2A**. The results presented in **FIGs. 2B to 2E** shows that a detectable BRET signal was obtained using the two BRET donors tested (Rluc8 and RlucII), with RlucII typically giving a better dynamic window relative to Rluc8. The results presented in **FIGs. 2F and 2G** demonstrate that the Rap1GAP(1-442) construct is sensitive to phosphorylation, as evidenced by the lower responses measured when cells were pre-treated with forskolin (which promotes an increase cAMP production and activation of protein kinase A leading to phosphorylation of different proteins), which could affect the assay. C-terminal truncated variants (1-420 and 1-436) and different mutants comprising combinations of Ser to Ala and Ser to Asp substitutions were made at putative phosphorylation sites (residues Ser 437, 439 and 441) were generated and tested. C-terminal truncated variants (Δ CT) of RAP1GAP were insensitive to the effects of forskolin, but the window of this shorter fragment (Rap1GAP 1-420) is significantly lower than that of Rap1GAP (1-442) (**FIG. 2H**). Among the other mutants tested, the only mutants still influenced by forskolin were Rap1GAP (SS-AD), Rap1GAP (SSS-TTT) and Rap1GAP (SS-AA), with the latter showing a dynamic window comparable to Rap1GAP (1-442)-RlucII (**FIGs. 2J-2Q**). Rap1GAP (SSS-AAA) was used for the other experiments described below.

G protein profiling of the Dopamine D4 receptor (D4R) upon dopamine-promoted stimulation was obtained with Rap1GAP (SSS-AAA)-RlucII translocation to the plasma

membrane, each G protein (Gi1, Gi2, Gi3, GoA & GoB) showing dose-response curves with distinct pharmacological characteristics (**FIG. 2R**). Gz activation was used to profile several dopamine receptor ligands (A412 997, Dopamine, L741 742 and Way-100635) on the 5 dopamine receptors, and the results are depicted in **FIGs 2S to 2W**. Dopamine was shown to activate all the receptors (EC_{50} : D1R = 380 nM, D2R = 2.6 nM, D3R = 0.50 nM, D4R = 0.11 nM, D5R = 51 nM), the known D4R antagonist L741,742 did not show agonist nor inverse properties on any of the dopamine receptors. WAY-100635 only showed agonist properties on D4R (EC_{50} = 0.83), whereas A412,997 activated both D3R (EC_{50} = 281 nM) and D4R (EC_{50} = 0.04 nM), but not D1R, D2R or D5R. The results depicted in **FIGs. 2X-2Z** show that the assay is robust and compatible with high-throughput screening (HTS), with Z' factor evaluated to 0.812, 0.703 and 0.607 for Gi2, GoA and Gz activation, respectively.

FIGs. 3A-3D show that an RGS domain of members of the Regulator of G-protein signaling (RGS) proteins such as RGS17, 19 and 20 may be used as an alternative to Rap1GAP to monitor G protein activation. RlucII-tagged constructs based on the RGS domain of the RGS17 (residues 64-210), RGS19 (residues 70-217) and RGS20 (residues 242-388) proteins are presented in **FIG. 3A**. Using only the G α -binding RGS domain of these proteins advantageously allows for a cytosolic localization and translocation to different compartments upon G protein activation, with no influence of other domains present in the full-length protein or palmitoylation sites present at the N-terminal part of these proteins. Dose-response curves obtained with RGS(RGS17)-RlucII (**FIG. 3B**), RGS(RGS19)-RlucII (**FIG. 3C**) and RGS(RGS20)-RlucII (**FIG. 3D**) are presented for D4R/Dopamine-mediated activation of Gi1, Gi2, Gi3, GoA, GoB and Gz activation at the PM. Similar results (coupling and EC_{50}) were obtained with the 3 RGS constructs, confirming that these RGS-based constructs may be used to monitor Gi and Go activation. The results were similar to those obtained with the Rap1GAP-based constructs, except that coexpression of Gz did not lead to a signal distinguishable from the mock condition, and the dynamic window for Gi3 was smaller with the RGS-based constructs relative to the Rap1GAP-based constructs.

Example 4: Systems and assays for monitoring activation of the G proteins of the G12/13 family.

The optimization and use of a PDZRhoGEF-based BRET system for monitoring activation of G proteins of the G12/13 family is described in **FIGs. 4A-G**. A fragment of PDZRhoGEF comprising the G12/13 binding domain (residues 281-483, PDZRG) was tagged in C-terminal with the BRET donor RlucII (**FIG. 4A**). The dynamic window for measuring activation of G12 and 13 was shown to be directly dependent on the level of expression of the G α subunit, but the level of expression did not affect the potency for I-BOP/TPaR-mediated activation of G12 and 13 as evidenced by the comparable LogEC50 values obtained with the different amounts of

G12 and 13 (**FIGs. 4B and 4C**). PDZRG-RlucII was used to profile TPαR ligands on G12 and G13 activation at the PM. Cells were stimulated with known full agonists (U46619, I-BOP, CTA2), with one partial agonist (U51605) and the antagonists I-SAP and SQ 29,558. The results depicted in **FIGs. 4D and 4E** show that SQ 29,558 is not acting as a TPαR agonist. Consistent with their known activity/properties, U46619, I-BOP and CTA2 are full agonists on G12 and 13 activation, confirming the validity of the assay to monitor G12/13 activation. Interestingly, U51605 and I-SAP were shown to act as biased ligands. U51605 was demonstrated to be a partial agonist on G12 (40% of Max) activation, and almost a full agonist on G13 (93% of Max). In contrast, I-SAP stimulation fails to induce significant G12 activation in the assay (**FIG. 4D**), but a partial agonist activity (53% of Max) was detected on G13 (**FIG. 4E**). Finally, Z' factors evaluated to 0.645 for G12 activation and 0.812 for G13 activation were obtained (**FIGs. 4F and 4G**), again indicating that the assay is robust and compatible with high-throughput screening applications based on G12/G13 modulation.

The optimization and use of a P115RhoGEF-based BRET system for monitoring activation of G proteins of the G12/13 family is described in **FIGs. 5A-E**. A fragment of P115RhoGEF comprising the G12/13 binding domain (residues 1-244, P115RG) was tagged in C-terminal with the BRET donor RlucII (**FIG. 5A**). P115RG-RlucII was used to profile TPαR ligands on G12 and G13 activation at the PM. Cells were stimulated with U46619, I-BOP, CTA2, U51605, I-SAP and SQ 29,558. The results depicted in **FIGs. 5B and 5C** are consistent with those obtained with PDZRG-RlucII and show that SQ 29,558 is not acting as a TPαR agonist, U46619, I-BOP and CTA2 are full agonists on G12 and 13 activation, U51605 and I-SAP were shown to act as biased ligands. U51605 was demonstrated to be a partial agonist on G12 (14% of Max) and G13 (60% of Max) activation, whereas I-SAP stimulation was a partial agonist on G13 (19% of Max) and neutral on G12 activation. Finally, Z' factors evaluated to 0.703 for G12 activation and 0.743 for G13 activation were obtained (**FIGs. 5D and 5E**), again indicating that the assay is robust and compatible with high-throughput screening applications based on G12/G13 modulation.

Example 5: Systems and assays for monitoring activation of the G proteins of the Gq family.

The optimization and use of a P63RhoGEF-based BRET system for monitoring activation of G proteins of the Gq family (Gq, G11, G14 and G15) is described in **FIGs. 6A-S**. A fragment of P63RhoGEF comprising the Gq binding domain (residues 295-502, P63RG) was tagged in C-terminal with the BRET donor RlucII (**FIG. 6A**). The full length P63RhoGEF protein is associated with the PM by its N-terminus. As with PDZRG-RlucII and P115RG-RlucII, using a fragment comprising only the G protein binding domain of P63RhoGEF advantageously allows for a cytosolic localization and translocation to different compartments upon G protein activation.

Optimization for monitoring G protein activity at the PM and at early endosomes (EE) using different membrane markers (rGFP-CAAX for PM and rGFP-FYVE for EE) are presented in **FIGs. 6B-6E** (for PM) and **FIGs. 6F-I** (for EE). Mock represents responses obtained from endogenous G proteins, in cells not transfected with a recombinant G α . The dynamic window for measuring activation of members of the Gq family was shown to be dependent on the level of expression of the G α subunit. As presented in **FIG. 6B** for Gq and **FIG. 6D** for G14, the dynamic window increases as more G α is cotransfected. For G15 (**FIG. 6E**), the effect is already maximum at 5ng of co-transfected constructs encoding G α 15, and transfecting more had no significant effect. For G11 (in **FIG. 6C**), increasing the level of transfected DNA over 5 ng was shown to lead to a decrease of the dynamic window. Similar results were obtained for the monitoring of G protein activity at EE (**FIGs. 6F-I**).

Optimized conditions were used to profile the TP α R ligands U46619, I-BOP, CTA2, U51605, I-SAP and SQ 29,558 at the PM (**FIGs. 6J-6M**) and EE (**FIGs. 6N-6Q**). As shown in **FIGs. 6J-6M**, U46619, I-BOP and CTA2 are full agonists on Gq (**FIG. 6J**), G14 (**FIG. 6K**), G11 (**FIG. 6L**) and G15 (**FIG. 6M**); U51605 is a partial agonist (32% of I-BOP max response for Gq, 25% for G14, 24% for G11 and 10% for G15); I-SAP (only 7% on Gq) and SQ 29,558 fail to induce significant activation of all four G proteins at the PM. Similar results (potency and efficacy) were obtained when monitoring G protein activity at EE (**FIGs. 6N-6Q**). For U51605, a higher efficacy was observed at EE for Gq (71% of I-BOP max response), G11 (61%) and G14 (75%), but no response for G15. Finally, Z' factors evaluated to 0.840 and 0.879 for Gq and G11 activation at the PM respectively, were obtained (**FIGs. 6R** and **6S**), again indicating that the assay is robust and compatible with high-throughput screening applications based on Gq modulation.

The optimization and use of a GRK2-based BRET system for monitoring activation of G proteins of the Gq family (Gq, G11, G14 and G15) is described in **FIGs. 7A-7F**. Two RGS(GRK2) construct are presented. A fragment of GRK2 comprising the Gq binding domain (RGS domain) (residues 30-203, RGS(GRK2)) was tagged at the N-terminal (RlucII-RGS(GRK2)) or C-terminal (RGS(GRK2)-RlucII) with the BRET donor, RlucII. The full length GRK2 protein has a Pleckstrin (PH) domain and a G $\beta\gamma$ -interacting domain that modulate its recruitment to the PM. Using only the Gq-binding RGS domain of GRK2 advantageously allows for a cytosolic localization and translocation to different compartments upon Gq protein activation, with no influence of PIP₂ or free G $\beta\gamma$ subunits levels that could be modulated through activation of other non-Gq G proteins, for example. In **FIGs 7B** and **7C**, dose-response curves obtained with RlucII-RGS(GRK2) are presented for Gq, G11, G14 and G15 activation at the PM, by two Gq-coupled receptors, AT1AR stimulated with angiotensinII (**FIG. 7B**) and TP α R stimulated with U46619 (**FIG. 7C**). As depicted in **FIGs. 7D** and **7E**, similar results (coupling and EC₅₀) were obtained with RGS(GRK2)-RlucII, albeit with dynamic windows that were generally lower relative to RlucII-RGS(GRK2). Z' factor evaluated to 0.785 was obtained when assessing recruitment of RlucII-RGS(GRK2) to the PM

following activation of TPαR with 100 nM U46619, confirming that the assay is robust and compatible with high-throughput screening applications based on Gq modulation.

Example 6: Systems and assays for monitoring activation of G proteins by non-receptor guanine nucleotide exchange factors (GEF).

G protein activation can be achieved via non-receptor guanine nucleotide exchange factors (GEF) such as GIV (Gα-interacting vesicle-associated protein, also known as Girdin), NUCB1 (nucleobindin1, also known as calnuc), NUCB2 and DAPLE (Dishevelled-associating protein). GIV/Girdin activity is associated with RTKs (*e.g.*, EGFR) and integrin modulation of Gi activity. DAPLE is associated with rac and Gi-activation through wnt/Frizzle receptors (GPCRs). GEF-mediated activation of WT Gi proteins can be monitored using the systems/assays for monitoring activation of the G proteins of the Gi family described herein, such as the systems/assays using Rap1Gap (SSS-AAA)-RlucII. However, as GPCRs have been shown to transactivate RTKs such as EGFR and IGFR, it could be useful to be able to discriminate between GPCR and GEF-mediated activation of G proteins, for HTS applications and ligand profiling studies. To achieve this objective, a group of mutant Gai2 proteins having C-terminal mutations (**FIG. 8A**) were designed and tested. EGFR-mediated activation of WT and mutant Gi2 was compared to GPCR (BKB2R) response in dose response curves. Deleting the last 2 residues of Gai2 (**FIGs. 8B, 8C**) or the last 5 residues of Gai2 (**FIGs. 8D, 8E**) and GaoB (**FIGs. 8F, 8G**) was sufficient to prevent GPCR-mediated G protein activation while maintaining EGFR-mediated activation of Gi2 and GoB. For Gi2, both deletions changed the basal activity as compared to the WT G proteins for cells co-expressing EGFR. Substitutions were made at 2 conserved leucine residues (positions -7 and -2) to identify a residue that would lead to a similar basal activity with EGFR but with a mutant still inactive upon GPCR activation. The L-7G mutant showed a different basal activity than the WT with EGFR (**FIGs. 8H, 8I**). Results with L-2G mutant of Gai2 showed a similar basal activity than WT with EGFR but a different one with BKB2R (**FIGs. 8J, 8K**). For the GaoB L-2G mutant, the basal activity was similar to the WT protein for both receptors (**FIGs. 8L, 8M**). Additional L-2 mutant Gai2 proteins were tested (L-2D, L-2P and L-2R). While their dynamic window, as measured with agonist-induced translocation of Rap1GAP(SSS-AAA)-RlucII to the PM, is smaller than with the WT proteins, differences in basal activity were much closer relative to the deletion or L-2G mutants of Gai2 protein (**FIGs. 8N-8S**). Z' factor evaluated to 0.70 with WT Gai2 (**FIG. 8T**) and to 0.73 with the L-2P Gai2 mutant (**FIG. 8U**) were obtained, which indicates a robust assay for monitoring non-GPCR-mediated G protein activation using both mutant and WT G proteins. **FIGs 8V-8X** show that Rap1GAP(SSS-AAA)-RlucII can be used to monitor GPCR (**FIGs 8V** with SNC80-mediated DOR activation and in **FIG. 8W**, Quinpirole-mediated activation of D2R) as well as GEF-mediated Gprotein activation (through EGFR stimulation; **FIG. 8X**) while RGS(RGS17)-RlucII-based sensor only monitors GPCR-mediated

activation as there is no significant increase in the BRET signal in the presence of escalating concentrations of the agonist EGF. As GPCR activation can lead to RTK transactivation, these results provide evidence that WT $G\alpha/RGS(RGS17)$ -RlucII and mutant $G\alpha/Rap1GAP(SSS-AAA)$ -RlucII could be used to distinguish GPCR-mediated from RTK/GEF-mediated Gprotein activation.

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Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to". The singular forms "a", "an" and "the" include corresponding plural references unless the context clearly dictates otherwise.

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WHAT IS CLAIMED IS:

1. A system for measuring modulation of G protein activation in a G α protein subunit family-selective manner, said system comprising a cell expressing:

(i) a first component comprising a G α subunit interacting polypeptide (GASIP) tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein:

if said G α protein subunit family is G i , said GASIP comprises the G protein-binding domain of RAP1 GTPase Activating Protein 1 (Rap1GAP) or of a Regulator of G-protein signaling (RGS) protein;

if said G α protein subunit family is G q , said GASIP comprises the G protein-binding domain of Rho guanine nucleotide exchange factor 25 (P63RhoGEF); and

if said G α protein subunit family is G12/13, said GASIP comprises the G protein-binding domain of Rho guanine nucleotide exchange factor 11 (PDZRhoGEF) or Rho guanine nucleotide exchange factor 1 (P115RhoGEF); and

(ii) a second component comprising a plasma membrane (PM)-targeting moiety, an endosomal-targeting moiety or a Golgi-targeting moiety tagged with a bioluminescent donor molecule or a fluorescent acceptor molecule;

wherein if said GASIP is tagged with said fluorescent acceptor molecule, said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said bioluminescent donor molecule, and if said GASIP is tagged with said bioluminescent donor molecule, said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said fluorescent acceptor molecule;

and wherein there is no protein-protein interaction between (i) the PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety and (ii) the GASIP.

2. The system of claim 1, wherein said GASIP comprises the G protein-binding domain of Rap1GAP.

3. The system of claim 2, wherein said G protein-binding domain of Rap1GAP comprises residues 1 to 442 of Rap1GAP set forth in SEQ ID NO:8, or a variant thereof in which one or more of the serine residues at positions 437, 439 and 441 are mutated or absent.

4. The system of claim 3, wherein said G protein-binding domain of Rap1GAP comprises residues 1 to 420 or 1 to 436 of Rap1GAP.

5. The system of claim 1, wherein said GASIP comprises the G protein-binding domain of an RGS protein.

6. The system of claim 5, wherein said RGS protein is Regulator of G-protein signaling 17 (RGS17), Regulator of G-protein signaling 19 (RGS19) or Regulator of G-protein signaling 20 (RGS20).

7. The system of claim 6, wherein said G protein-binding domain comprises residues 64 to 210 in the sequence of RGS17 set forth in SEQ ID NO:17, residues 70-217 in the sequence of RGS19 set forth in SEQ ID NO:18, or residues 242-388 in the sequence of RGS20 set forth in SEQ ID NO:19.

8. The system of claim 1, wherein GASIP comprises the G protein-binding domain of P63RhoGEF.

9. The system of claim 8, wherein said G protein-binding domain of P63RhoGEF comprises residues 295 to 502 in the sequence of P63RhoGEF set forth in SEQ ID NO:25.

10. The system of claim 1, wherein said GASIP comprises the G protein-binding domain of PDZRhoGEF or P115RhoGEF.

11. The system of claim 10, wherein said G protein-binding domain of PDZRhoGEF comprises residues 281 to 483 in the sequence of PDZRhoGEF set forth in SEQ ID NO:21.

12. The system of claim 10, wherein said G protein-binding domain of P115RhoGEF comprises residues 1 to 244 in the sequence of P115RhoGEF set forth in SEQ ID NO:23.

13. The system of any one of claims 1 to 12, wherein said GASIP is tagged with said bioluminescent donor molecule and said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety is tagged with said fluorescent acceptor molecule.

14. The system of any one of claims 1 to 13, wherein said PM targeting moiety is a PM protein or a peptide that localizes to the PM.

15. The system of claim 14, wherein said PM protein or peptide comprises (a) a palmitoylation, myristoylation, and/or prenylation signal sequence and/or (b) a polybasic sequence.

16. The system of claim 14 or 15, wherein said PM targeting moiety comprises the amino acid sequence GCMSCCKCVLS set forth in SEQ ID NO:60, GCMGLPCVVM set forth in SEQ ID NO:61, CVKIKKCIIM set forth in SEQ ID NO:62, KKKKKKSKTKCVIM set forth in SEQ ID NO:63, KNGKKKRKSLAKRIRERCCIL set forth in SEQ ID NO: 45, CMSCKCCIL set forth in SEQ ID NO:4, or SPKKGLLQRLFKRQHQNNSSKS set forth in SEQ ID NO:5.

17. The system of any one of claims 1 to 13, wherein said endosomal targeting moiety is an endosomal protein or a peptide that localizes to the endosomes.

18. The system of claim 17, wherein said endosomal protein or peptide comprises a FYVE domain.
19. The system of claim 18, wherein said endosomal targeting moiety comprises residues 739 to 806 in the sequence of human endofin set forth in SEQ ID NO:39.
- 5 20. The system of any one of claims 1 to 13, wherein said Golgi targeting moiety is Nitric oxide synthase, endothelial (eNOS1) or a fragment thereof comprising residues 1 to 73 in the sequence of human eNOS1 set forth in SEQ ID NO:46.
21. The system of claim 20, wherein said Golgi targeting moiety comprises residues 1 to 73 in the sequence of human eNOS1 set forth in SEQ ID NO:46.
- 10 22. The system of any one of claims 1 to 21, wherein (a) said first component further comprises a linker between (i) said GASIP and (ii) said bioluminescent donor molecule or fluorescent acceptor molecule; and/or said second component further comprises a linker between (i) said PM-targeting moiety, endosomal-targeting moiety or Golgi-targeting moiety and (ii) said bioluminescent donor molecule or fluorescent acceptor molecule.
- 15 23. The system of claim 22, wherein said linker is a peptide linked of 5 to 25 amino acids.
24. The system of any one of claims 1 to 23, further comprising a third component that is a cell surface receptor that signals through said G protein.
25. The system of claim 24, where said cell surface receptor is a G protein-coupled receptor (GPCR), a receptor tyrosine kinase (RTK) or an integrin receptor.
- 20 26. The system of any one of claims 1 to 25, further comprising a fourth component that is a recombinant G α subunit polypeptide.
27. The system of claim 26, wherein said G protein activation is non-receptor guanine nucleotide exchange factor (GEF)-mediated G protein activation, wherein said recombinant G α subunit polypeptide comprises at least one mutation in the carboxy (C)-terminal domain of said
- 25 G α subunit polypeptide, and wherein said C-terminal domain corresponds to the last seven residues of said G α subunit polypeptide.
28. The system of claim 27, wherein said GEF is guanine nucleotide-binding (G) protein α subunit (G α)-interacting vesicle-associated protein (GIV/Girdin), and wherein said GASIP comprises the G protein-binding domain of Rap1GAP as defined in any one of claims 1 to 4.
- 30 29. The system of claim 28, wherein said GEF is activated by a receptor tyrosine kinase (RTK).

30. The system of any one of claims 1 to 29, wherein said bioluminescent donor molecule is *Renilla* luciferase protein (rLuc) and said fluorescent acceptor molecule is a *Renilla* green fluorescent protein (rGFP).

31. A host cell expressing the components of the system defined in any one of claims 1 to 30.

5 32. A method for determining whether an agent modulates the activation of a G protein of interest, said method comprising:

(a) contacting the system of any one of claims 1 to 30 with a substrate for said bioluminescent donor molecule; and

10 (b) measuring the Bioluminescence Resonance Energy Transfer (BRET) signal in the system in the presence and absence of said agent;

wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates the activation of said G protein of interest.

33. A method for determining whether an agent modulates non-receptor guanine nucleotide exchange factor (GEF)-mediated G protein activation, said method comprising

15 (a) contacting the system of any one of claims 27 to 29 with a substrate for said bioluminescent donor molecule; and

(b) measuring the BRET signal in the system in the presence and absence of said agent; wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates non-receptor GEF-mediated G protein activation.