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LETTER

Novel Genetically Encoded Biosensors Using Firefly Luciferase

Frank Fan[†], Brock F. Binkowski[†], Braeden L. Butler, Peter F. Stecha, Martin K. Lewis, and Keith V. Wood*

Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin 53711. [†]These authors contributed equally to this work.

ABSTRACT Genetically encoded biosensors have proven valuable for real-time monitoring of intracellular phenomena, particularly FRET-based sensors incorporating variants of green fluorescent protein. To increase detection sensitivity and response dynamics, we genetically engineered firefly luciferase to detect specific intermolecular interactions through modulation of its luminescence activity. This concept has been applied in covalent, noncovalent, and allosteric design configurations. The covalent design gives sensitive detection of protease activity through a cleavage-dependent increase in luminescence. The noncovalent and allosteric designs allow reversible detection of the small molecules rapamycin and cAMP, respectively. These sensors allow detection of molecular processes within living cells following addition of the luciferin substrate to the growth medium. For example, the cAMP sensor allows monitoring of intracellular signal transduction associated with G-protein coupled receptor function. These and other luminescent biosensors will be useful for the sensitive detection of cellular physiology in research and drug discovery.

*Corresponding author, keith.wood@promega.com

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The observation of dynamic molecular processes within living cells is a long-standing desire of cell biology research. Accordingly, recent years have brought increased efforts toward engineering reporter proteins to serve as intracellular biosensors. For example, numerous FRET-based sensors have been configured to detect a range of physiological mediators such as Ca^{2+}, cAMP, and phosphorylation (*1, 2*). These biosensors typically comprise an allosterically responsive domain linking a pair of fluorescent proteins, where the conformational change influences the relative orientation and separation of the adjoining FRET energy donor and acceptor.

Although this approach has proven useful for cellular imaging, its suitability for macroscopic measurements can be limited by insufficient detection sensitivity and dynamic range. In contrast, luciferase reporter proteins are well-known for providing high sensitivity and a wide dynamic range, and they are commonly used for rapid quantitation within multiwell microplates. This has been especially valuable in automated screening applications where microplates may contain 1536 wells or more. Moreover, as intracellular probes, luciferases can be used effectively at much lower expression levels than fluorescent proteins, thereby minimizing their potential influence on cellular physiology. Luciferases are also commonly used for molecular imaging in living organisms, such as mice.

The beneficial properties of luciferases as intracellular probes have, nonetheless, been limited largely to their use as genetic reporters. To broaden their applicability for elucidating cellular phenomena, we have set out to develop new strategies for configuring firefly luciferase as a biosensor of intracellular molecules or events. Firefly luciferase is a 61 kDa monomeric enzyme that catalyzes the oxidation of firefly luciferin in the presence of Mg·ATP and molecular oxygen to generate oxyluciferin, CO_2, and AMP with the concomitant emission of yellow-green light. Crystal structures of luciferase reveal two domains, connected through a hingelike region, that rotate and close together upon substrate binding (Figure 1, panel a) (*3, 4*). Our biosensor design strategies modeled this conformational change as the closing of a simple hinge, where each of the strategies was intended to restrict or modulate the motion of this hinge.

Because the N- and C-termini are located on opposite sides of the hinge region, the first design strategy was to restrict hinge motion simply by connecting these termini together, thus impeding the luminescent reaction (Figure 1, panel b). This was done by creating a circularly permuted form of firefly luciferase, where the native N- and C-termini are relocated to a new position in the protein structure (*5*). To identify sites tolerant of structural manipulations, we used transposon mutagenesis to create a library of mutant luciferases containing random insertions of five amino acids. One mutant from this library, retaining 75% of the parental luminescence activity, contained a polypeptide insertion following Pro233 (Figure 1, panel a). With this location for the new N- and C-termini, a gene encoding a circularly per-

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muted luciferase (CP_{234}—Luc) was created by positioning the coding region for amino acids 234—544 ahead of the region encoding 4—233, where amino acids 4 and 544 are connected by a polypeptide linker (Figure 1, panel b). Several protease sites were separately incorporated into this polypeptide linker (Figure 2, panel a), and the genes were expressed in cell-free translation reactions. The translation products yielded very little luminescence, several thousand-fold below the parental luciferase expressed under equivalent conditions (Figure 2, panel b). However, luminescence activity increased between 185- and 2610-fold following protease treatment, raising the light intensity to 3—16% of the parental luciferase.

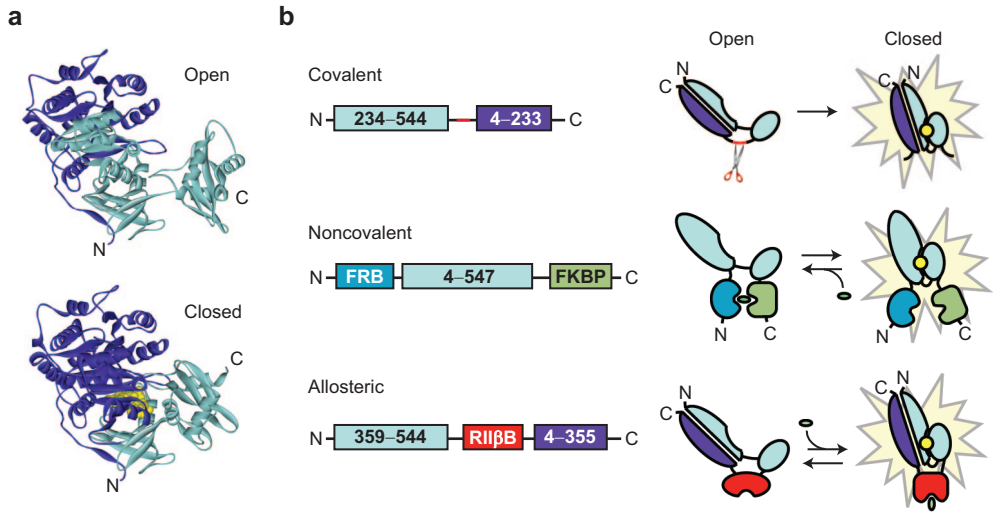

Figure 1. Design strategies for biosensors using firefly luciferase. a) Molecular models of firefly luciferase in the absence of substrates (open conformation) or bound to an analog of the luciferyl-adenylate reaction intermediate (closed conformation, analog colored yellow) generated using PDB files 1LCI and 2D1S, respectively. The smaller C-terminal domain of luciferase (residues 441—544) is predicted to rotate and translocate toward the larger N-terminal domain during the catalytic cycle. Residues 4—233 and 234—544 are colored blue and gray, respectively, for the "open" conformation. Residues 4—355 and 359—544 are colored blue and gray, respectively, for the "closed" conformation. Nonvisible residues at the N- and C-termini of PDB file 1LCI were typically excluded from the various biosensor design strategies. b) Schematic representation of the three design strategies used to generate luciferase biosensors. *Covalent*. Fusion of the wild-type N- and C-termini with a polypeptide containing a protease cleavage site inhibits formation of the closed conformation. Cleavage by protease relieves this constraint, allowing increased luminescence. *Noncovalent*. Association of polypeptides FRB and FKBP12 in the presence of rapamycin inhibits formation of the closed conformation, causing decreased luminescence. *Allosteric*. The conformational change of an analyte binding domain modulates luminescence, *e.g.*, cAMP binding to RIIβB promotes increased luminescence.

We further characterized the utility of these protease biosensors by evaluating a CP_{234}—Luc construct containing a caspase 3 cleavage site (CP_{234}—Luc/DEVDG). After cell-free expression and fluorescent labeling, the time dependence of protease cleavage was monitored by both luminescence activity and SDS-PAGE (Supplementary Figure 1). Increased sample luminescence correlated with the appearance of the expected 36 and 25 kDa protein fragments, whereas no increase was observed in the absence of caspase 3 or with CP_{234}—Luc lacking the DEVDG cleavage site (data not shown). In separate experiments, luminescence activation was linearly proportional to the amount of caspase 3 for greater than 3 orders of magnitude (Figure 2, panel c), and the luminescent assay was over 1000-fold more sensitive than was detection by SDS-PAGE (Supplementary Figure 1). To optimize the response of this sensor, the length of the polypeptide linker containing the DEVDG cleavage sequence was systematically varied (Supplementary Figure 2). Longer linkers yielded greater basal luminescence with diminished activation by protease treatment, consistent with our model of conformational constraint. An optimal overall linker length between 11 and 13 amino acids was empirically determined for maximal relative activation by caspase 3.

The CP_{234}—Luc/peptide biosensors provide a unique tool for the study of protease activity: they are easily generated using molecular biology techniques; they can display protease specificity determinants present on both sides of the scissile peptide bond; and they provide the sensitivity and large dynamic range characteristic of luminescent assays. Assays using these sensors can be readily employed to quantify protease activity, analyze substrate specificity, or rank the potency of protease inhibitors (Supplementary Figure 3), and we are currently investigating their use in living cells.

Our second design strategy for biosensor development restricts hinge closure through a noncovalent mechanism. In this concept, rather than connecting the protein termini through a peptide linkage, they are instead joined noncovalently by interacting protein/peptide domains (Figure 1, panel b). As a proof of concept, we fused FRB and FKBP12

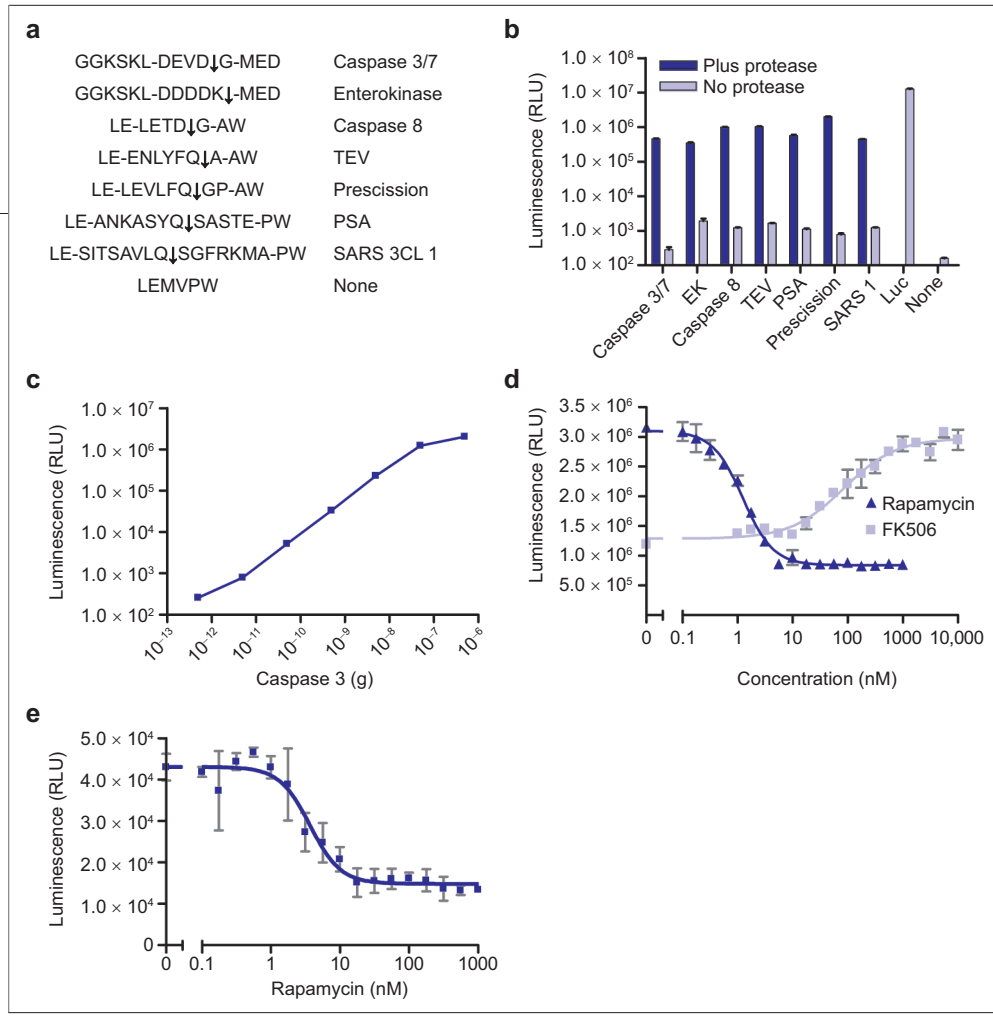

Figure 2. Covalent and noncovalent biosensors. a) Primary structure of protease biosensors. Each of the polypeptides shown is fused to luciferase residues 544 and 4 as shown in Figure 1. Protease recognition sites are denoted by hyphens and scissile peptide bonds are indicated by arrows. b) Cleavage-dependent activation of protease biosensors expressed *in vitro*. c) Dose-dependent detection of caspase 3 activity using the CP_{234}−Luc/DEVDG biosensor expressed *in vitro*. d) Rapamycin induced decrease and FK506 mediated increase in FRB-Fluc-FKBP12 activity following expression *in vitro*. Samples were treated with rapamycin alone or varying concentrations of FK506 and 1.5 nM rapamycin for 10 min and 2.5 h, respectively. e) Rapamycin-mediated decrease in FRB-Luc-FKBP12 activity following transient expression in HEK293 cells. Luminescence was measured from living cells 2 h after addition of varying concentrations of rapamycin. For b and c, error bars represent standard error, $n = 3$. For d and e, error bars represent standard error of the mean, $n = 3$. Error bars are not visible for the data of panel c.

protein domains to the N- and C-termini of firefly luciferase, respectively (Figure 1, panel b). These protein domains associate in the presence of rapamycin, and this association can be blocked by the addition of excess FK506. Following cell-free expression of FRB-Luc-FKBP12, a dose-dependent decrease in luminescence activity was detected with increasing concentrations of rapamycin, with a maximal reduction of 4-fold (Figure 2, panel d). The IC50 for rapamycin was 1 nM, 12-fold lower than the published value of the dissociation constant *in vitro* (*6*), likely owing to the covalent tethering of FRB and FKBP. Decreased luminescence was not evident using FRB-Luc, Luc-FKBP12, or Luc alone (Supplementary Figure 4) and was reversed by addition of FK506 (Figure 2, panel d). Rapamycin also had no effect in a mixture of FRB-Luc and Luc-FKBP12, signifying that the effect on FRB-Luc-FKBP12 was likely not the result of intermolecular complex formation (Supplementary Figure 4).

To evaluate applicability in living cells, we transiently expressed FRB-Luc-FKBP12 in HEK293 cells to observe changes in luminescence upon addition of rapamycin. Luminescence can be readily detected in cells expressing luciferase simply by adding luciferin to the culture medium. The results indicated a dose-dependent response to rapamycin with IC50 of 4 nM and a maximal decrease in luminescence of 3-fold, similar to results obtained *in vitro*. No response to rapamycin was apparent in cells expressing the parental Luc, FRB-Luc, or Luc-FKBP12 (data not shown). Together, the results obtained for this model system indicate the potential utility of biosensor design strategies that involve interacting domains appended to the termini of firefly luciferase.

In the final design strategy, an allosteric domain is placed near the hinge region so that luminescence activity will be modulated by conformational changes in this domain (Figure 1, panel b). We applied this strategy for the detection of cAMP by utilizing the cAMP-binding domain B from protein kinase A regulatory subunit type IIβ (RIIβB). This structurally conserved domain, which had been used previously in the development of a FRET-based biosensor (*7*), undergoes a conformational change that includes capping of the cAMP binding site by a C-terminal α helix (*8*). However, in contrast to our results presented above where restricting the luciferase termini yielded predictable decreases in luminescence, it was more difficult to predict the potential relationship between allosteric conformation, hinge flexibility, and luminescence activity in this new configuration.

We fused the RIIβB domain between the luciferase 234−544 and 4−233 fragments of CP_{234}−Luc using peptide linkers of varying lengths (Supplementary Figure 5). Cell-

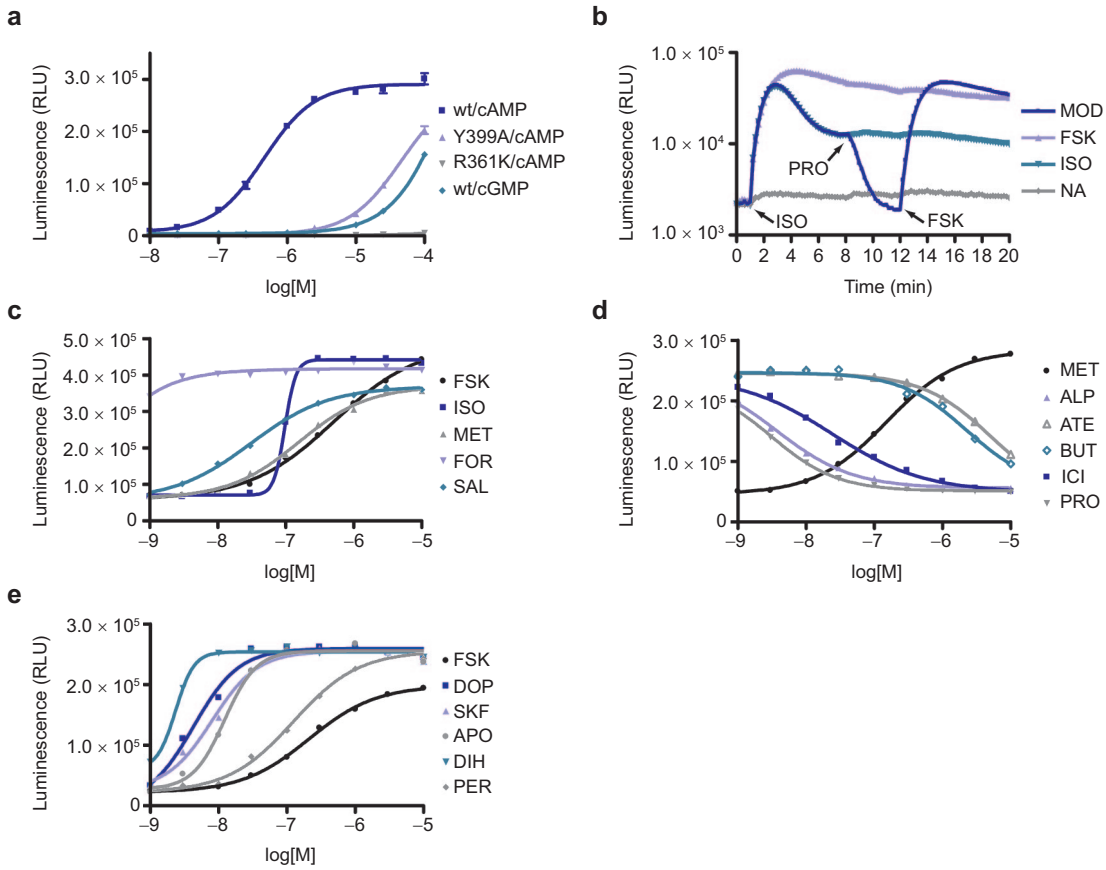

Figure 3. Allosteric cAMP biosensor. a) CP_{359}—Luc/RIIβB dose response and selectivity following expression *in vitro*. Error bars represent standard error of the mean, $n = 3$. b) Signal kinetics and reversibility in living cells at 37 °C. HEK293 cells transiently expressing CP_{359}—Luc/RIIβB were treated with 10 μM isoproterenol (ISO) or 10 μM forskolin (FSK) alone. Modulated cells were treated sequentially with 10 μM ISO, 10 μM propranolol (PRO), and 10 μM FSK ($n = 3$). c) Agonists to the endogenous β2-adrenergic receptor. d) Antagonists to the endogenous β2-adrenergic receptor. e) Agonists to exogenous dopamine receptor D1. For c, d, and e, RT measurements were taken from living cells 12.5 min after compound addition.

free expression of constructs containing Gly/Ser rich peptide linkers of 4, 10, and 20 amino acids showed 16-, 36-, and 19-fold activation of luminescence activity in the presence of 100 μM cAMP *in vitro*, whereas a control construct with a random 42 amino acid peptide linker showed no response. Optimization of linker lengths and the site of circular permutation resulted in a construct with 12 and 0 amino acids at the N- and C-termini, respectively, with circular permutation at residue 359 (CP_{359}—Luc/RIIβB) (Supplementary Figures 6—8). This optimized construct displayed greater than 70-fold increase in luminescence in the presence of 100 μM cAMP *in vitro* (EC_{50} 0.5 μM). In addition, activation by cAMP was selective over cGMP and was reduced by point mutations R361K or Y399A (Figure 3, panel a), mutations that have been shown previously to reduce the affinity of RIIβB or related domains for cAMP (*8, 9*).

We characterized the intracellular performance of CP_{359}—Luc/RIIβB using HEK293 cells stably or transiently expressing the biosensor. Following transient transfection of cells and equilibration with luciferin at 37 °C, endogenous adenylate cyclase was activated by addition of 10 μM forskolin (FSK) giving >25-fold increase in luminescence within 3.5 min (Figure 3, panel b). Similarly, activation of the endogenous β2-adrenergic receptor by addition of 10 μM isoproterenol (ISO) caused >20-fold increase within 2 min. The reversibility of the response was demonstrated by the sequential addition of 10 μM ISO, 10 μM propranolol (PRO; β-adrenergic antagonist), and10 μM FSK, giving responses similar to ISO or

FSK alone. Comparable results were seen for experiments conducted at lower temperatures, albeit with reduced signal to background and slower kinetics (Supplementary Figure 9). Although the biosensor gives the predicted responses to these stimuli, further experimentation is required to examine the quantitative nature of these responses.

The action of agonists and antagonists on either transgenic or endogenous GPCRs can be investigated using the CP_{359}−Luc/RIIβB biosensor in a simple assay format. For example, with HEK293 cells stably expressing the biosensor, assays ranking the potency of agonists and antagonists of the endogenous β2-adrenergic receptor were performed in only 12.5 min (Figure 3, panels c and d). Similarly, agonists of the dopamine D1 receptor were ranked in a double stable cell line (Figure 3, panel e). Importantly, the potency ranking of the various D1 agonists matched those of independent assays using either a luciferase reporter gene under the control of tandem cAMP response elements (*10*) or a lytic assay for cAMP based on activation of exogenous protein kinase A (*11*). Moreover, the biosensor EC50 values closely matched those determined using the lytic assay.

Although numerous FRET/BRET-based biosensors have been developed for studying molecular aspects of cellular physiology, their use can be hindered by a relatively small change in the emission ratio and laborious conditions for experimental measurement. Moreover, the sensitivity of fluorescent measurements can often be limited by cellular autofluorescence. In contrast, the bioluminescent sensors described here have increased response dynamics with S/B values extending several orders of magnitude in some cases, and the data can be collected using a simple assay format.

Alternative approaches have been described for creating biosensors using modified luciferases. These have generally relied on intermolecular fragment complementation to detect protein/protein interactions (*12*–*15*). Recently, Paulmurugan *et al*. developed an intracellular sensor by inserting the ligand binding domain (LBD) of the human estrogen receptor into the Renilla luciferase structure (*16*). By this design, light output is modulated upon binding to specific ligands, presumably due to conformational changes in the LBD that influence the efficiency of protein folding. Our approach employs fusion of polypeptides or protein domains with firefly luciferase or circularly permuted luciferase mutants. Although we have used a simple model involving the flexibility of a hinge associated with enzyme catalysis in our design strategies, we cannot currently rule out mechanisms based on complementation of protein fragments similar to Paulmurugan *et al*. and Kim *et al*. (*16*–*18*).

In summary, we demonstrate the use of three independent design strategies to create biosensors using firefly luciferase. The response dynamics of these luminescent sensors makes them well suited for microplate-based assay formats and potentially whole animal imaging. We envision that the incorporation of additional allosteric domains will further broaden the range of luciferase biosensors beyond those described here.

METHODS

Unless specified, all reagents are products of Promega Corporation (http://www.promega.com).

Gene Construction. See Supporting Information.

Covalent Biosensor Assays. Plasmid DNA encoding CP_{234}−Luc/peptide fusions was expressed using TNT T7 Coupled Wheat Germ Extract for 1 h at 30 °C. Following expression, 10 μL of TNT reaction was combined with 10 μL of 2× cleavage buffer and the following proteases were added to the respective biosensor constructs: caspase 3 (100 ng, Millipore), enterokinase (2.5 units, EKMax, Invitrogen), caspase 8 (10 units, Biomol), TEV (10 units, AcTEV, Invitrogen), PreScission (2 units, GE Healthcare), GST-SARS-CoV 3CL (6 μg). Following incubation at the appropriate temperature for 1 h, luminescence was measured from 5 μL of cleavage reaction following injection of 100 μL of Luciferase Assay Reagent using a Glomax luminometer (0.5 s integration time). *caspase 3 dose response.* The CP_{234}−Luc/DEVDG biosensor was expressed using the TNT T7 Coupled Wheat Germ Extract System and labeled using the Fluorotect GreenLys *in vitro* Translation Labeling System for 1 h at 30 °C. Following expression, 10 μL of cell-free translation reaction was combined with 10 μL of 2× reaction buffer containing serial dilutions of caspase 3 (100 mM HEPES pH = 7.5, 200 mM NaCl, 0.2% CHAPS, 2 mM EDTA, 20% glycerol, 20 mM DTT). Samples were incubated for 2.5 h at 30 °C, and luminescence was measured from 5 μL of each sample as before. In addition, 5 μL of sample was resolved on an SDS-PAGE gel (Supplementary Figure 1, panel c).

Noncovalent Biosensor Assays. *In Vitro Rapamycin Dose Response.* Plasmid DNA encoding the FRB-Luc-FKBP12 fusion protein was expressed using TNT T7 Coupled Wheat Germ extract for 1 h at 30 °C. Following expression, 150 μL of the TNT reaction was combined with 250 μL of 2× binding buffer (100 mM HEPES pH = 7.5, 200 mM NaCl, 0.2% CHAPS, 2 mM EDTA, 20% glycerol, 20 mM DTT) and 50 μL of dH_2O. Aliquots of this solution were combined with 10× stock solutions of rapamycin (Sigma) in DMSO. Following incubation at RT for 10 min, luminescence was measured from 5 μL aliquots of each reaction by injection of 100 μL of Luciferase Assay Reagent using a Glomax luminometer (1 s integration time).

In Vitro FK506 Dose Response. Alternatively, following expression and dilution of the same samples, aliquots were initially combined with 10× stock solutions of FK506 (Sigma) in DMSO. Following incubation at RT for 10 min, a 10× stock solution of rapamycin was added to each sample to a final concentration of 1.5 nM. Luminescence was measured as before following incubation at RT for 2.5 h.

Cell Culture Assays. HEK293 cells transiently expressing FRB-Luc-FKBP12 were treated with CO_2 independent media (Invitrogen) + 10% FBS containing 2 mM endotoxin-free luciferin (Luciferin-EF) for 2 h at RT (15,000 cells per well; 96-well plate). Following equilibration, cells were treated with 10× stocks of rapamycin in CO_2 independent media + 10% FBS and luminescence was measured after 2 h using a Glomax luminometer (1 s integration time).

Allosteric Biosensor Assays. *In vitro.* Plasmid DNA encoding CP_{359}−Luc/RIIβB and control constructs (R361K and Y399A point mutations in RIIβB) was expressed *in vitro* using the TNT T7 Coupled Reticulocyte Lysate System for 1 h at 30 °C. Following expression, 9 μL of TNT reaction was combined with 1 μL of a 10× stock solution of cAMP or cGMP in H_2O, the mixture was incubated at RT for 15 min, and luminescence was measured from 2 μL of sample following injection of 100 μL of Luciferase Assay Reagent using a Glomax luminometer (0.5 s integration time).

Signal Response in Living Cells. HEK293 cells transiently expressing CP_{359}−Luc/RIIβB were treated with CO_2 independent media + 10% FBS containing 5 mM Luciferin-EF for 2 h at 37 °C (15,000 cells per well; 96-well plate). Following equilibration, cells were treated with 10 μM isoproterenol, 10 μM forskolin, or left untreated. Modulated cells were treated with the regime of 10 μM isoproterenol, 10 μM propranolol, followed by 10 μM forskolin at the indicated time points.

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Compounds were added by manual addition of 10−100× stock solutions in CO_2 independent media (serum-free) preheated to 37 °C and luminescence was measured using a Varioskan (Thermo Scientific) luminometer at 37 °C (1 s integration time).

GPCR Interrogation in Living Cells. HEK293 cells stably expressing CP_{359}−Luc/RIIβB were treated with CO_2 independent media + 10% FBS containing 5 mM Luciferin-EF for 2 h at RT. Following equilibration, cells were treated with varying concentrations of agonists from 10× stocks serially diluted in CO_2 independent media (serum free). Alternatively, cells were treated with varying concentrations of antagonists from 10× stocks serially diluted in CO_2 independent media (serum free) containing metaproterenol (1 μM final concentration). Luminescence was measured from individual wells 12.5 min after compound addition using a Mithras luminometer (Berthold Technologies) at a steady-state temperature of ~28 °C (1 s integration time). A double stable cell line was used for experiments on dopamine receptor D1.

Conflict-of-interest Disclosure: The authors declare competing financial interests.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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