Published in final edited form as:

Methods Cell Biol. 2017 ; 142: 145–157. doi:10.1016/bs.mcb.2017.08.001.

Rapid kinetic BRET measurements to monitor G protein activation by GPCR and non-GPCR proteins

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Abstract

Heterotrimeric G proteins are central hubs of signal transduction whose activity is controlled by G-protein coupled receptors (GPCRs) as well as by a complex network of regulatory proteins. Recently, Bioluminescence Resonance Energy Transfer (BRET)-based assays have been used to monitor real-time activation of heterotrimeric G proteins in cells. Here we describe the use of a previously established BRET assay to monitor G protein activation upon GPCR stimulation and its adaptation to measure G protein activation by non-GPCR proteins, such as by cytoplasmic guanine nucleotide exchange factors (GEFs) like GIV/Girdin. The BRET assay monitors the release of free $G\beta\gamma$ from $G\alpha$ - $G\beta\gamma$ heterotrimers as a readout of G protein activation, which is readily observable upon agonist stimulation of GPCRs. To control the signal input for non-GPCR activators, we describe the use of a chemically-induced dimerization (CID) strategy to promote rapid membrane translocation of proteins containing the $G\alpha$ Binding and Activating (GBA) motif found in some non-receptor GEFs. The assay described here allows the kinetic measurement of G protein activation with sub-second temporal resolution and to compare the levels of activation induced by GPCR agonists versus those induced by the membrane recruitment of non-receptor G protein signaling activators.

Keywords

Heterotrimeric G protein; GPCR; Bioluminescence Resonance Energy Transfer (BRET); GEF; GIV/Girdin; protein-protein interaction

1 Introduction

Heterotrimeric G proteins act as molecular “on/off” switches which control virtually all aspects of human physiology [1,2]. In the inactive state, the nucleotide-binding α subunit is bound to GDP and to the $G\beta\gamma$ dimer. Upon stimulation by extracellular ligands, G-protein-coupled receptors (GPCRs), which are guanine nucleotide exchange factors (GEFs), promote the exchange of GDP for GTP on $G\alpha$. The active $G\alpha$ -GTP dissociates from the $G\beta\gamma$ dimer, allowing them both to interact with downstream effector molecules. The intrinsic GTPase activity of $G\alpha$ allows it to return to the inactive, GDP-bound state and to reassociate with $G\beta\gamma$, thus completing the classical G protein cycle.

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Other than GPCRs, there are “accessory proteins” that modulate G protein activation/inactivation [3,4]. Among them, non-receptor GEFs have been found to mimic the function of GPCRs in G protein activation. These are cytoplasmic factors that, like GPCRs, bind to inactive G α -GDP and promote nucleotide exchange. Only in a subset of non-receptor GEFs has it been possible to ascribe the GEF activity to a defined sequence element. This element is named the G α -Binding and Activating (GBA) motif and has been identified in the mammalian proteins GIV/Girdin [5], DAPLE [6], CALNUC [7] and NUCB2 [7] as well as in the worm-restricted protein GBAS-1 from *C. elegans* [8]. GIV, the first identified and best characterized GBA protein, contributes to the regulation of multiple cellular processes including cell motility, autophagy and mitosis, as well as to many pro-metastatic phenotypes in a GBA motif-dependent manner [9]. Likewise, the GBA motif of DAPLE also regulates phenotypes associated with cancer progression [6]. Although the GBA motif of CALNUC and NUCB2 binds and activates G α i *in vitro* [7], the exact biological functions associated with it are unknown. However, it is known that CALNUC binds to G α i on the surface of the Golgi apparatus [10] and it regulates the intracellular localization of G proteins [11], which might be related to the function of the GBA motif.

Monitoring real-time activation of G proteins has been simplified by the development of Bioluminescence Resonance Energy Transfer (BRET)-based assays. BRET relies on the transfer of energy from a donor of luminescence (typically a luciferase protein) to an acceptor of luminescence (typically a fluorescent protein) (Figure 1A). Since this transfer is dependent on the close proximity (< 10 nM) of the two proteins, proteins of interest can be fused to donor or acceptor molecules to determine the kinetics of their association or dissociation [12]. In 2009, the Lambert laboratory developed a BRET-based assay to monitor binding of free G $\beta\gamma$ to the c-terminal domain of G protein-coupled receptor kinase 3 (GRK3) as a proxy for G protein activation. In brief, G β and G γ subunits tagged with a split fluorescent protein are used to reconstitute G $\beta\gamma$ fused to the BRET acceptor whereas GRK3 is fused to *Renilla* luciferase as the BRET donor. Upon G protein activation by a GPCR, fluorescently-labeled G $\beta\gamma$ dissociates from G α -GTP and binds to the luciferase-fused GRK3 construct resulting in increased BRET [13]. This system has been recently improved by the Martemyanov laboratory [14]. Essentially, they replaced the original *Renilla* luciferase by a new generation luciferase called nanoluciferase (Nluc) that is derived from the organism *Oplophorus gracilirostris* [15]. The luminescence of this luciferase when used in combination with the substrate furimazine results in a signal many fold higher than any previously reported luciferase. In the G protein activation BRET assay, this translated in significantly higher sensitivity, improved signal-to-noise ratio and better reproducibility, allowing for the measurement of G protein activation even with notoriously difficult GPCR-G protein pairs [14].

Monitoring real-time G protein activation by non-receptor GEFs has some challenges compared to GPCR-mediated activation. The main one is that the activity of GPCRs can be easily controlled by the addition of extracellular ligands that directly activate them, while non-receptor GEFs are cytoplasmic proteins that are not directly activated by extracellular ligands. We recently envisioned a method to control the activation of G proteins by GBA motif-containing factors with extracellular ligands using a synthetic biology approach (Figure 1B). We combined the Nluc-based BRET system described above along with a

chemically-induced dimerization (CID) strategy to induce the rapid translocation of the GBA motif of GIV to membranes while simultaneously monitoring G protein activation [16]. The CID system we used is based on rapamycin-induced dimerization of the FK506-binding protein (FKBP) and FKBP rapamycin-binding (FRB) domain of mTOR [17–19]. These two domains only heterodimerize in the presence of the cell-penetrating small molecule rapamycin (or rapamycin analogs), resulting in the formation of a ternary FKBP-rapamycin-FRB complex. By expressing FRB fused to a membrane localization signal (e.g., the first 11 residues of the Lyn kinase, Lyn11), one can induce the translocation of an FKBP-fused GBA construct (e.g., FKBP-GIV) to the plasma membrane by adding rapamycin [20]. By using this approach along with numerous controls, we recently showed that membrane translocation of GIV is sufficient for it to activate G proteins [16], suggesting that membrane recruitment is an important mechanism by which other GBA proteins regulate G protein signaling.

One major advantage of controlling GBA-mediated action by CID is that it allows the direct comparison of G protein activation levels with those elicited by GPCRs using a unified quantitative readout (i.e., BRET). Here, we describe the steps required to measure G protein activation using BRET in response to stimulation of a prototypical Gi-coupled GPCR (i.e., the muscarinic M4 receptor, M4R) as well as in response to the translocation of a GIV construct to the plasma membrane.

2 Materials

1. HEK293T cells (ATCC®, CRL-3216)
2. 100 mm tissue culture dishes (Thermo Scientific BioLite, 130182)
3. Flat bottom, 6-well cell culture plates (Corning Costar, 3516)
4. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, 11965-092) supplemented with Bovine Calf Serum (10%, Hyclone, SH30072.03), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (1%) (purchased as a 100X mixture, Corning, 30-009-CI). This is referred to as “complete medium” in the protocol described below.
5. Phosphate Buffered Saline (PBS) 1X, without calcium or magnesium (Corning, 21-040-CV)
6. 0.1% (w:v) gelatin. Prepared in sterile water from 2% (w:v) gelatin (Sigma, G1393).
7. 1 M CaCl₂
8. HEPES-buffered saline (HBS) 2X: 280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄. Adjust pH to 7.08. Store aliquots at –20°C.
9. Cell scrapers, 25 cm (Denville Scientific, T0125)
10. Polystyrene culture test tubes, 12 x 75 mm (Fisherbrand, 14-956-3A)

11. Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.37 mM NaH₂PO₄, 24 mM NaHCO₃, 10 mM HEPES pH 7.4, 0.1% glucose). Keep at 4°C for short term storage (<1 month) or freeze aliquots at -20°C for long term storage.
12. Nano-Glo luciferase assay substrate (Promega, N1110). Store at -20°C.
13. White opaque 96-well plates (Perkin Elmer OptiPlate-96, 6005290)
14. POLARstar Omega microplate reader (BMG LABTECH) or similar plate reader suitable for dual emission luminescence detection
15. Mammalian cell lysis buffer: 20 mM HEPES, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% [v:v] Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate, and 0.5 mM Na₃VO₄. Adjust pH to 7.2. Store aliquots at -20°C. Add fresh protease inhibitor cocktail (Sigma-Aldrich, S8830) before use.
16. 5X Laemmli sample buffer: 156 mM Tris-HCl pH 6.8, 5 % (w:v) SDS, 25% (v:v) glycerol, 25% (v:v) 2-mercaptoethanol, 0.025% (w:v) bromophenol blue

Plasmids

Mammalian vectors expressing the following constructs are required:

1. masGRK3ct-NLuc. Kindly provided by K. Martemyanov (Scripps Research Institute, Florida) [14]
2. Venus (156-239)-Gβ1 (VC-Gβ1). Kindly provided by N. Lambert (Augusta University) [13]
3. Venus (1-155)-Gγ2 (VN- Gγ2). Kindly provided by N. Lambert (Augusta University) [13]
4. 3xHA-hM4R. Purchased from the cDNA resource center at Bloomsburg University (Catalog # MAR040TN00)
5. Gαi3 [21]
6. Lyn11-FRB-myc [16]
7. mRFP-FKBP-GIV (residues 1660-1870, containing the GBA motif) [16]
8. Empty plasmid (pcDNA3.1) (Invitrogen, V790-20)

3 Methods

The following procedure describes how to monitor G protein activation in response to the stimulation of a GPCR as well as in response to the rapamycin-induced translocation of a non-receptor G protein activator to the plasma membrane. As examples, we stimulate the Gα_{i/o}-coupled M4 muscarinic acetylcholine receptor (M4R) with the agonist carbachol, and recruit the non-receptor GEF, GIV/Girdin, to the membrane. The procedure consists of four parts: (1) transfection, (2) harvesting cells, (3) BRET measurement, and (4) data analysis.

3.1 Transfection of cells

1. Working under sterile conditions in a laminar flow hood, add 1 mL of 0.1% gelatin, pre-warmed to 37°C, to the wells of flat-bottom 6-well plates (*see Note 1*). Swirl to coat the bottom of the wells evenly, and incubate for 5 min. Aspirate to remove the gelatin, and allow the wells to dry for approximately 20 minutes.
2. Seed 400,000 HEK293T cells in each gelatin-coated well in a final volume of 2 mL of pre-warmed complete culture medium. Incubate HEK293T cells at 37°C with 5% CO₂.
3. 16–24 hours after seeding, aspirate the medium in the wells and replace with 1.5 mL of fresh complete medium. Return cells to 37°C with 5% CO₂ while the next steps to prepare the transfection reagents are performed.
4. In this example, we will transfect cells with four conditions requiring the following plasmids (*see Note 2*): (1) Donor only: masGRK3ct-Nluc (BRET donor), 3xHA-hM4R, and Lyn11-FRB-myc; (2) Donor and acceptor: masGRK3ct-Nluc, VC-Gβ1 and VN-Gγ2 (Bimolecular fluorescence complementation-based BRET acceptor), 3xHA-hM4R, and Lyn11-FRB-myc; (3) Donor, acceptor, and Gαi3: masGRK3ct-Nluc, VC-Gβ1 and VN-Gγ2, Gαi3, 3xHA-hM4R, and Lyn11-FRB-myc, and (4) experimental condition: masGRK3ct-Nluc, VC-Gβ1 and VN-Gγ2, Gαi3, mRFP-FKBP-GIV (1660–1870), 3xHA-hM4R, and Lyn11-FRB-myc (*see Note 3*). Prepare and label four polystyrene test tubes, corresponding to each transfection condition. In a separate polystyrene tube, prepare a transfection master mix by adding 528 μL of sterile water and 72 μL of 1 M CaCl₂ (*see Note 4*).
5. Add the components common to all transfection conditions to the master mix: masGRK3ct-Nluc, 3xHA-hM4R, and Lyn11-FRB-myc plasmid DNA. Mix by pipetting and transfer 120 μL of the mix to the polystyrene tube corresponding to the donor-only transfection condition (*see Note 5*).
6. To prepare condition 2 (donor and acceptor), add VC-Gβ1 and VN-Gγ2 plasmid DNA to the remaining volume of the master mix. Mix by pipetting and transfer 120 μL to the polystyrene tube corresponding to condition 2.

¹Based on our observations, gelatin-coating promotes cell adhesion and improves transfection efficiency.

²The amounts of DNA for each component must be determined empirically, with particular attention to the ratio of Gα to Gβγ [13,14,22]. We use the following amounts of DNA per well: masGRK3ct-Nluc (0.1 μg), VC-Gβ1 (0.2 μg), VN-Gγ2 (0.2 μg), 3xHA-hM4R (0.2 μg), Gαi3 (1.0 μg), Lyn11-FRB-myc (3.0 μg), and mRFP-FKBP-GIV (1660-1870) (0.5 μg).

³Conditions 1–3 serve as controls. The BRET ratio (i.e., luminescence at 528 nm, corresponding to the maximum for the acceptor emission, divided by the luminescence at 460 nm, corresponding to the maximum for the donor emission) of the donor-only condition should be low due to the absence of acceptor. The BRET ratio of the donor and acceptor condition should be higher due to the close proximity of donor and acceptor upon binding of free Gβγ (in the absence of Gαi3) to GRK3. The BRET ratio of the donor, acceptor, and Gαi3 condition should be lower than in the donor plus acceptor condition because Gαi3 binds to Gβγ and precludes its binding to GRK3, thereby diminishing the donor/acceptor association. The difference in BRET between the latter two controls serves as the theoretical dynamic range of the BRET assay. In practice, this range is never completely reached, as even potent G protein activators do not result in the complete dissociation of Gβγ trimers [13,14,22].

⁴For each condition, 120 μL of diluted CaCl₂ are required (105.6 μL of sterile water plus 14.4 μL of 1 M CaCl₂). It is useful to prepare an excess of master mix, such as for *n*+1 conditions.

⁵We disregard small volumes (< 5%) contributed by plasmid DNA from downstream calculations of pipetting volumes.

7. To prepare condition 3 (Donor, acceptor, and G α i3), add G α i3 DNA to the master mix. Mix by pipetting and transfer 120 μ L to the corresponding polystyrene tube.
8. To prepare condition 4, transfer 120 μ L of the master mix to the corresponding experimental condition tube. In addition, add mRFP-FKBP-GIV (1660–1870) plasmid DNA (*see* Note 6).
9. Equalize total transfected DNA in each transfection with pcDNA3.1 vector.
10. Mix each calcium/DNA transfection mixture by pipetting and incubate for 5 minutes.
11. Prepare and label a second set of four polystyrene tubes, one per condition. Add 120 μ L of 2X HBS to each tube.
12. Add each calcium/DNA transfection mixture dropwise over a tube containing 2X HBS, while vortexing. Incubate the resulting calcium phosphate DNA solutions for 20–30 minutes.
13. Add the calcium phosphate DNA solutions dropwise over the cell wells. Mix by gently swirling the wells. Incubate cells at 37°C with 5% CO₂.
14. Aspirate media after 5–6 hours and replace with 2 mL of fresh complete medium.

3.2 Harvesting cells

1. Approximately 22–28 hours after transfection, wash cells with 2 mL of warm PBS and add 1 mL of PBS per well. Harvest cells by gentle scraping and collect in microcentrifuge tubes.
2. Count cells using a hemocytometer (*see* Note 7).
3. Centrifuge the tubes containing cells for 5 min at 550 rcf. Carefully aspirate supernatant.
4. Resuspend cell pellets in Tyrode's solution (pre-warmed at room temperature) to achieve a density of 10⁶ cells per mL (*see* Note 8).

3.3 BRET measurement

3.3.1 Measurement of basal BRET ratios—Measuring the basal BRET ratios, without any agonist addition, can serve as a quick quality control and provides a quick measure of possible effects of regulators on G protein activity prior to agonist stimulation and/or membrane recruitment.

⁶For initial experiments, it is useful to transfect cells with a range of concentrations of non-GPCR activator DNA in order to identify the dynamic range of the BRET signal induced by the activator.

⁷Once reproducible experimental conditions have been established, cell counting can be skipped, as BRET measurements are ratiometric and tolerate variations in the total number of cells within an acceptable range (+/– 30% in the number of cells).

⁸We have found that cell suspensions can be kept for up to 3 or 4 hours without appreciable decay in the observed BRET responses.

1. Resuspend cells and add 25 μ L of each condition to the wells of a white opaque 96-well plate.
2. Prepare the working substrate solution by diluting Nano-Glo substrate at a 1:150 dilution in Tyrode's solution (*see* Note 9).
3. Transfer 75 μ L of the working substrate solution to each well, and mix the wells by tapping the side of the plate. Incubate in the dark for 2 minutes.
4. Measure luminescence at both 460 nm and 528 nm wavelengths in a plate reader capable of detecting dual emission. Read wells in succession using a "plate mode" protocol (*see* Note 10).

3.3.2 Measurement of BRET kinetics

1. Rinse the tubing and injector first with 4.5 ml of 70% (v:v) ethanol and then with 4.5 ml of sterile deionized water. Prime the injector by pumping 1.5 mL of a 20X (2 mM) carbachol or a 20X (10 μ M) rapamycin solution through the tubing, depending on the condition being tested. Set up the plate reader software to inject 5 μ L of this agonist solution per well (*see* Note 11).
2. Resuspend the cells corresponding to one of the conditions. Add 25 μ L of the cell suspension to the well of a white opaque 96-well plate.
3. Prepare the working substrate solution by diluting Nano-Glo substrate at a 1:150 dilution in Tyrode's solution.
4. Transfer 75 μ L of the working substrate solution to the well, and mix by pipetting. Incubate in the dark for 2 minutes at 28 °C (*see* Note 12).
5. Measure luminescence at both 460 nm (maximum for donor emission) and 528 nm (maximum for acceptor emission) wavelengths in a plate reader capable of detecting dual emission every 0.24 s (integration time = 0.24 s) at 28 °C by using the "single well mode". Measurements are carried out for 4 minutes and carbachol or rapamycin are injected 30 s after the start (*see* Note 13). Repeat the BRET kinetics measurement individually for each condition.
6. Levels of expressed proteins can be determined by immunoblot analysis using the remaining cells. Centrifuge cell suspensions at 14,000 rcf for 30–60 s.

⁹Prepare 75(*n*+1) μ L of Tyrode's buffer, where *n* is the number of samples to measure.

¹⁰We measure emission at 3 intervals, separated by about 30 s, with an integration time of 0.24 s. This is sufficient to quickly determine the average basal BRET ratio for each sample and assess if the BRET ratio signal is stable with time.

¹¹We use a final concentration of 0.5 μ M rapamycin for membrane recruitment of FKBP-tagged proteins, and 100 μ M carbachol to stimulate the M4R. These concentrations are sufficient to achieve maximal effects on the desired targets (M4R stimulation or FRB-FKBP dimerization).

¹²We typically carry out these experiments at a temperature slightly higher than room temperature to minimize possible variations from day to day. This preincubation with the substrate is done inside the plate reader to ensure that there is no change in temperature between substrate preincubation and BRET measurement. If desired, the temperature can be set to a different value (e.g., 37 °C).

¹³We use a BMG LABTECH POLARstar Omega microplate reader, although similar microplate readers equipped with filters to detect luminescence at 460 nm and 528 nm can be used. The POLARstar Omega allows for simultaneous dual emission detection, which allows for more rapid measurement of BRET kinetics. Emission can be detected by integration of the signal over an interval as short as 0.02 s, which is ten-fold shorter than the interval we use. In the Omega software, we use the following protocol to measure kinetics: Dual Luminescence method; Well mode; *Filter settings*: Simultaneous dual emission; Emission filter: 460–80 / 535–35; Gain: 2000 / 2000; Measurement start time: 30 s, No. of intervals: 1000; Measurement interval time: 0.24 s; Interval time: 0.24 s; Injection start time: 60 s (30s after measurement start); Injection Volume: 5 μ L.

Aspirate the supernatant and lyse cells on ice by resuspending in mammalian lysis buffer. Centrifuge the cell lysates at 14,000 rcf for 10 min. Transfer the supernatants to new microcentrifuge tubes containing Laemmli sample buffer and mix by pipetting. Boil lysates for 5 min. Separate proteins by SDS-PAGE and analyze by immunoblotting.

7. Optionally, acceptor protein expression can be estimated by measuring YFP fluorescence (*see* Note 14).

3.4 Data analysis

1. To determine the BRET ratio for each time point, divide the emission at 528 nm (acceptor maximum) by the emission at 460 nm (donor maximum) (*see* Note 15).
2. To simplify data interpretation, BRET ratios can be corrected relative to the pre-agonist basal values ($BRET_{baseline}$) (*see* Note 16) by subtracting the average pre-agonist BRET ratio corresponding to the first 30 s of measurement from the BRET ratio of each time point (*see* Note 17).
3. When analyzing experimental replicates, calculate the average and standard error of $BRET_{baseline}$ between multiple experiments for each time point.
4. Export these data-corrected $BRET_{baseline}$ values to a graphing software such as GraphPad Prism 6. Plot $BRET_{baseline}$ over time. An example of G protein activation induced by carbachol compared to activation induced by membrane recruitment of the GBA motif of the non-receptor GEF, GIV, is presented in Figure 2.

Acknowledgments

This work was supported by NIH grants R01GM108733, R01GM112631, American Cancer Society grants RSG-13-362-01-TBE and IRG-72-001-36, and the Karin Grunebaum Foundation (to MG-M).

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¹⁴Transfer 100 μ L of cells to a well, excite at 460 nm and record emission at 528 nm.

¹⁵This calculation can be performed automatically by the plate reader software, such as BMG LABTECH MARS, allowing for a reduced data export to spreadsheet software.

¹⁶The goal of this data correction is to facilitate the visualization of the effect of agonist or rapamycin, which can be obscured by small differences in the variability of basal BRET ratio from experiment to experiment and from condition to condition. These minor variations are acceptable and should not have a significant impact on the magnitude of the BRET response ($BRET_{baseline}$) from experiment to experiment for each respective condition.

¹⁷This data correction can also be performed automatically by the plate reader software.

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Figure 1. Chemically-induced dimerization strategy to monitor G protein activation by non-GPCR proteins using BRET

(A) A simplified diagram of the overlapping donor emission and acceptor excitation spectra of molecules to explain the principle of BRET. BRET relies on energy transfer from a bioluminescent donor molecule such as Nanoluc (a luciferase) to an acceptor fluorophore such as Venus. If the donor emission wavelength (blue line) overlaps with the excitation wavelength of an acceptor (black line), BRET can occur if donor and acceptor are in close physical proximity. The transfer of energy results in the emission of light by the acceptor molecule (yellow line). (B) Membrane translocation of an FKBP-GIV construct is paired with a cell-based BRET assay to monitor G protein activation. The FKBP-rapamycin-FRB system relies on the rapamycin-induced heterodimerization of the FRB and FKBP domains. We have adapted this system by co-expressing FRB fused to a membrane localization signal (Lyn11) together with a FKBP-fused domain of GIV with GEF activity (input control system, left). Addition of rapamycin results in membrane recruitment of GIV, which in turn results in G protein activation [16]. Upon G protein activation, $G\alpha$: $G\beta\gamma$ trimers dissociate and free $G\beta\gamma$ dimers associate with the C-terminal domain of GRK3. The close proximity between Venus-tagged $G\beta\gamma$ and GRK3-NLuc results in an increased BRET signal (output control system, right). This image (Panel B) was originally published in the Journal of Biological Chemistry. Parag-Sharma et al. Membrane Recruitment of the Non-receptor Protein GIV/Girdin ($G\alpha$ -interacting, Vesicle-associated Protein/Girdin) Is Sufficient for

Activating Heterotrimeric G Protein Signaling. *J Biol Chem.* 2016; 91 (53):27098–27111. © the American Society for Biochemistry and Molecular Biology.

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Figure 2. Basal and kinetic measurements of BRET ratios

(A) Basal BRET ratios in unstimulated cells. HEK293T cells were transfected with the indicated plasmids and BRET measured in the absence of stimulation. **(B)** Comparative analysis of the kinetics of G protein activation induced by stimulation of the M4R with carbachol (black) or by membrane recruitment of the non-receptor GEF, GIV (red). HEK293T cells transfected with masGRK3ct-Nluc, VC-Gβ1 and VN-Gγ2, Gαi3, mRFP-FKBP-GIV (1660–1870), 3xHA-hM4R, and Lyn11-FRB-myc were stimulated with either 100 μM carbachol (black) or 0.5 μM rapamycin (red) as indicated. $\text{BRET}_{\text{baseline}}$ was determined by subtracting the average pre-stimulation BRET ratio from the BRET ratio at each subsequent time point. This figure has been adapted from results originally published in the Journal of Biological Chemistry. Parag-Sharma et al. Membrane Recruitment of the Non-receptor Protein GIV/Girdin (Gα-interacting, Vesicle-associated Protein/Girdin) Is Sufficient for Activating Heterotrimeric G Protein Signaling. *J Biol Chem.* 2016; 91 (53): 27098–27111. © the American Society for Biochemistry and Molecular Biology.