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Bioluminescence Resonance Energy Transfer assay to characterize Gi-like G protein subtype- dependent functional selectivity

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Abstract

G protein-coupled receptors (GPCRs) comprise the single most targeted protein class in pharmacology. G protein signaling transduces extracellular stimuli such as neurotransmitters into cellular responses. Although preference for a specific GPCR among different G protein families (e.g. Gs-, Gi- or Gq-like proteins) is often well studied, preference for a specific G protein subtype (e.g. Gi1, Gi2, Gi3, Go1 and Go2) has received little attention. Due to tissue expression differences and potentially exploitable functional differences, G protein subtype-dependent functional selectivity is an attractive framework to expand GPCR drug development. Herein we present a bioluminescence resonance energy transfer (BRET)-based method to characterize functional selectivity among Gi-like protein subtypes.

Keywords

GPCR; G protein subtype; BRET; catecholamine; functional selectivity

INTRODUCTION

Bioluminescence resonance energy transfer (BRET) allows detection of proximity change between a bioluminescent donor and a fluorescent acceptor within roughly 10 nm. Upon successful respective fusion of two proteins to a luciferase and a green fluorescent protein (GFP) variant, the specificity as well as ligand-induced relative movement between the two can provide a useful pharmacological assay (Marullo and Bouvier, 2007). Although in general it is an easily executed procedure, it requires structural understanding of the target constructs and the correct equipment for signal detection.

Particularly within G protein coupled receptor (GPCR) pharmacology, BRET has been incorporated as a reliable method to investigate ligand-induced receptor activities such as apparent G protein coupling and β arrestin recruitment (Sánchez-Soto et al., 2016). These different coupling events can be scrutinized to uncover a ligand-specific biased profile. The concept of effector coupling bias, also called functional selectivity, has identified G protein

versus β arrestin bias or G protein family bias and has attracted a potential new approach for drug development (Kenakin, 2011). Aside from the receptor selectivity (e.g. among different catecholamine receptor subtypes), biased signaling should allow an additional strategy for targeting specific cell populations with specific ligands. For this reason, G protein subtypes of the same family are also appealing targets to obtain ligands with biased profile, due to their potential tissue- and cell type- specific expression patterns.

G protein-linked catecholamine receptors play essential roles in neurotransmission. In particular, Gi family-coupled receptors (i.e. α_2 adrenergic receptor family and dopamine D₂-like receptors) are involved in several major neuropsychiatric disorders and constitute widely studied therapeutic targets. With luciferase-fused Gi-like subtype constructs, Gi subtype functional selectivity can be investigated for individual catecholamine receptor species. In this unit, we present a BRET-based pharmacology assay to study and analyze biased profile within Gi subtype activation exemplified by α_2 adrenergic receptors (Sanchez-Soto *et al.*, in preparation).

BASIC PROTOCOL. G protein activation BRET assay

This method monitors relative conformational changes between GPCRs and their cognate G proteins in the presence of receptor ligands by measuring a BRET signal between G α and G γ subunits. It requires the use of donor- and acceptor-fused G-protein subunit constructs. In this protocol, we use *Renilla* luciferase- fused G α_i -like and Venus (green fluorescent protein variant)-fused γ_2 subunits (Fig. 1). Agonist activation of the receptor induces an active conformational change in the heterotrimeric G protein, which results in a decrease of the constitutive BRET signal, consistent with the relative distance change between the G α_i and γ_2 subunits (Gales et al., 2005; Gales et al., 2006).

Upon activation, G α_i -like proteins interact with adenylyl cyclase and inhibits its cyclic AMP production in addition to the involvement of other signaling pathways (Wettschureck and Offermanns, 2005). The G α_i subtype family is made of G α_{i1} , G α_{i2} , G α_{i3} , the splice variants G α_{o1} and G α_{o2} and G α_z . Taking advantage of their high sequence homology, luciferase (substrate-activated donor) can be inserted at the same position (amino acid 91) for the five G α_i/o protein subunits. Together with their high homology, the use of the same insertion scheme allows a reliable comparison of the different G α_i/o protein subtypes in their ability to undergo conformational changes. Due to their wide expression in the brain, β_1 and γ_2 subunits were used to create the heterotrimeric complex with all the G α_i/o protein subtypes. The potential for different effects with other β and γ subunits should always be considered. The fluorescent acceptor Venus is fused to the N-terminus of the γ_2 subunit, to minimize steric issues. To study selective G α_i protein activation by the receptor of interest, cells are transfected with four different constructs: unmodified receptor, Rluc-fused G α_i , β_1 and Venus-fused γ_2 . The co-transfection of β_1 is recommended as its expression presumably improves the stoichiometry and formation of the heterotrimeric G protein.

The protocol described herein (termed G protein activation BRET assay) can be used to analyze ligand- mediated activation of specific G α_i protein subtypes. Receptors and G

protein subtypes can be transiently or stably transfected in different mammalian cell lines and a wide variety of GPCRs can be used. Here we use a transient transfection protocol in the readily transfectable HEK293T cell line due to the need for a reliable expression level, and we analyze α_{2A} and α_{2C} adrenergic receptor-mediated Gai/o protein subtype activation.

Materials

HEK293T cells (ATCC, cat. No. CRL-3216)

HEK293T culture medium (Supplemented DMEM, recipe described in later section)
DMEM (Gibco, cat. No. 11960044)

Fetal bovine serum (FBS; Atlanta Biologics, cat. No. S11150) Antibiotic/antimycotic 100x (Gibco, cat. No. 15240062)

L-Glutamine 200 mM (Gibco, cat. No. 25030081)

Dulbecco's phosphate-buffered saline (DBPS; Gibco, cat. No. 14130144) Trypsin (Gibco, cat. No. 25300054)

Mammalian expression plasmids:

Plasmid encoding unfused receptor of choice, e.g., pcDNA3.1- α_{2A}

Plasmid encoding donor-fused Gai-like subunit, e.g., pcDNA3.1-Gai1-Rluc8

Plasmid encoding unfused G β subunit, e.g., pcDNA3.1-G β 1

Plasmid encoding acceptor-fused G γ subunit, e.g., pcDNA3.1-G γ 2-Venus 1 μ g/
 μ l polyethylenimine (PEI, see recipe)

DPBS BRET buffer (see recipe).

5 mM coelenterazine H (see recipe)

10-cm tissue-culture plates (USA scientific, cat. No. CC7682-3614)

Compound plate (agonist), 96-well clear U-bottom plates (Greiner Bio-One, cat. No. 650101)

Compound plate (antagonist), 96-well clear V-bottom plates (Greiner Bio-One, cat. No. 651101) BRET assay plate, white 96-well flat bottom plates (Greiner Bio-One, cat. No. 655075) Fluorescence plate, black 96-well flat bottom plates (Greiner Bio-One, cat. No. 655076)

12-Channel multichannel pipette, 10–100 μ l (USA scientific, cat. No. 7112-1100) or 30–300 μ l (USA scientific, cat. No. 7112-3300)

Pipette basin (USA scientific, cat. No. 2330-2220)

Manual repeater pipette (Eppendorf, cat. No. EPR-1000R)

Plate reader for luminescence, fluorescence and BRET (Mithras LB 940, Berthold Technologies) Software for data analysis (e.g. Microsoft Excel and Graphpad Prism)

Microcentrifuge (Eppendorf, 5415R)

Counter top centrifuge with 96 well plate adaptor (Thermo, Sorvall Legend XTR)
Standard cell culture operating room

NOTE: All mammalian tissue culture must be conducted using aseptic techniques in a laminar flow hood. Cells should be maintained in a humidified incubator at 37°C in 5% CO₂.

This protocol entails the transfection of five 10 cm plates with α_{2A} adrenergic receptor and one of the G α i/o subtypes for each plate. For analysis of how to compare different receptors and G α i/o protein subtypes, go to *Statistical analysis* and *Analysis of functional selectivity of activation of G protein subtype* sections.

Day 1. Split cells

- 1 Aspirate media from a fully confluent 10-cm plate of HEK293T cells (~2.5 x 10⁷ cells). A passage of ~2 x 10⁵ cells would reach full confluence in 7 days. Wash with 2 ml DPBS and aspirate.
- 2 Add 1–2 ml trypsin to the plate and incubate at room temperature for 30 sec to 1 min to detach cells.
- 3 Add 4 ml supplemented DMEM and transfer cells to a 15-ml conical tube with a serological pipet.
- 4 Centrifuge for 4 min at 900 x g, room temperature.
- 5 Aspirate medium and resuspend cells in 10 ml supplemented DMEM.
- 6 Count the cells using a hemocytometer under the microscope.
- 7 Seed 3 x 10⁶ HEK293T cells into a 10-cm tissue culture plate in a total volume of 10 ml supplemented DMEM.
- 8 Place cells in an incubator at 37°C and 5% CO₂.

Day 2. Transfect cells

- 9 After 24 h of incubation at 37°C and 5% CO₂, inspect cells under microscope. They should be at 50–70% confluence. The experiment should be discontinued if the confluence is low since it might indicate non-physiological conditions. In addition, the signal to measure might be undetectable.
- 10 Prepare plasmids for transient transfection in water by combining the appropriate amount of each plasmid, including receptor, G α -Rluc, G β 1 and G γ 2-Venus in a 1.5 ml microcentrifuge tube (typically 5 μ g receptor, 0.5 μ g G α i/o-Rluc8, 4.5 μ g G β 1, and 5 μ g G γ 2-Venus) and add non-supplemented DMEM to a final volume of 1 ml. Vortex briefly to mix.
- 11 Vortex the thawed 1 μ g/ μ l PEI stock solution stored in the freezer.
- 12 Add 2 μ l of PEI for every 1 μ g of DNA. Vortex for 5–10 sec.
- 13 Incubate at room temperature for 15 min.

- 14 Using a P1000 pipette, slowly add the 1 ml mixture into the medium in the 10-cm plate containing cells. Gently rock the plate and return it to the incubator.
- 15 After 6 h (up to 12 h) aspirate the transfection media and replace it with 10 ml of fresh supplemented DMEM.

Day 4. Perform BRET assay—There is no procedural step on Day 3 as the cells need ~48 h of expression from the moment of transfection. The experiment exemplified here consists of a dose-response study for norepinephrine-mediated G protein activation of different Gαi/o subtypes on cells transiently expressing α_{2A} adrenergic receptor (from 100 μM to 100 pM, or from 10⁻⁴ to 10⁻¹⁰). A protocol scheme is shown in Fig. 2.

As none of the following steps require aseptic technique, they can be performed outside of the tissue culture hood.

Prepare agonist dilution series

- 16 Prepare a 10 mM stock solution of the agonist using appropriate diluent (e.g. water or DMSO).
- 17 Dilute 22.2 μl of the 10 mM stock into 978 μl of DPBS BRET buffer (final concentration of 222 μM).
- 18 Prepare 10-fold dilution series to a minimal concentration of 222 pM (a total of 7 tubes, from 222 μM to 222 pM). For example, serially transfer 100 μl to 900 μl DPBS BRET buffer to each tube, mixing well each tube.

45 μl of compound will be added to a final volume of 100 μl. Therefore, each dilution must be 2.2x higher than the final assay concentration.

- 19 Ligands will be distributed in triplicate into a U-bottom 96-well plate in the following order:

Distribute 160 μl of DPBS BRET buffer or increasing concentrations of ligands into the first two rows for 3 dose-response curves (= 3 curves x 45 μl + 25 μl [dead volume]). Add the BRET buffer as a vehicle control into first three wells of the first row (i.e. A1–A3). Add the lowest concentration 10⁻¹⁰ M ligand into the next three wells (i.e. A4–A6), the next lowest 10⁻⁹ M into the next three wells (i.e. A7–A9) until the highest concentration 10⁻⁴ M in the last three wells of the second row (i.e. B10–B12).

Repeat the distribution of vehicle and ligand following the same scheme as above with 115 μl for 2 dose-response curves (= 2 curves x 45 μl + 25 μl [dead volume]) starting from the third row (i.e. C1–C3 for vehicle, C4–C6 10⁻¹⁰ M, etc.)

This serial dilution is distributed into 4 rows (48 wells) of the compound plate with increasing concentrations ordered from left to right: row 1 and 2 will serve for 3 dose-response curves and rows 3 and 4 will serve for 2 dose-response curves.

Prepare cells

- 20 Inspect transfected cells under microscope. They should be at 80–90% confluence. Confirm a low proportion (< 20% surface area) of dead cells for each transient transfection.
- 21 Aspirate the media and add 2 ml of DPBS BRET buffer using a manual repeater pipette. With a 1- ml pipette detach and resuspend the cells off the plates.
- 22 Using a multichannel pipette, transfer 80 µl of cell suspension of each 10 cm plate into 24 wells (2 rows) of the BRET assay plate (white 96 well plate). At the end, five 10 cm plates will fill up one entire white plate (i.e. 8 rows) plus 2 rows of the second white plate.
- 23 For each 10-cm plate of one transfection combination, aliquot 80 µl into 2 wells of a fluorescence plate (black 96 well plate). Fluorescence values will be used as a readout for the expression level of the transfection.
- 24 Centrifuge the white and black plates for 4 min at 900 x g at RT in a table top centrifuge with plate adaptors. Using a multichannel pipette, aspirate the media and add fresh DPBS BRET buffer using a solution basin (45 µl per well for the white 96 well plate and 50 µl for the black plate).

Perform BRET experiment

- 25 Dilute 15 µl of 5 mM Coelenterazine H into 1485 µl DPBS BRET buffer, to a final concentration of 50 µM.

10 µl will be added to a final volume of 100 µl therefore the final assay concentration is 5 µM. Coelenterazine H is light sensitive and must be stored in a dark tube or wrapped in aluminum foil.
- 26 Read the 96-well fluorescence plate (black plate) using a plate reader (485 nm excitation 530 nm emission) to determine fluorescence level, thus proper transfection.
- 27 Using a repeating pipette inject 10 µl of 50 µM Coelenterazine H into each well of the first 96-well BRET assay plate containing cells (white 96 well plate). Alternatively, for antagonist detection mode, 1 µM antagonist (e.g. yohimbine) in minimal volume (e.g. 10 µl) can be added to each well prior to the Coelenterazine H addition.

Inject the Coelenterazine at 1 well per second since that is the amount of time it will take the plate reader to read one well.

Inject in the pattern that the plate reader will read across the plate (e.g. in a serpentine fashion across columns or rows, horizontally or vertically).
- 28 2.5 minutes after the addition of Coelenterazine H, begin to inject 45 µl of the agonist one row at a time using a multichannel pipette.

Stagger the injection into each row by the length of time it will take the plate reader to read an entire row or column (around 18 sec).

- 29 Read the plate 2, 10 and 20 min after agonist addition using a BRET plate reader (Rluc8 at 485 nm and Venus at 530 nm).
- 30 Repeat the same procedure for the second BRET assay plate.

Data analysis

- 31 Export the raw data for Rluc8 (485 nm) and Venus (530 nm) into a Microsoft Excel spreadsheet (Fig. 3).

Ensure that the luminescence values for the 485 nm filter are not saturated by optimizing the transfection of the donor-fused G α protein (see Optimization of transfection conditions).
- 32 Calculate the BRET ratio for each well by dividing the 530 nm signal by the 485 nm signal.
- 33 On a Microsoft Excel spreadsheet organize the data according to the G α subtype transfected, treatment, concentration and time.
- 34 Import the data into a XY-graph in GraphPad Prism with three replicates. Introduce the concentration in log values on the X column.
- 35 To calculate the ligand-induced effect, subtract the baseline BRET ratio from the rest of the values.

The baseline values correspond to the first three vehicle-containing wells.
- 36 Fit the data to a non-linear regression curve using the sigmoidal dose-response function and extract the EC₅₀ and E_{max}.

SUPPORT PROTOCOL. Optimization of transfection conditions

In this assay, the following cDNA amount was used for each construct on a 10-cm cell culture

plate: 5 μ g cDNA of receptor

0.5 μ g cDNA of G α i/o-Rluc8

4.5 μ g cDNA of G β 1

5 μ g cDNA of G γ 2-Venus

The PEI:cDNA ratio used in this protocol is 2:1 (w/w) (i.e. 2 μ g of PEI per every μ g of cDNA). Therefore, in this case, 30 μ g of PEI are used to transfect 15 μ g of cDNA. This ratio may be changed in order to increase the efficiency of the transfection. It should be considered, however, that PEI is cytotoxic and the increased concentration could lead to cell death and/or non-reliable results.

The amount of cDNA for each plasmid can be modified to obtain an appropriate expression level, since it depends on the identity of the constructs and the efficiency of the transient transfection. Therefore, to be able to compare the activation of different G α i/o protein subtypes (drug-induced BRET values) the expression level of the Rluc-fused alpha subunit should be similar across all G α subtypes (determined by the luminescence level). It is recommended that the transfection amount used for each Rluc-fused G α subunit be the same. However, if large differences in luminescence are observed, optimization of the transfection amount for each G protein subtype should be performed. Importantly, if the amount of cDNA coding for Rluc-fused G α i/o is too high, it could lead to saturating luminescence values. With our equipment, luminescence values vary from ~100,000 to ~2,000,000 units, with 20,000,000 units as the ceiling count.

To avoid a significant influence of spare receptors (excess amount of receptor to that required to produce a full efficacy), the minimal amount of receptor cDNA that proportionately increases the surface receptor expression as well as the $E_{\max}$ of the BRET should be determined. One way to estimate the surface receptor amount is by titration of the receptor DNA and analysis of the surface receptor expression in parallel with drug-induced BRET. Using a fluorescence activated cell sorter (FACS), antibody staining against the N-terminally tagged epitope can be performed on the remaining cells after setting aside the cells needed for a dose-response BRET curve. One example is shown in Fig. 4, where 5 μ g of α_{2A} receptor exhibits a robust dynamic range for agonist-induced BRET (A) and a reliable surface receptor expression level quantified by FACS (B).

REAGENTS AND SOLUTIONS

HEK293T culture medium

DMEM (Gibco, cat. No. 11960044) supplemented with:

10% fetal bovine serum (FBS, Atlanta Biologics, cat. No. S11150)

5 ml Antibiotic/Antimycotic 100x (Gibco, cat. No. 15240062)

5 ml L-Glutamine 200 mM (Gibco, cat. No. 25030081)

Store at 4°C.

Polyethylenimine (PEI) 1 μ g/ μ l

Heat 50 ml of ddH₂O in a beaker to 55–60°C. Dissolve 50 mg of PEI (branched, MW ~25,000; Sigma-Aldrich, cat. No. 408727) by stirring over heat, about 30 to 45 min. PEI is light sensitive so cover the beaker. Add concentrated HCl to the solution dropwise to adjust the pH to 7.2. Filter the solution using a 0.2-micron filter and aliquot into 1.5-ml centrifuge tubes. Store at –20°C up to a year.

DPBS BRET buffer

DPBS with 0.1% glucose (w/v) and 200 μ M sodium bisulfite, used to reduce catechol oxidation, pH 7.4. Prepare fresh the day of the experiment.

COMMENTARY

Background information

G protein subtype selectivity in pharmacology—Gi family proteins are crucial mediators in regulating various physiological responses (Wettschureck and Offermanns, 2005). In the context of the central nervous system, Gi-coupled catecholamine receptors, namely α_2 adrenergic receptor family and D₂-like dopamine receptors, have been studied extensively as pharmacological targets for neuropsychiatric disorders (Arnstein, 2011; Beaulieu and Gainetdinov, 2011).

As mentioned earlier, distinct expression patterns among G protein subtypes potentially allow specific ligands to target a specific tissue or cell type. This approach may be able to address alleviation of undesired effects. Unfortunately, the wealth of current literature on tissue-specific and cell type-specific expression of G protein subtypes is still limited and needs further investigation to accommodate the idea into realization. Nonetheless, differences of the relative abundance of the Gi-like subtypes in different brain areas have become apparent (Jiang et al., 2001). Besides the expression issue, some studies already suggest that there are functional differences within the Gi-like subtypes such as effector coupling (Hille, 1994; Robinson and Caron, 1997). In addition, the drug design studies particularly for D₂-like receptors have started to demonstrate Gi-like subtype functionally selective compounds (Bonifazi et al., 2017; Herenbrink et al., 2016; Hiller et al., 2013; Moller et al., 2014). In principle, given the homologous structures of the Gi-coupled catecholamine receptors, the similar opportunity of drug design awaits for the α_2 adrenergic receptors, which are targets for several neuropsychiatric disorders such as attention deficit hyperactivity disorder (Arnsten, 2011).

Another example of subtype functional selectivity can be investigated for the Gs family by exploiting the distinct brain-region expression differences between Gs and Golf. Gs and Golf have near reciprocal patterns of expression in the cortex and striatum, whereby Gs and Golf are enriched in the cortex and the striatum, respectively (Hervé, 2011). The dopamine D₁ receptor couples to both Gs and Golf and its ligands can potentially have unique biased agonism between the two Gs-like subtypes. In a separate study, we have embarked on identifying such a compound and characterized biased functions for D₁ receptor agonists (Yano *et al.*, 2017, in preparation).

BRET as a tool to study ligand-mediated G protein activation

BRET-based protein interaction assays are easy-to-use highly versatile methods. The lack of need for costly reagents (e.g., radioactive materials) should be appealing to many laboratories. As mentioned in this protocol, besides the transient transfection, the experimental part itself can be completed within several hours. Moreover, when the procedure is strictly followed, the day-to-day experimental variability is quite low. Since the readout (i.e. BRET ratio) is based on the ratiometric values of the donor and acceptor, it is not easily influenced by the fluctuation of protein expression. Therefore, the assay is amenable for a high throughput approach whereby multi-conditional assays can be achieved in parallel, including real-time kinetic follow up.

G protein activation BRET constitutes a unique GPCR functional assay that allows real-time monitoring and does not require disruption of the cells, which provides an advantage to other commonly used functional assays such as cAMP assay and GTP γ S binding assay. The readout of conformational movement (i.e., rearrangement of heterotrimeric G protein) and immediate downstream effector activity (i.e., cAMP inhibition) have been tightly correlated (Gales et al., 2005; Sánchez-Soto et al., 2016), establishing it as a reliable pharmacological assay. Because the targeted G proteins are specifically labeled with a luciferase and a GFP variant, the selective monitoring of specific G protein subtypes can be made possible unlike other assays. As it detects the ligand-induced G protein conformational change, its readout is undiluted by other mechanistic interpretation such as effector coupling efficiency or related enzymatic activity. In addition, multi-parametric comparisons among the G protein subtypes or receptor species are possible because of the highly quantitative nature (e.g., luminescence, fluorescence, FACS receptor detection to monitor expression of each component). Furthermore, with both agonist and antagonist modes of detection, G protein activation BRET is suited for in-depth quantitative characterization of GPCR ligands.

Critical parameters and troubleshooting

Negative controls—In the G protein activation BRET, transfected receptors are unmodified wild type constructs (i.e. not fused to fluorescent or luminescent probe). HEK293 cells are known to express an extensive array of GPCRs in various degrees, such as adrenergic receptors (Atwood et al., 2011), that could contribute with a significant part of the ligand-mediated G protein activation being measured if the ligand is not selective. Therefore, the transfected receptor-mediated activity has to be isolated from that of an endogenously expressed receptor. It is therefore fundamental to use a selective antagonist for the investigated receptor, which should fully counteract the signal mediated by the agonist of the transfected receptor. An additional control experiment is to analyze the ligand-mediated G protein activation in cells transfected with only the G protein subunits but not the receptor. Then the endogenous receptor-driven drug-induced BRET change can be accounted and subtracted from the receptor-transfected results. If there is a significant off-target activation of endogenously expressed receptors that interferes with the results, selective antagonists of the “non-specific” receptors may be used (Sánchez-Soto et al., 2016).

Anticipated results

For α_{2A} and α_{2C} adrenergic receptors, the basal BRET values (BRET ratio without an added ligand) are around 1.15–1.25 units for all G α i/o protein subtypes. As it is higher than non-interacting protein pairs (not shown), it is indicative of a basal interaction between the G α and the G γ subunits, consistent with the “closed” conformation of the inactive heterotrimeric G protein complex (Gales et al., 2005; Gales et al., 2006). The basal BRET values may change, although not drastically, depending on the luminescent and fluorescent levels due to the variable donor and acceptor expression as well as the parameter settings of the plate reader.

The maximal drug-induced change in BRET ratio ($E_{\max}$) may vary between ~0.015 and ~0.08 depending on the expression level as well as the coupling efficiency of receptors and G α i/o protein subtypes. Since the $E_{\max}$ differences can be intrinsic to the particular receptor-

Gαi/o combination, normalization to a reference drug is suited for data interpretation instead of the raw BRET ratio comparison across different receptor-Gαi/o combinations.

Time considerations

This protocol is generally performed over a period of four days. It consists of a cell culture phase, including cell seeding, transient transfection and expression of the coding DNAs for 48 h and an experimental phase lasting less than 6 h. If stable cell lines are available, only seeding and carrying out the assay are needed, reducing the overall time. The actual ligand-induced experiment at the last step (on day 4) can require from 30 min to hours, depending on the number of plates and the number of different compounds being used. The time it takes to read a full 96-well plate may vary for different plate readers, although decent photon integration reading should be able to keep it under ~ 3 min. The time it takes to analyze the data depends on the number of ligand samples, transfection conditions, as well as the organization levels (e.g. less than a couple of hours for 20–30 dose response curves). Ideally, a programmable reiteration method such as macro for Microsoft Excel should be integrated and saved as a template for spreadsheet and/or graphing software when the data is analyzed.

Statistical analyses

To study potency and efficacy of receptor ligands and compare their behavior on different Gαi/o protein subtypes and GPCRs, sigmoidal concentration-response curves are generated and EC₅₀ and E_{max} values are obtained. Dose-response curves may be pulled together using the Row stats function in GraphPad to obtain an average dose-response curve. EC₅₀ values obtained from each independent curve may be pulled together to obtain an average EC₅₀ ± Standard Error of Mean (S.E.M.) E_{max} values may also be obtained from each independent curve by subtracting top-bottom, and the values can be averaged. One-way ANOVA with post-hoc analysis can be performed to look for differences between EC₅₀ and/or E_{max} values of different compounds, Gαi/o protein subtypes and GPCRs.

Analysis of G protein subtype-dependent functional selectivity

As an example, the following tables summarize the results obtained from α_{2A} and α_{2C} - mediated Gi-like subtype activation by norepinephrine (NE) and the selective α₂ adrenergic receptor agonist clonidine (Sánchez- Soto *et al.*, 2017, in preparation). Here, statistics are used to highlight the differences between α_{2A} and α_{2C} for each Gi-like subtype, as denoted by uppercase symbols. Since the raw BRET ratios can be combination- dependent (i.e., variable BRET ratios by combinations of different receptor species and G protein subunits), caution is warranted with the interpretation of quantitative comparisons. Notice we do not attempt to make any comparison over more than two parameters (e.g. α_{2A}-Gαi1 NE vs. α_{2C}-Gαi2 clonidine). As seen in Table 2, E_{max} may be compared with appropriate normalization to a reference agonist.

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Significance

Using the assay protocol described herein, biased activation of different subtypes of Gi-like G proteins can be assessed quantitatively for catecholamine receptors and other G protein-coupled receptors. The assay can be used as a useful pharmacological tool to determine the potencies and efficacies of ligands to exert differential signaling bias and functional selectivity through most of the Gi-like protein subtypes.

Figure 1. G protein activation BRET assay

A. Schematic representation of G protein activation BRET assay where *Renilla* Luciferase (RLuc) is fused to the α subunit and Venus is fused to the γ subunit of the heterotrimeric G α i/o protein. BRET signal change between the two subunits is monitored upon ligand binding to the receptor. B–C. Dose-response results for α_{2A} adrenergic receptor-mediated G α i1 (B) and G α o1 (C) protein activation (Clonidine and Norepinephrine). BRET ratios with vehicle were subtracted from the BRET ratio for each agonist concentration. Data were fitted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means $\pm$ S.E.M. of at least 3 experiments performed in triplicate (Sanchez-Soto et al., in preparation).

Figure 2. Schematic flow of BRET operation

A serial dilution of ligand is prepared in a 96-well plate (dark gray). Substrate coelenterazine (light gray) is added to an assay plate with cells in the buffer (lighter gray). The ligand is transferred by a multichannel pipet into the assay plate immediately after. The assay plate is inserted to the plate reader for BRET measurement.

Figure 3. Representative view of data output in Microsoft Excel
Numbers are given in three groups according to the plate layout at the top; luminescence counts (Rluc) read at 485 nm, fluorescence counts (eYFP) read at 530 nm, and the BRET ratio between them (Ratio).

Figure 4. Optimization of the receptor transfection in the G protein activation BRET

A. HEK293T cells were transiently transfected with 0.25, 1, 5 and 10 µg of α_{2A} adrenergic receptor together with 0.5 µg G α i1-Rluc8, G β 1, and G γ 2-Venus using PEI (PEI:DNA ratio of 2:1). Norepinephrine-induced changes in BRET at 10 min were analyzed and baseline-corrected for a vehicle control. B. Surface expression of α_{2A} adrenergic receptor was detected by anti- N-terminus epitope immunostaining and subsequent FACS assay. 5 µg of α_{2A} R was deemed to be the most robust condition with fewer spare receptors among the tested (i.e. second highest BRET E_{max} and FACS detection) (Sanchez-Soto et al., in preparation).

Table 1

Potency (EC_{50} values, in nM) of NE and clonidine for α_{2A} and α_{2C} coupled to the different G α i/o subtypes. EC_{50} values were obtained from a sigmoidal concentration-response function fit by nonlinear regression analysis and are expressed as means $\pm$ S.E.M. of 2 to 11 experiments performed in triplicate. In parenthesis, EC_{50} value corresponds to a low efficacy combination (i.e. E_{max} lower than 50% shown in Table 2).

G α Subunit	Receptor	NE	Clonidine
		$EC_{50}(nM)$	
G α i1	α_{2A}	$11 \pm 2^{**}$	3 ± 1
	α_{2C}	90 ± 30	6 ± 2
G α i2	α_{2A}	$1.3 \pm 0.3^*$	2.0 ± 0.8
	α_{2C}	0.4 ± 0.2	3 ± 1
G α i3	α_{2A}	0.6 ± 0.2	1.0 ± 0.2
	α_{2C}	0.4 ± 0.2	4 ± 1
G α o1	α_{2A}	$3.0 \pm 0.5^*$	$2.0 \pm 0.4^*$
	α_{2C}	19 ± 6	(12 ± 5)
G α o2	α_{2A}	$6 \pm 1^*$	$4.0 \pm 0.2^{**}$
	α_{2C}	50 ± 10	11 ± 2

Statistical differences between α_{2A} and α_{2C} adrenergic receptors were calculated for each G α subtype by non-paired, two-tailed Student's t test;

* and **
p<0.05, respectively.

Table 2

Efficacy ($E_{\max}$ values, as percentages of NE $E_{\max}$ values) of NE and clonidine for α_{2A} and α_{2C} coupled to the different Gai/o subtypes. $E_{\max}$ values were obtained from a sigmoidal concentration-response function fit by nonlinear regression analysis and are expressed as means $\pm$ S.E.M. of 2 to 11 experiments performed in triplicate. In italics, $E_{\max}$ value lower than 50% is shown.

Ga Subunit	Receptor	NE	Clonidine
		$E_{\max}$ (% of NE)	
Gai1	α_{2A}	100 ± 5	120 ± 5
	α_{2C}	100 ± 2	$64 \pm 4^{**}$
Gai2	α_{2A}	100 ± 10	89 ± 6
	α_{2C}	100 ± 7	107 ± 4
Gai3	α_{2A}	100 ± 10	112 ± 8
	α_{2C}	100 ± 9	85 ± 5
Gao1	α_{2A}	100 ± 7	110 ± 10
	α_{2C}	100 ± 10	$41 \pm 3^{**}$
Gao2	α_{2A}	100 ± 9	120 ± 2
	α_{2C}	100 ± 8	$70 \pm 8^*$

Statistical differences between NE and clonidine for each receptor and Gai/o protein subtype were calculated for each Ga subtype by one-way ANOVA, followed by Dunnett post hoc test;

* and ** $p < 0.05$ and $p < 0.01$, respectively.