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Detecting GPCR signals with optical biosensors of Gα-GTP in cell lines and primary cell cultures

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Abstract

G protein-coupled receptors (GPCRs) are the largest class of transmembrane receptors and mediate a wide variety of physiological processes. GPCRs respond to a plethora of extracellular ligands and initiate signaling pathways inside cells via heterotrimeric G proteins (Gαβγ). Because of the critical role GPCRs play in regulating biological processes and as pharmacological targets, the availability of tools to measure their signaling activity are of high interest. Live-cell biosensors that detect the activity of G proteins in response to GPCR stimulation have emerged as a powerful approach to investigate GPCR/G protein signaling. Here, we detail methods to monitor G protein activity through direct measurement of GTP-bound Gα subunits using optical biosensors based on bioluminescence resonance energy transfer (BRET). More specifically, this protocol describes the use of two types of complementary biosensors. The first protocol explains how to use a multi-component BRET biosensor that relies on expression of exogenous G proteins in cell lines. This protocol yields robust responses that are compatible with end-point measurements of dose-dependent ligand effects or with kinetic measurements of sub-second resolution. The second protocol describes the implementation of unimolecular biosensors that detect the activation of endogenous G proteins in either cell lines expressing exogenous GPCRs, or in primary cells upon stimulation of endogenous GPCRs. Overall, using the biosensors as described in this protocol will help users characterize the mechanisms of action of many pharmacological agents and natural ligands that modulate GPCR and G protein signaling with high precision.

Basic Protocol 1: Using bi-molecular BRET biosensors to monitor Gα-GTP formation of tagged Gα in live cells.

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CONFLICT OF INTEREST STATEMENT:
The authors declare no conflicts of interest.

Keywords

G Protein Coupled Receptors; G protein; GTPase; bioluminescence energy transfer (BRET); optical biosensor

INTRODUCTION:

G protein-coupled receptors (GPCRs) are the largest class of transmembrane receptors in the human genome (~800 genes) and as such initiate a wide variety of signaling pathways (e.g. responses to most neurotransmitters or to more than half of all hormones) (Rosenbaum, Rasmussen, & Kobilka, 2009; Weis & Kobilka, 2018; Wingler & Lefkowitz, 2020; Wootten, Christopoulos, Marti-Solano, Babu, & Sexton, 2018). The biomedical relevance of GPCRs is well-established, as exemplified by the fact that they are the target for ~40% of clinically-approved drugs and for many other pharmacological agents either in clinical trials or at different stages in the drug discovery pipeline (Hauser, Attwood, Rask-Andersen, Schiöth, & Gloriam, 2017; Sriram & Insel, 2018). GPCRs signal primarily via heterotrimeric G proteins (Gαβγ), although some responses are mediated by arrestins (Weis & Kobilka, 2018; Wingler & Lefkowitz, 2020). Receptor stimulation leads to conformational changes in heterotrimeric G proteins that promote the exchange of GDP for GTP on the Gα subunit, which leads to the concomitant dissociation (or rearrangement) of Gβγ dimers. Both Gα-GTP and “free” Gβγ then activate their respective effectors to initiate a cascade of events leading to cellular responses (Gilman, 1987; Oldham & Hamm, 2008). Since GPCRs and G proteins initiate such a wide range of signaling mechanisms, measuring their activity with precision and accuracy is of high importance to gain insights into biological processes or the mechanism of action of many widely used drugs.

To monitor GPCR-mediated G protein activation, the field has traditionally relied on measuring downstream readouts such as second messengers (e.g., cAMP, intracellular Ca^{2+}) or gene reporters (Olsen & English, 2022; Salahpour et al., 2012). While these have proven useful, they are subject to amplification and crosstalk events, which can skew results. To overcome these limitations, biosensors that directly measure activation at the level of the G proteins have been developed. A widespread approach consists of measuring the dissociation of Gαβγ heterotrimers into Gα and Gβγ (Bunemann, Frank, & Lohse, 2003; Gales et al., 2006; Hollins, Kuravi, Digby, & Lambert, 2009; Janetopoulos, Jin, & Devreotes, 2001; Masuho, Martemyanov, & Lambert, 2015; Olsen et al., 2020). However, all these methodologies fail to measure the initial step of the G protein activation cycle, which is the loading of GTP on Gα, and require the expression of multiple exogenous components, including G proteins. Detecting Gα-GTP would not only be a more direct way

to measure GPCR-mediated activation, but it would also permit an adequate interrogation of other mechanism of G protein regulation, like those mediated by non-receptor regulators of G proteins, including GoLoco motif-containing guanine-nucleotide dissociation inhibitors (GDIs) or different guanine-nucleotide exchange factors (GEFs) (Garcia-Marcos, 2021; Maziarz et al., 2020; Tall, Krumins, & Gilman, 2003; Willard, Kimple, & Siderovski, 2004). Moreover, overcoming the requirement for expression of multiple exogenous components would expand the applicability of G protein biosensors to cells that are not easily transfected (i.e. primary cells) without compromising the fidelity of the readouts.

We recently described new types of BRET-based biosensors of G protein activity that permit the direct detection of Gα-GTP in cells, even in the absence of exogenous G protein and in difficult-to-transfect primary cells expressing only endogenous GPCRs (Maziarz et al., 2020). To achieve this, short peptides or protein domains that were found to specifically bind to GTP-bound Gα subunits were leveraged as detector modules into BRET biosensors. In one biosensor design, the detector modules are fused to nanoluciferase (Nluc) (Hall et al., 2012; Machleidt et al., 2015), serving as the BRET donor with YFP-tagged Gα subunit serving as the BRET acceptor. When the G protein is activated (i.e. when it is loaded with GTP), the detector module will bind to the Gα subunit, bringing the BRET donor and BRET acceptor in close proximity and thereby leading to an increase in BRET (Maziarz et al., 2020).The detector modules were also leveraged to generate a unimolecular biosensor design that does not require the expression of an exogenous G protein and can detect endogenous G protein activity without interfering with signaling from GPCRs to effectors downstream of G proteins (Maziarz et al., 2020).

Here, we describe detailed protocols on how to use these two optical biosensor designs. We start by explaining how to use the bi-molecular biosensor to detect G protein activation with sub-second resolution in a kinetic assay format (Basic Protocol 1) and how to obtain dose-dependence stimulation curves for a particular GPCR in an end-point assay format (Alternate Protocol 1). Then, we detail methodologies to use the unimolecular biosensor to detect endogenous G protein activity in cell lines expressing exogenous GPCRs (Basic Protocol 2) and in primary mouse cells expressing endogenous GPCRs (e.g. cortical neurons; Alternate Protocol 2). While these protocols focus on specific GPCRs and G proteins as examples, they can be easily extrapolated to other GPCRs and G proteins detected by biosensors of the same class.

BASIC PROTOCOL 1

Basic protocol title:

Using bi-molecular BRET biosensors to monitor Gα-GTP formation of tagged Gα in live cells.

Introductory paragraph:

This protocol describes a multi-component biosensor that can detect the formation of Gα-GTP species in a highly transfectable cell line (i.e. HEK293T). If the protocol is conducted properly, the user should be able to measure formation of Gα-GTP of different G protein

families reproducibly and with sub-second temporal resolution. The protocol is specific for Gαi3-GTP and its cognate GPCR the $\alpha 2_A$-adrenergic receptor as a prototypical example. The general principles and procedures are widely applicable to previously described Gα-GTP biosensors developed for G proteins of other families and to a wide variety of GPCRs (Maziarz et al., 2020). The biosensor design and representative data are shown in Figure 1.

Materials:

- HEK293T cells (ATCC CRL-3216)
- DMEM, high glucose (Gibco 11965–092)
- Bovine Calf Serum (FCS; HyClone SH30073.03)
- Penicillin Streptomycin L-Glutamine, 100X (PSG; Corning 30–009-Cl)
- 1X PBS (Corning 21–040-CV)
- 0.05% Trypsin (see Reagents and Solutions)
- 0.1% gelatin solution (see Reagents and Solutions)
- 2X HEPES Buffered Saline (see Reagents and Solutions)
- 2 M Calcium Chloride Dihydrate (Fisher BP510–100) in H_2O
- Sterile H_2O
- Plasmids:
  - pcDNA3.1-Gαi3-YFP (internal b/c loop) (Marivin et al., 2016)
  - pcDNA3.1-mas-KB-1753-Nluc (Maziarz et al., 2020)
  - pcDNA3-$\alpha 2_{A/D}$-AR (Oner et al., 2010)
- BRET buffer (see Reagents and Solutions)
- 20X 1 μM Brimonidine (see Reagents and Solutions)
- 20X 25 μM Yohimbine (see Reagents and Solutions)
- Nano-Glo Luciferase Assay Substrate (Promega N1110)
- Cell culture hood
- 10-cm tissue culture dishes (Thermo Scientific 130182)
- 6-well tissue culture treated plates (Corning 3516)
- Hemocytometer (Hausser Scientific 3520)
- 5 ml polystyrene tubes (Fisherbrand FB149563A)
- Cell scraper (Thermo Scientific 179693)
- POLARstar Omega (BMG LABTECH) with dual emission detection and 2 injectors

  - Other plate readers with similar capacities should also serve this purpose

- White 96-well assay plates (PerkinElmer 6005290)

Protocol steps with *step annotations*:

Culturing HEK293T cells:

1. Prepare complete culture media for HEK293T cells by making DMEM, 10% FCS, 1X PSG media (e.g., add 50 ml FCS and 5.5 ml of 100X PSG to 500 ml bottle of DMEM)

2. Wash the stock 10-cm plate with 5 ml of PBS

   Add PBS slowly against the wall of the dish to avoid detaching cells

3. Add 1 ml of 0.05% trypsin, and incubate at 37 °C for 1 min or until cells are completely detached.

4. Resuspend detached cells in DMEM, 10% FCS, 1X PSG medium

   For a 2-day split, resuspend 1 ml of trypsinized cells in 6 mls of fresh medium (1:7 split); for a 3-day split, resuspend 1 ml of trypsinized cells in 13 mls of fresh medium (1:14 split)

5. Add 1 ml of diluted cells on a 10-cm plate containing 9 ml of fresh media

   Never let cells reach over 95% confluency. The rate of cell growth and adequate splitting ratios may differ depending on specific experimental conditions and may require optimization.

Seeding HEK293T for experiment:

6. Add 1 ml 0.1% gelatin to each well of a 6-well plate and let it sit for 5 minutes

   Spread the gelatin by tilting plate up/down and left/right so that the entire surface of the well is covered

7. Aspirate gelatin and leave the plates without lid to air-dry

8. Trypsinize cells as described above (see steps 2–4) and dilute in complete medium

9. Count cells using hemocytometer and seed 350,000 cells in a final volume of 2 ml for on each well

10. Rock the plate to spread cells evenly and place it back in incubator until the following morning

Calcium Phosphate Transfection: Other methods of transfection (e.g. PEI or lipofectamine) can also be used, but will not be detailed here

11. Warm up solutions needed in 37 °C water bath: culture media, 2X HBS, 2 M $CaCl_2$, and sterile H_2O

12. Change media of cells in 6-well plate by removing the 2 ml of media and adding 1.5 ml of fresh medium

13. Add 122 µl 2X HBS to a 5 ml polystyrene tube

14. Add 105.6 µl H_2O and 14.4 µl of 2 M $CaCl_2$ to another polystyrene tube

15. Add the following amounts of plasmid DNA: 200 ng for pcDNA3-$\alpha 2_{A/D}$-AR; 500 ng for pcDNA3.1-Gαi3-YFP (internal b/c loop); and 25 ng pcDNA3.1-mas-KB-1753-Nluc to the tube with H_2O/$CaCl_2$ mix

    These amounts may need to be empirically optimized to account for transfection variability across users, but a ratio of 1:10 or 1:20 for BRET donor : acceptor is a good starting reference.

16. Briefly vortex tube with DNA and wait 5 minutes

17. Add dropwise the entire volume in the tube with DNA (i.e. the tube prepared in steps 14–15) to the tube with 2X HBS (i.e. the tube prepared in step 13) while vortexing for 10 seconds

    Performing this step adequately is critical for proper precipitate formation and subsequent transfection efficiency.

18. Incubate tubes at room temperature for 25 minutes

19. Add volume of the transfection mixture (see step 17) dropwise to cells and mix by rocking the plate

20. Replace media with fresh 2 ml after ~6 hours of incubation at 37 °C in a CO_2 incubator

21. Use cells for experiments the following day

Luminescence measurements:

22. Take the 6-well plate out of the incubator, aspirate cell media, and carefully add 2 ml of PBS without disturbing the cell monolayer

    Add PBS slowly and against the wall of the well to avoid detaching cells

23. Aspirate PBS and add 1 ml of PBS

24. Use cell scraper to detach cells and collect the resuspended cells using a p1000 pipet

25. Transfer cells to a 1.5 ml microcentrifuge tube and spin at 550 × g for 5 minutes

26. Aspirate supernatant and gently resuspend cells in ~900 µl BRET Buffer

    Keep cells in the dark once they are resuspended in BRET buffer to avoid photobleaching of the fluorescent component of the biosensor.

27. Prepare a 20X solution of Brimonidine and a 20X solution of Yohimbine in BRET buffer to achieve a final concentration of 1 µM and 25 µM, respectively, in the assay. Prime injection pumps in plate reader with these solutions

28. Add 50 µl BRET buffer to one well of a 96-well assay plate

29. Add 50 µl of cells to same well

    Gently resuspend cells before adding them as cells may settle at bottom of the tube over time.

30. Add 0.5 µl Nano-Glo to the well containing cells and gently mix by pipetting up and down twice

31. Place plate in plate-reader equilibrated at 28 °C and wait for 2 min before starting measurement

32. Set integration time at 0.24 s

    Integration times can be adjusted based on the system/biosensor expression you work with. Higher integration times will allow for higher counts at the expense of time resolution.

33. Measure simultaneously emission centered at 450 nm with a bandpass of 80 nm (nanoluciferase luminescence) and emission centered at 535 nm with a bandpass of 30 nm (YFP emission)

34. Inject 5 µl of the brimonidine solution 30 s after starting the measurement and 5 µl of the yohimbine solution at 90 s

35. Stop measurements at 240 s

    The kinetics of activation and deactivation can proceed at different rates depending on the GPCR and G protein under investigation, so the times of injection, duration of the measurement, and the frequency of time points need to be adjusted accordingly.

BRET data processing:

36. Export raw luminescence values from both the 535 nm channel (BRET acceptor) and the 450 nm channel (BRET donor)

37. Divide raw luminescence counts in the acceptor channel by the raw luminescence counts in the donor channel for each time point to obtain a BRET ratio value per point.

38. Average the BRET ratio values for the 30 s preceding injection of the agonist to determine the basal BRET signal.

39. Subtract this average from all data points to get a difference in BRET relative to baseline [ΔBRET (baseline)].

ALTERNATE PROTOCOL 1

Alternate protocol title:

Measurement of GPCR dose-dependent Gα-GTP responses in end-point format.

Introductory paragraph:

The following protocol provides details on how to obtain a dose-dependent stimulation curve for the $\alpha 2_A$-AR by measuring formation of Gαi3-GTP. Instead of acquiring kinetic information as detailed in Basic Protocol 1, this assay processes multiple conditions in parallel using endpoint measurements. This protocol can easily be scaled up to investigate several GPCRs and dose dependent-responses to different agonists simultaneously. Dose-dependent curves can then be used to extract pharmacological parameters of the GPCR responses such as efficacy and EC_{50} (Figure 2).

Additional Materials:

Reagents:

- Coelenterazine 400a (CTZ400a; GoldBio C-320)

Hardware

- Round-bottom clear 96-well plate

Protocol steps with *step annotations*:

Culturing HEK293T cells:

1. Prepare complete culture media for HEK293T cells by making DMEM, 10% FCS, 1X PSG media (e.g., add 50 ml FCS and 5.5 ml of 100X PSG to 500 ml bottle of DMEM)
2. Wash the stock 10-cm plate with 5 ml of PBS

   Add PBS slowly against the wall of the dish to avoid detaching cells
3. Add 1 ml of 0.05% trypsin, and incubate at 37 °C for 1 min or until cells are completely detached.
4. Resuspend detached cells in DMEM, 10% FCS, 1X PSG medium

   For a 2-day split, resuspend 1 ml of trypsinized cells in 6 mls of fresh medium (1:7 split); for a 3-day split, resuspend 1 ml of trypsinized cells in 13 mls of fresh medium (1:14 split)
5. Add 1 ml of diluted cells on a 10-cm plate containing 9 ml of fresh media

   Never let cells reach over 95% confluency. The rate of cell growth and adequate splitting ratios may differ depending on specific experimental conditions and may require optimization.

Seeding HEK293T for experiment:

6. Add 1 ml 0.1% gelatin to each well of a 6-well plate and let it sit for 5 minutes

   Spread the gelatin by tilting plate up/down and left/right so that the entire surface of the well is covered
7. Aspirate gelatin and leave the plates without lid to air-dry

8. Trypsinize cells as described above (see steps 2–4) and dilute in complete medium
9. Count cells using hemocytometer and seed 350,000 cells in a final volume of 2 ml for on each well
10. Rock the plate to spread cells evenly and place it back in incubator until the following morning

Calcium Phosphate Transfection: Other methods of transfection (e.g. PEI or lipofectamine) can also be used, but will not be detailed here

11. Warm up solutions needed in 37 °C water bath: culture media, 2X HBS, 2 M $CaCl_2$, and sterile H_2O
12. Change media of cells in 6-well plate by removing the 2 ml of media and adding 1.5 ml of fresh medium
13. Add 122 µl 2X HBS to a 5 ml polystyrene tube
14. Add 105.6 µl H_2O and 14.4 µl of 2 M $CaCl_2$ to another polystyrene tube
15. Add the following amounts of plasmid DNA: 200 ng for pcDNA3-α$2_{A/D}$-AR; 500 ng for pcDNA3.1-Gαi3-YFP (internal b/c loop); and 25 ng pcDNA3.1-mas-KB-1753-Nluc to the tube with H_2O/$CaCl_2$ mix

    These amounts may need to be empirically optimized to account for transfection variability across users, but a ratio of 1:10 or 1:20 for BRET donor : acceptor is a good starting reference.
16. Briefly vortex tube with DNA and wait 5 minutes
17. Add dropwise the entire volume in the tube with DNA (i.e. the tube prepared in steps 14–15) to the tube with 2X HBS (i.e. the tube prepared in step 13) while vortexing for 10 seconds

    Performing this step adequately is critical for proper precipitate formation and subsequent transfection efficiency.
18. Incubate tubes at room temperature for 25 minutes
19. Add volume of the transfection mixture (see step 17) dropwise to cells and mix by rocking the plate
20. Replace media with fresh 2 ml after ~6 hours of incubation at 37 °C in a CO_2 incubator
21. Use cells for experiments the following day

Luminescence measurements:

22. Take the 6-well plate out of the incubator, aspirate cell media, and carefully add 2 ml of PBS without disturbing the cell monolayer

    Add PBS slowly and against the wall of the well to avoid detaching cells

23. Aspirate PBS and add 1 ml of PBS

24. Use cell scraper to detach cells and collect the resuspended cells in a 1.5 ml microcentrifuge tube using a p1000 pipet

25. Spin cells for 5 min at 550 × g and resuspend cells in 400 µl BRET buffer, then keep cells in dark until use.

26. Prepare brimonidine dilution by making a 5X 10 µM brimonidine in BRET in well #12 (right-most well) of a round-bottom 96-well plate

    The maximal dose used for dose dependence curves should be supra-maximal to reach saturation of GPCR/G protein activation (this will be shown as a plateau of max activation in the results).

27. Dilute brimonidine stepwise in ½ logs by serial 3.16X dilutions in BRET buffer

    This can be done by transferring 100 µl of the higher dose into 216 µl BRET buffer in the next well. Thorough mixing should be done for each dilution

28. Leave well #1 (left-most well) only with BRET buffer

    At this point you should have 12 different dilutions prepared in a row of a 96-well plate going from BRET buffer only (well #1) to a max dose of 10 µM brimonidine (well #12)

29. Transfer 20 µl of agonist dilutions to a row of a 96-well assay plate

30. Add 35 µl BRET buffer to each well

31. Prepare CTZ400a master mix by mixing 1 µl of CTZ400a with 22.5 µl BRET buffer for each well

    For example, for 12 wells, one could prepare a master mix for 15 wells (i.e. 336 µl BRET buffer + 15 µl CTZ400a)

32. Add 22.5 µl CTZ400a master mix going from well #12 to well #1 on your assay plate (i.e. high dose to low dose)

    Be careful not to touch pipet tip to the agonist dilution

33. Briefly mix cells and add 22.5 µl of cells again going from well #12 to well #1 on your assay plate

34. Start endpoint measurement on plate reader measuring each minute for a total of 5 minutes.

    Doing this protocol will give you 6 different time points (min 0, 1, 2, 3, 4 and 5) which covers the range of times at which most GPCR and G proteins will reach a maximum response.

BRET data processing:

35. Export raw values from both the 535 nm channel (YFP) and the 450 nm channel (Nluc)

36. Divide YFP values over Nluc to get a raw BRET ratio for each measurement

Having multiple time points in the protocol as suggested above can help in finding optimal time for GPCR activation (i.e. different GPCRs will reach peak activation at somewhat different time points).

37. Subtract the BRET ratio value corresponding to the condition with no agonist (i.e. well #1) from all condition to get a difference in BRET relative to no agonist [ΔBRET (no agonist)].

38. Use software to plot a three-parameter sigmoidal curve, assuming a Hill slope of 1

One software option is GraphPad Prism, but other software may be used.

39. Extract parameters from this non-linear fit such as EC_{50} (i.e. dose of agonist required for ½ max activation).

This protocol can easily be adjusted to measure multiple GPCRs at the same time, by using multiple rows of the 96-well plate. Higher throughput can also be achieved by using multi-channel pipets to dispense all reagents.

BASIC PROTOCOL 2:

Basic protocol title:

Using unimolecular BRET biosensors to study endogenous G protein activity.

Introductory paragraph:

This protocol details the use of a unimolecular biosensor, named BRET sensor with ER/K linker and YFP (BERKY), that permits detection of endogenous Gα-GTP (Maziarz et al., 2020). If properly followed, this protocol will allow the user to measure endogenous formation of Gα-GTP in cell lines with sub-second temporal resolution. Although different versions of BERKY sensors exist for detection of various G protein families, this protocol will explain the use of the BERKY sensor for Gαi-GTP, with overexpression of the $\alpha 2_A$-adrenergic receptor as a prototypical example. The principles and guidelines described here are applicable to other BERKY sensors and other GPCRs (Maziarz et al., 2020). These biosensors allow for direct measurement of G protein activity (i.e. formation of Gα-GTP) with high fidelity (i.e. no G protein overexpression required). This allows for a better representation of signaling mechanisms without introducing exogenous components that significantly distort the behavior of the system under study. Detecting endogenous responses can however be more challenging as the magnitude of responses is bound to be smaller compared to systems with G protein overexpression. The biosensor design and representative data are shown in Figure 3.

Materials:

Except the plasmids, the materials are the same as Basic Protocol 1.

- Plasmids:

- pcDNA3.1-Gαi*-BERKY3 (Maziarz et al., 2020)
- pcDNA3-$\alpha 2_{A/D}$-AR (Oner et al., 2010)

Protocol steps with *step annotations*:

Culturing HEK293T cells:

1. Prepare complete culture media for HEK293T cells by making DMEM, 10% FCS, 1X PSG media (e.g., add 50 ml FCS and 5.5 ml of 100X PSG to 500 ml bottle of DMEM)
2. Wash the stock 10-cm plate with 5 ml of PBS

   Add PBS slowly against the wall of the dish to avoid detaching cells
3. Add 1 ml of 0.05% trypsin, and incubate at 37 °C for 1 min or until cells are completely detached.
4. Resuspend detached cells in DMEM, 10% FCS, 1X PSG medium

   For a 2-day split, resuspend 1 ml of trypsinized cells in 6 mls of fresh medium (1:7 split); for a 3-day split, resuspend 1 ml of trypsinized cells in 13 mls of fresh medium (1:14 split)
5. Add 1 ml of diluted cells on a 10-cm plate containing 9 ml of fresh media

   Never let cells reach over 95% confluency. The rate of cell growth and adequate splitting ratios may differ depending on specific experimental conditions and may require optimization.

Seeding HEK293T for experiment:

6. Add 1 ml 0.1% gelatin to each well of a 6-well plate and let it sit for 5 minutes

   Spread the gelatin by tilting plate up/down and left/right so that the entire surface of the well is covered
7. Aspirate gelatin and leave the plates without lid to air-dry
8. Trypsinize cells as described above (see steps 2–4) and dilute in complete medium
9. Count cells using hemocytometer and seed 350,000 cells in a final volume of 2 ml for on each well
10. Rock the plate to spread cells evenly and place it back in incubator until the following morning

Calcium Phosphate Transfection: Other methods of transfection (e.g. PEI or lipofectamine) can also be used, but will not be detailed here

11. Warm up solutions needed in 37 °C water bath: culture media, 2X HBS, 2 M $CaCl_2$, and sterile H_2O

12. Change media of cells in 6-well plate by removing the 2 ml of media and adding 1.5 ml of fresh medium

13. Add 122 µl 2X HBS to a 5 ml polystyrene tube

14. Add 105.6 µl H_2O and 14.4 µl of 2 M $CaCl_2$ to another polystyrene tube

15. Add the following amounts of plasmid DNA: 50 ng for pcDNA3.1-Gαi*-BERKY3 and 200 ng pcDNA3-$\alpha 2_{A/D}$-AR to the tube with H_2O/$CaCl_2$ mix.

    The amount of biosensor may need to be adjusted based on lab-to-lab variability, but these amounts can be used as a starting reference. We recommend doing a titration of the transfected biosensor amount (e.g. 10 ng, 50 ng, 200 ng, 500 ng) and testing it side-by-side

16. Briefly vortex tube with DNA and wait 5 minutes

17. Add dropwise the entire volume in the tube with DNA (i.e. the tube prepared in steps 14–15) to the tube with 2X HBS (i.e. the tube prepared in step 13) while vortexing for 10 seconds

    Performing this step adequately is critical for proper precipitate formation and subsequent transfection efficiency.

18. Incubate tubes at room temperature for 25 minutes

19. Add volume of the transfection mixture (see step 17) dropwise to cells and mix by rocking the plate

20. Replace media with fresh 2 ml after ~6 hours of incubation at 37 °C in a CO_2 incubator

21. Use cells for experiments the following day

Luminescence measurements:

22. Take the 6-well plate out of the incubator, aspirate cell media, and carefully add 2 ml of PBS without disturbing the cell monolayer

    Add PBS slowly and against the wall of the well to avoid detaching cells

23. Aspirate PBS and add 1 ml of PBS

24. Use cell scraper to detach cells and collect the resuspended cells using a p1000 pipet

25. Transfer cells to a 1.5 ml microcentrifuge tube and spin at 550 × g for 5 minutes

26. Aspirate supernatant and gently resuspend cells in ~900 µl BRET Buffer

    Keep cells in the dark once they are resuspended in BRET buffer to avoid photobleaching of the fluorescent component of the biosensor.

27. Prepare a 20X solution of Brimonidine and a 20X solution of Yohimbine in BRET buffer to achieve a final concentration of 1 µM and 25 µM, respectively, in the assay. Prime injection pumps in plate reader with these solutions

28. Add 50 µl BRET buffer to one well of a 96-well assay plate

29. Add 50 µl of cells to same well

    Gently resuspend cells before adding them as cells may settle at bottom of the tube over time.

30. Add 0.5 µl Nano-Glo to the well containing cells and gently mix by pipetting up and down twice

31. Place plate in plate-reader equilibrated at 28 °C and wait for 2 min before starting measurement

32. Set integration time at 0.24 s

    Integration times can be adjusted based on the system/biosensor expression you work with. Higher integration times will allow for higher counts at the expense of time resolution.

33. Measure simultaneously emission centered at 450 nm with a bandpass of 80 nm (nanoluciferase luminescence) and emission centered at 535 nm with a bandpass of 30 nm (YFP emission)

34. Inject 5 µl of the brimonidine solution 30 s after starting the measurement and 5 µl of the yohimbine solution at 90 s

35. Stop measurements at 240 s

    The kinetics of activation and deactivation can proceed at different rates depending on the GPCR and G protein under investigation, so the times of injection, duration of the measurement, and the frequency of time points need to be adjusted accordingly.

BRET data processing:

36. Export raw luminescence values from both the 535 nm channel (BRET acceptor) and the 450 nm channel (BRET donor)

37. Divide raw luminescence counts in the acceptor channel by the raw luminescence counts in the donor channel for each time point to obtain a BRET ratio value per point.

38. Average the BRET ratio values for the 30 s preceding injection of the agonist to determine the basal BRET signal.

39. Subtract this average from all data points to get a difference in BRET relative to baseline [ΔBRET (baseline)].

ALTERNATE PROTOCOL 2

Alternate protocol title:

Using unimolecular BRET biosensors to study endogenous G protein activity in mouse cortical neurons.

Introductory paragraph:

This protocol provides a description on how to use BERKY sensors to detect endogenous G protein activity upon stimulation of GPCRs endogenously expressed in mouse cortical neurons. We will detail each step from production of lentiviral particles, to isolation, culture and transduction of neurons, to luminescence measurements to extract BRET data. This protocol will focus on the detection of endogenous Gαi-GTP after stimulation of endogenous α2-adrenergic receptors. If followed properly, users should be able to detect responses as shown in Figure 3. It is also important to note that this system can be applied for other BERKY sensors and for stimulation of other GPCRs in primary cells suitable for cell culture and lentiviral transduction (Maziarz et al., 2020).

[Additional] Materials:

Reagents for Lenti-X 293T cell culture are the same as for Basic Protocol 1. The reagents below are the necessary material for lentiviral packaging and infection of mouse cortical neurons.

- Lenti-X 293T cells (Takara Bio 632180)
- Plamids:
  - pLVX-hSynapsin-Gαi*-BERKY3 (Maziarz et al., 2020)
  - psPAX2 (Addgene #12260)
  - pMD2.G (Addgene #12259)
- Polyethylenimine (PEI; Polysciences Inc. 23966–1)
- Poly-L-lysine hydrobromide (Millipore Sigma P9155)
- Hank’s Balanced Salt Solution (HBSS; Corning 21–022-CV)
- DMEM, high glucose (Gibco 11965–092)
- Fetal Bovine Serum (FBS; Gibco 2614–079)
- Pennycillin/Streptomycin, 100X (Gibco 15140122)
- 2.5% Trypsin, 10X (Gibco, 15090–046)
- Neurobasal media (Gibco 21103049)
- B-27 supplement (Gibco 17504001)
- Glutamax-I, 100X (Gibco 35050061)
- AraC (Tocris Bioscience 4520/50)
  - Store as 10 mM stock in −20°C
- 10-cm tissue culture dishes (Thermo Scientific 130182)
- 150 mm diameter dish (Thermo Scientific 130183)
- 15 ml conical (Falcon 352099)
- Tabletop Centrifuge (Sorvall Legend XTR or other with same capacities)

- 0.45 μm PES filter (Fisherbrand 22363547)
- Ultracentrifuge 1.5 ml tubes (Beckman Coulter 357448)
- Centrifuge (Sorvall RC6+ or other with same capacities)
  - Rotor: F12–6×500
- −80 °C freezer
- 5 mm glass coverslip (World Precision Instruments 502040)
- 96-well plate (Thermo Scientific 130188)
- Surgical tools for mouse cortical neuron isolation (e.g. two 45° angled Dumont forceps and one pair of scissors)
- Stereomicroscope (Fisherbrand 03–000-038)

Protocol steps with *step annotations*:

Lentivirus packaging: This protocol describes lentiviral packaging of 1 plasmid. Adjust protocol for multiple constructs.

1. Culture Lenti-X 293T cells in same conditions as HEK 293T cells (see protocol 1 step 1) and expand culture to get four confluent 10-cm dishes
2. Seed ~2.5 million cells per dish in 4 gelatin coated 150 mm diameter dishes (see Basic Protocol 1 steps 1–10)
3. Add 108 μg of pLVX-Gαi*-BERKY3 DNA (27 μg DNA/dish) along with packaging plasmids: 72 μg psPAX2 DNA (18 μg DNA/dish) and 45 μg pMD2.G DNA (11.25 μg DNA/dish) to a 15 ml conical containing 6 ml DMEM
4. In a second 15 ml conical containing 6 ml DMEM, add PEI at a 2:1 PEI:DNA ratio (w/w)

   In this case, the total amount of DNA is 225 μg, so 450 μg of PEI should be added to the 15 ml conical for a 2:1 ratio
5. Add the PEI solution (prepared in step 4) to the DNA solution (prepared in step 3) using a serological pipet and vortex mixture for 10 seconds
6. Incubate at room temperature for 15 min then add 3 ml of the PEI/DNA mix in each of the 150 mm plates

   This protocol was adapted from (Longo, Kavran, Kim, & Leahy, 2013)
7. Remove media ~16 hours after transfection and add 30 ml of fresh media to each plate.
8. Twenty-four hours later, collect lentivirus-containing media with serological pipet and store at 4 °C. Add 32 ml of fresh media to each plate and collect media again 24 h later. Pool the first and second batch of media harvested.

   At this point you should have ~250–300 ml of lentivirus-containing media coming from the 4 dishes

9. Centrifuge all media for 5 min at 900 × g and filter through 0.45 μm sterile PES filter

10. Centrifuge the filtered media for ~18 hours at 17,200 × g and 4°C

    Weight out bottles of media to have all of them precisely balanced during this centrifugation step

11. Discard the supernatant and gently resuspend pellet in 1 ml of PBS

    Whenever resuspending viral pellet, do so gently. This can take some time of pipetting on the walls around the pellet before it gets into solution.

12. Transfer the resuspended virus suspension into an ultracentrifuge 1.5 ml tube and centrifuge for 1 hour at 50,000 × g and 4°C

13. Gently aspirate the supernatant and resuspend the pellet in 1 ml PBS

    Resuspend pellet gently as in step 8.

14. Centrifuge again for 1 hour at 50,000 × g and 4 °C

15. Gently aspirate supernatant and resuspend the pellet in ~400 μl PBS

16. Make single-use aliquots of 10 μl and store at −80 °C

    A typical aliquots volume is 10 μl. Avoid storing aliquots near the exterior of the freezer, as the small volume makes them particularly susceptible to thawing. After thawing, aliquots can be kept no longer than 1 week at 4 °C.

Preparation of 96-well plates for neuron culture:

17. Place 5 mm glass coverslip in wells making sure to leave at least 2 outer rows/columns empty for step 18

18. Sterilize coverslips by UV-radiation in the tissue culture hood for 5–10 min

19. Coat coverslips by adding 90 μl per well of 0.1 mg/ml poly-L-lysine hydrobromide and incubate overnight at room temperature

20. On the day of neuron isolation, wash wells twice with 150 μl of HBSS and add 100 μl DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/μl streptomycin and 2mM L-glutamine (complete isolation DMEM medium)

21. Add 100 μl sterile H_2O to the two outer rows and columns to avoid any evaporation of media the outermost wells containing cells.

22. Place plate in cell incubator set at 37 °C, 5% CO_2

Mouse cortical neuron culture and infection: Cortical neuron isolations are done as previously described (Kaech & Banker, 2006) with minor modifications.

23. Euthanize newborn mouse pups (P0) by decapitation

    Please ensure that proper animal handling as defined at your institution is followed

24. Remove brain from skull and place in cold HBSS

25. Use forceps to separate the cerebrum from other brain regions and remove meninges

Using a stereomicroscope is useful to precisely dissect out cerebrum and enrich for cortical neurons

26. Mince tissue into 1–2 mm pieces using sterile razor blade and digest in 0.25% Trypsin in HBSS for 5 min at 37°C while gently shaking

Prepare Trypsin by diluting 1 ml 2.5% Trypsin with 9 ml HBSS

27. Let tissue settle by gravity at bottom of tube for 2 min, carefully aspirate the supernatant and add 10 ml of fresh HBSS

Repeat this step for a total of 3 washes with HBSS to completely remove Trypsin

28. Resuspend tissue in 3 ml complete isolation DMEM medium for 4–5 cortices. To mechanically disrupt tissue, use 10 ml serological pipet first, then use p1000 to gently break down tissue.

29. Pass the tissue suspension through 45 μm strainer to remove large tissue fragments, and wash strainer with additional complete isolation DMEM medium.

Final volume should not be more than ~2 ml per cortex

30. Count cells and plate 75,000 cells per well (100 μl) on poly-L-lysine coated coverslips in 96-well plates. This is day 1 in vitro (DIV1).

You should now have cells in 200 μl complete isolation DMEM medium in each well.

31. *Incubate cells at* 37 °C, 5% CO_2

32. Replace one half of the media (100 μl) ~5 hrs after seeding with complete neural media (neurobasal media, B-27 supplement, 1X Glutamax-I) supplemented with 100 U/ml penicillin and 100 mg/μl streptomycin.

33. After 2 days, change half of the media by removing 100 μl in each well and adding 100 μl of complete neural media supplemented with 5 μM AraC (DIV3)

Continue replacing half of the media in the well with complete neural media every 2 days.

34. On DIV7, thaw an aliquot of frozen pLVX-hSynapsin-Gαi*-BERKY3 virus stock and dilute it 1:100 in complete neural medium. Transduce neurons by replacing half of media (100 μl) with the virus solution to achieve a final dilution of 1:200.

The dilution of lentivirus is determined empirically based on the typical transduction efficiencies obtained by following the packaging protocol described above. Titers will vary across laboratories, users, and lentiviral constructs, so the titers may need to be optimized on a case-by-case basis.,

35. After 1 hour incubation in the cell incubator, change half of the media with complete neural media

    Continue replacing half of the media in the well with complete neural media every 2 days.

Luminescence measurements:

36. Use neurons on DIV 12–16 for luminescence measurements.

    The optimal time for measurement will have to be determined empirically by users to achieve adequate expression of the biosensor and neuron differentiation.

37. Prepare a 20X solution of Brimonidine (α2-adrenergic agonist) and a 20X solution of Yohimbine (α2-adrenergic antagonist) in BRET buffer to achieve a final concentration of 5 μM and 25 μM, respectively, in the assay. Prime injection pumps in plate reader with these solutions

38. Aspirate complete neural media from 1 well and wash with 200 μl of BRET buffer

39. In a white opaque 96-well assay plate, add 100 μl BRET buffer and mix in 0.5 μl Nano-Glo

40. Use fine tweezers to transfer the coverslip from culture plate to the assay plate

41. Place the white opaque plate in plate-reader equilibrated at 28 °C and wait 2 min before starting measurement. Return the culture plate to the incubator until the following measurement.

42. Set integration time at 0.96 s

    Longer integration times are required in cortical neurons to obtain more luminescence signal and reduce noise in the measurements at the expense of time resolution.

43. Measure simultaneously emission centered at 450 nm with bandpass of 80 nm (nanoluciferase luminescence) and emission centered at 535 nm with a bandpass of 30 nm (YFP emission)

44. Inject 5 μl of the brimonidine solution 30 s after starting the measurement and 5 μl of the yohimbine solution at 90 s

45. Stop measurements at 240 s

    The kinetics of activation and deactivation can proceed at different rates depending on the GPCR and G protein under investigation, so the times of injection, duration of the measurement, and the frequency of time points need to be adjusted accordingly.

BRET data processing:

46. Export raw luminescence values from both the 535 nm channel (BRET acceptor) and the 450 nm channel (BRET donor)

47. Divide raw luminescence counts in the acceptor channel by the raw luminescence counts in the donor channel for each time point to obtain a BRET ratio value per point.

48. Average the BRET ratio values for the 30 s preceding injection of the agonist to determine basal BRET signal.

49. Subtract this average from all data points to get a difference in BRET relative to baseline [ΔBRET (baseline)].

REAGENTS AND SOLUTIONS:

BRET Buffer

- Dissolve chemicals in H_2O
- 140 mM NaCl (for 1 L, add 8.18 g)
- 5 mM KCl (for 1 L, add 0.373 g)
- 1 mM $MgCl_2$ (for 1 L, add 0.203 g)
- 1 mM $CaCl_2$ (for 1 L, add 0.147 g)
- 0.37 mM NaH_2PO_4 (for 1 L, add 4.44 g)
- 20 mM HEPES (for 1 L, add 4.77 g)
- 0.1% (w/v) glucose (for 1 L, add 1 g)
- Adjust pH to 7.4
- Filter sterilize by passing through 0.45 μm filer
- Store up to 12 months at −20 °C

2X HEPES Buffered Saline

- Dissolve chemicals in H_2O
- 280 mM NaCl (for 500 ml, add 8.18 g)
- 50 mM HEPES (for 500 ml, add 5.96 g)
- 1.5 mM Na_2HPO_4 (for 500 ml, add 201.05 mg)
- Adjust pH to 7.08 by adding some basic solution

  The pH will be below 7.08 when starting, add NaOH solution slowly as is it critical not to surpass a pH of 7.08.
- Store up to 8 months at −20 °C

0.05% Trypsin

- Dilute 0.25% Trypsin, 2.21 mM EDTA (Corning 25–053-Cl) to a concentration of 0.05% Trypsin in HBSS and store in aliquots at −20 °C.

For example, replace 100 ml of HBSS from a 500 ml bottle with 100 ml of 0.25% Trypsin, 2.21 mM EDTA (5-fold dilution)

0.1% Gelatin Solution

- Dilute 2% Gelatin, Type B (Sigma-Aldrich G1393) 20-fold in sterile H_2O to make 0.1% gelatin.

20X 1 μM Brimonidine

- From the stock brimonidine powder (Ark Pharm H-009668), make 50 mM aliquots dissolved in DMSO and store at −20 °C
- To make 20X 1 μM Brimonidine, dilute an aliquot 1:2500 the day of the assay
- Make agonist concentration fresh for each experiment

20X 25 μM Yohimbine

- From the stock yohimbine powder (Alfa Aesar J60185), make 100 mM aliquots dissolved in DMSO and store at −20 °C
- To make 20X 25 μM yohimbine, dilute an aliquot 1:200 the day of the assay
- Make agonist concentration fresh for each experiment

COMMENTARY:

Background Information:

Optical biosensors have long been used to study signaling pathways in cells. Sensors that rely on fluorescence or bioluminescence resonance energy transfer (FRET or BRET, respectively) can be leveraged to study GPCR/G protein activity with high precision and sensitivity (Lohse, Nuber, & Hoffmann, 2012; Olsen & English, 2022). These biosensor systems rely on the association or dissociation between one protein fused to a RET donor, and another protein fused to an RET acceptor (Salahpour et al., 2012; P. Wu & Brand, 1994; Y. Wu & Jiang, 2022), such that association facilitates the RET event. When the two proteins interact and bring the donor and acceptor in close proximity (<10 nm) there is an energy transfer from the donor to the acceptor, so light emission by the acceptor increases while light emission by the donor decreases (Salahpour et al., 2012; P. Wu & Brand, 1994; Y. Wu & Jiang, 2022). When designing biosensors, many factors should be considered, like relative spatial orientation and spectral overlap between donor and acceptor. In the field of GPCR signaling, many different types of FRET and BRET sensors have been designed, with acceptor/donor pairs located on GPCRs, G protein subunits, or specific interacting proteins (Bunemann et al., 2003; Gales et al., 2006; Hollins et al., 2009; Janetopoulos et al., 2001; Lohse et al., 2012; Masuho et al., 2015; Maziarz et al., 2020; Olsen et al., 2020; Olsen & English, 2022; Salahpour et al., 2012; P. Wu & Brand, 1994; Y. Wu & Jiang, 2022).

The above protocols detail how to use two different types of biosensors that monitor the initial step in G protein activation (i.e. formation of Gα-GTP). The bi-molecular sensor (Basic Protocol 1 and Alternate Protocol 1) requires the expression of exogenous

G proteins, which can affect the fidelity of the readout but allows for robust and highly reproducible detection of GPCR responses in cell lines. Although this type of biosensor is not suitable for systems that are not easy to transfect because of the need to introduce multiple genetic components at once, it can be easily used to study many different GPCR/G protein combinations in a kinetic measurement format (Basic Protocol 1; Fig. 1) or in an endpoint format to scale up for higher throughput (Alternate Protocol 1; Fig. 2). If fidelity of the readout is essential for a particular application, the unimolecular (BERKY) biosensor described in Protocol 2 for the detection of endogenous G protein activity are preferred (Fig. 3). These can be implemented either in cell lines with overexpressed GPCRs (Basic Protocol 2), or in cells expressing endogenous GPCRs, including primary cultures after lentiviral gene delivery (Alternate Protocol 2). It is important to note that while we described the use of BERKY biosensors in primary mouse neurons, their use could be expanded to other cell types that are more relevant to the user's research. Overall, the methods described in this article allow for direct measurement of G protein activity in numerous experimental settings.

Critical Parameters:

There are a number of parameters that are crucial to collect reliable and replicable data with the protocols described here. One critical aspect is the level of expression of the BRET biosensor components. For the bi-molecular sensor, we suggest starting with a 1:10 or even 1:20 BRET donor:acceptor ratio, although these will need to be titrated to find an optimal amount dependent on lab-to-lab variability. In essence, expression of the acceptor should be in great excess to expression of the donor, and the expression of the donor should be as low as possible while obtaining reproducible luminescence signals well above the background (>10–20 times over background). For BERKY unimolecular biosensors, the donor:acceptor ratio is already imposed by the design of the construct, but the goal should still be to express as little as possible while obtaining reproducible luminescence signals and measurable BRET responses. For BERKY sensors specifically, expression of high levels of the biosensor construct leads to constitutive high intermolecular BRET due to crowding at membranes that greatly suppresses the dynamic range of potential responses.

Endogenous responses detected by BERKY sensors are small by definition. The first question to be asked by the user is whether detection of endogenous responses is critical for a particular application. If not, bi-molecular biosensors will provide more robust results with less need for optimization. If detecting endogenous responses is required, several parameters must be considered to obtain reproducible results with BERKY sensors. In addition to carefully titrating levels of expression of the sensor, we highly recommend the use of instruments that allow simultaneous detection of emission from the donor and the acceptor. This reduces noise and increase the confidence in detecting BRET changes of small magnitude like the ones expected for endogenous G protein responses. Performing kinetic measurements (as opposed to end-point measurements) also allows to visually inspect the shape of responses and gain confidence in the readout. For this, we also prefer instruments that allow reading while injecting agonists or antagonists in real time— i.e., without stop/resume steps that can introduce artifacts in reading due to shifts in detection that occur inadvertently. The latter can obscure existing responses or create artificial ones. Inspecting the progression of the response with time can help identify sharp or unusual changes in

signal that are reading artifacts. In this regard, using controls like GPCR antagonists or G protein inhibitors is useful to determine if *bona fide* responses are being detected regardless of their magnitude (Maziarz et al., 2020).

Troubleshooting:

Table 1 shows common issues that can arise when using bi-molecular Ga-GTP biosensors (Basic Protocol 1 and Alternate Protocol 1) and what can be done to fix them. Table 2 describes troubleshooting methods when using BERKY biosensors both in cell lines and in neurons (Basic Protocol 2 and Alternate Protocol 2). Some troubleshooting strategies are applicable for both protocols and present in both tables.

Understanding Results:

If followed properly these protocols will allow for detection of G protein activity in many different experimental settings. The first protocol that we describe allows for sub-second resolution measurements of Gαi3-GTP in HEK293T cells (Fig. 1). After doing the measurement and exporting the data, user should be able to get traces that show $\alpha 2_A$-AR responses after agonist injection that decrease upon antagonist injection (Fig. 1C–D). From the raw BRET ratio (Fig. 1C), one can determine the basal BRET value by looking at the 30 s before agonist injection for a particular G protein and detector module pair (in our example, the value is ~0.07, Fig. 1C). This value may differ based on lab-to-lab variability, but it is important to have a reference value to aim for to ensure that donor and acceptor are expressed at acceptable ratios that do not lead to high constitutive BRET. Different combinations of GPCRs, G protein and detector modules will also affect basal BRET ratios. The ΔBRET trace shown is an indication of the agonist mediated GPCR response (Fig. 1D). In other words, it represents how much the GPCR can activate the G protein above the baseline level.

Another way to utilize the bi-molecular sensor is to do endpoint dose-dependence measurements (Alternate Protocol 1; Fig.2). This allows for testing of multiple conditions at once, at the expense of losing the temporal resolution of kinetic measurement. While we only show the example for one GPCR ($\alpha 2_A$ -AR), it is important to note that the protocol can be scaled up to test many GPCRs (for example, one could do one GPCR in each row of a 96-well assay plate; Fig. 2A). Similar to kinetic measurements, the basal raw BRET ratio (with no agonist) is subtracted to all points on the graph to get the ΔBRET responses as the dose of agonist is increased (Fig. 2B). From these points, a 3-parameter sigmoidal curve can be graphed and an EC_{50} value can be determined. The EC_{50} value corresponds to the dose of agonist at which 50% of the maximal response is achieved (in our example trace the EC_{50} value is 8.64 nM).

Finally, BERKY biosensors allow for kinetic measurements, but some differences should be noted compared to the bi-molecular sensors. Measuring endogenous G protein responses can be more challenging, as the amplitude of the response is bound to be smaller than when overexpressing exogenous G proteins. In HEK293T cells, for example, a maximal ΔBRET value ~4 units can be expected after activation of endogenous Gi proteins by the

$\alpha 2_A$-AR stimulated with brimonidine (Fig. 3C). In systems were the GPCR is also expressed endogenously, the magnitude of the responses is even smaller (Fig. 3 D–E).

Time Considerations:

Basic Protocol 1, Basic Protocol 2 and Alternate Protocol 1: : With HEK293T cells already in culture, the entire experimental protocol takes 3 days:

- Day 1: Seed cells into 6-well plate
- Day 2: Transfect cells in the morning; change media ~6 hours later
- Day 3: Measure cell luminescence

Alternate Protocol 2: Time considerations for primary cortical neurons are different. After isolation of cells from neonatal tissue, cultures need 12–16 days for neuron differentiation and sensor expression. This should be taken into account when planning experiments and troubleshooting (i.e. it is key to have a constant supply of neonatal pups to be able to adjust and troubleshoot quickly). Luminescence recordings can be made all in a single day or spread out over multiple days if need be.

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DATA AVAILABILITY STATEMENT:

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request.

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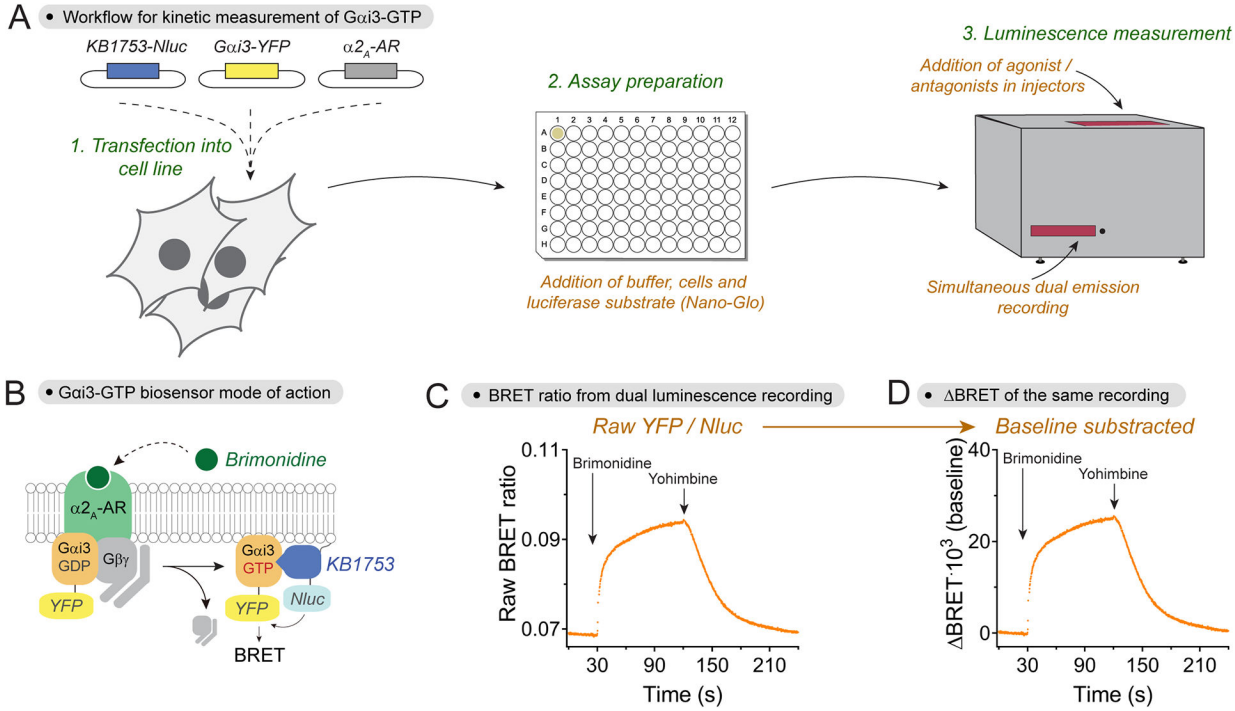

Figure 1. Kinetic measurements of Gαi-GTP levels in HEK293T cells with a bi-molecular BRET biosensor.

(**A**) Schematic of experimental protocol going from cell transfection to assay setup and luminescence recording in plate reader. (**B**) Schematic of bi-molecular sensor design and mode of action in cells. After addition of agonist, the GPCR will promote exchange of GDP to GTP on a YFP-tagged Gα, which will then interact with a Nanoluciferase-fused detector module. This brings the BRET donor (luciferase) and BRET acceptor (YFP) close enough to allow resonance energy transfer from the donor to the acceptor. (**C**) Detection of changes in Gαi3-GTP levels after stimulation of $\alpha 2_A$-AR with 1 μM brimonidine and inhibition with 25 μM yohimbine. The trace represents the raw BRET ratio from one single experiment. (**D**) Single trace of the same measurement as in C but now represented as ΔBRET. The BRET ratio of the 30 s prior to agonist injection in (C) was averaged and subtracted to all data points to yield the ΔBRET values represented here.

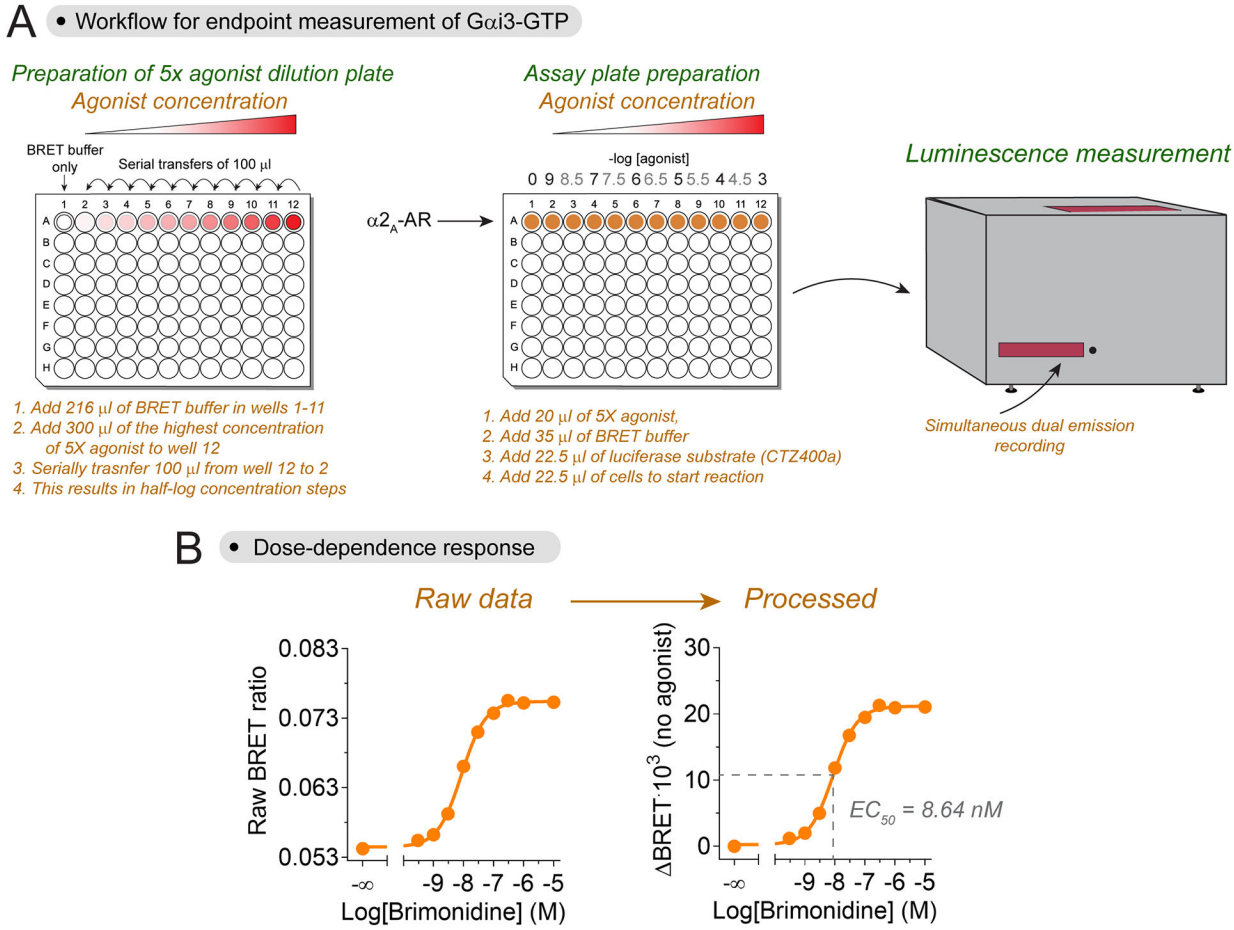

Figure 2. Endpoint measurements of Gαi-GTP levels in HEK293T cells with a bi-molecular BRET biosensor.

(**A**) Schematic of assay setup in 96-well plate and luminescence recording in plate reader. For dose-dependence curves, the agonist can be plated in increasing concentration from left to right, and luciferase substrate (CTZ400a) can be added before measuring luminescence as endpoint measurements. (**B**) Representative result when stimulating the $\alpha 2_A$-AR with increasing doses of brimonidine (0 – 10 μM). The BRET ratio of the well with buffer only is subtracted from all data points to obtain the ΔBRET values represented here.

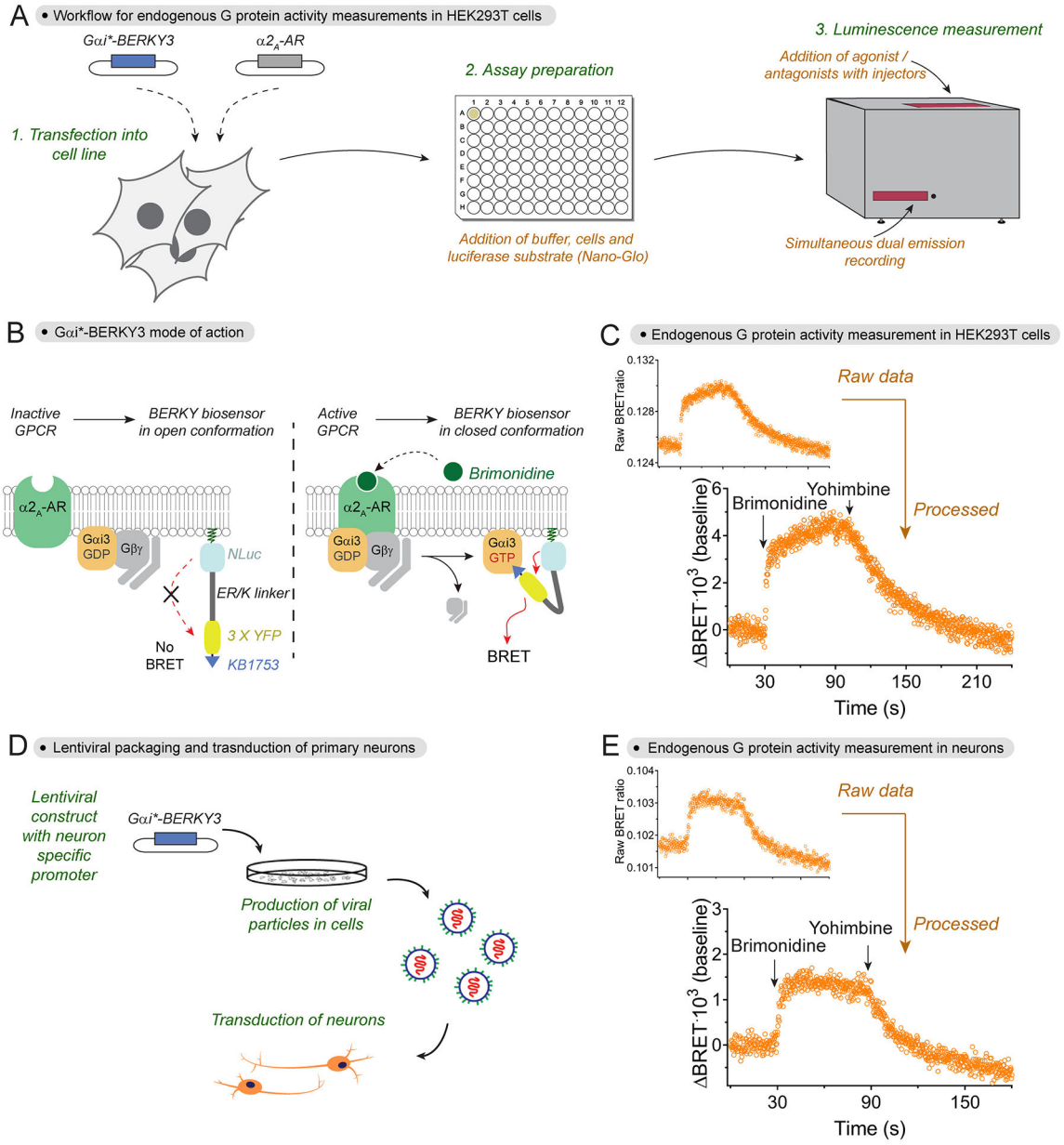

Figure 3. Kinetic measurements of endogenous Gαi-GTP levels in HEK293T cells and primary neurons with a BERKY BRET biosensor.

(**A**) Schematic of experimental protocol in cell lines going from cell transfection to assay setup and luminescence recording in plate reader. (**B**) Schematic of unimolecular BERKY sensor design and mode of action in cells. Injection of an agonist will lead to formation of Gα-GTP. Binding of the detector module (KB1753) in the BERKY sensor to Gα-GTP will promote a switch of the ER/K bi-stable linker from an open to a closed conformation, which in turn will bring the BRET donor and BRET acceptor in close proximity to favor the resonance energy transfer event. (**C**) Detection of changes in endogenous Gαi-GTP levels in HEK293T cells after stimulation of $\alpha 2_A$-AR with 1 μM brimonidine and inhibition with 25 μM yohimbine. The traces represent raw BRET ratio (top) and processed data of ΔBRET over baseline (bottom) of a single representative measurement. (**D**) Schematic of lentiviral

packaging of BERKY biosensor constructs and transduction of primary mouse neurons. (**E**) Detection of changes in endogenous Gαi-GTP levels in primary neurons after stimulation of α2-AR with 5 μM brimonidine and inhibition with 25 μM yohimbine. The traces represent raw BRET ratio (top) and processed data of ΔBRET over baseline (bottom) of a single representative measurement.

Table 1.

Troubleshooting guide for measurements of Gα-GTP formation using bi-molecular biosensors

Problem	Possible Cause	Solution
Drifting BRET ratio prior to agonist injection	Sensor expression too low or too high	Adjust transfected amounts of DNA and ratio of acceptor:donor (we recommend transfecting 10–20 times more of the acceptor than the donor)
Drifting BRET ratio prior to agonist injection	Luciferase substrate has deteriorated over time	Adjust volume of substrate added to each well or take fresh aliquots
Weak luminescence signal	Poor efficiency of transfection	Make sure all transfection reagents (2X HBS, 2M $CaCl_2$, plasmids) for transfection have been made correctly and have not gone through extensive freeze thaw cycles
Weak luminescence signal	Poor cell health	Make sure that cell lines do not reach confluency before passaging
High luminescence but no response detected after agonist injection	Biosensor expression is too high	Reduce the amount of DNA transfected
No change in BRET detected after ligand injection	Agonist or antagonist has deteriorated	Remake fresh ligand dilutions and avoid freeze thaw cycles by making single use aliquots
No response detected after agonist injection	GPCR weakly expressed or desensitized	Increase the amount of GPCR plasmid transfected. In some cases (e.g., mGluRs), starving cells in medium with low or no serum can prevent desensitization
Injection artifact (i.e. sharp shift in BRET ratio incompatible with G protein activation rates)	The ligand used interferes with the luminescence readout	Decrease concentration of the ligand if possible or seek alternative ligand
Injection artifact (i.e. sharp shift in BRET ratio incompatible with G protein activation rates)	Volume injected is too large	Reduce volume injected
Injection artifact (i.e. sharp shift in BRET ratio incompatible with G protein activation rates)	Speed of injection is too fast	Reduce injection speed

Table 2.

Troubleshooting guide for measurements of Gα-GTP formation using BERKY biosensors

Problem	Possible Cause	Solution
Drifting BRET ratio prior to agonist injection	Sensor expression too low or too high	Adjust transfected amounts of DNA / dilution of lentiviral constructs used for primary cell trasnduction
Drifting BRET ratio prior to agonist injection	Luciferase substrate has deteriorated over time	Adjust volume of substrate added to each well or take fresh aliquots
Weak luminescence signal and noisy responses	Biosensor expression might be too low in a particular cell type	Increase integration time to gain confidence on luminescence readout per unit of time while decreasing the noise of the measurement
Weak luminescence signal	Poor efficiency of transfection or transduction	Make sure all transfection reagents (2X HBS, 2M $CaCl_2$, plasmids) for transfection have been made correctly and have not gone through extensive freeze thaw cycles Make sure that lentiviral packaging has been performed adequately by benchmarking expression in another system with positive controls
Weak luminescence signal	Poor cell health	Make sure that cell lines do not reach confluency before passaging For lentiviral transductions, decrease the titer and/or time of exposure to the virus Increase the number of neurons seeded. Cell density can affect the viability of neurons after transduction
High luminescence but no change in BRET detected after agonist injection	Biosensor expression is too high	Reduce the amount of DNA transfected or increase dilution of lentiviral particles
No response detected after ligand injection	Agonist or antagonist has deteriorated	Remake fresh ligand dilutions and avoid freeze thaw cycles by making single use aliquots
No response detected after agonist injection	GPCR absent, weakly expressed, or desensitized	Increase the amount of GPCR plasmid transfected. In some cases (e.g., mGluRs), starving cells in medium with low or no serum can prevent desensitization Perform measurement with neurons at a later DIV post-transduction. Neuronal differentiation can enable GPCR responses
Injection artifact (i.e. sharp shift in BRET ratio incompatible with G protein activation rates)	The ligand used interferes with the luminescence readout	Decrease concentration of the ligand if possible or seek alternative ligand
Injection artifact (i.e. sharp shift in BRET ratio incompatible with G protein activation rates)	Volume injected is too large	Reduce volume injected
Injection artifact (i.e. sharp shift in BRET ratio incompatible with G protein activation rates)	Speed of injection is too fast	Reduce injection speed