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# Monitoring G protein activation in cells with BRET

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## Summary

Live-cell assays based on fluorescence and luminescence are now indispensable tools for the study of G protein signaling. Assays based on fluorescence and bioluminescence resonance energy transfer (FRET and BRET) have been particularly valuable for monitoring changes in second messengers, protein-protein interactions, and protein conformation. Here we describe a BRET assay that monitors the release of free Gβγ dimers after activation of heterotrimers containing Gα subunits from all four G protein subfamilies. This assay provides useful kinetic and pharmacological information with reasonably high throughput using standard laboratory equipment.

### Keywords

Bioluminescence resonance energy transfer (BRET); transfection; luciferase; venus fluorescent protein; G protein coupled receptors (GPCRs)

## 1. Introduction

The availability of genetically-encodable fluorescent and luminescent proteins has revolutionized functional studies of G protein-coupled receptors (GPCRs) and heterotrimeric G proteins. In addition to simple visualization of receptors and downstream signaling molecules by microscopy, an wide array of sensors and fusion proteins have been developed to monitor virtually every step of several signaling pathways in real time and in living cells [1]. Many of these tools are based on fluorescence or bioluminescence resonance energy transfer (FRET or BRET), both of which report the proximity and orientation of energy donors and acceptors on a molecular scale. In many cases both FRET and BRET versions of a given sensor or fusion protein are available, and the choice of method is determined by the particular requirements of the experiment. The primary advantage of FRET for monitoring molecular events in cells is the ability to image single cells, and thus to gain spatial information in addition to FRET signals. The luciferases used most often for BRET produce too few photons for imaging with reasonable temporal resolution, although the advent of brighter engineered luciferases is likely to change this situation [2]. The primary advantage

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of BRET is sensitivity. Because no excitation light is introduced into the sample background signals and direct excitation of the energy acceptor are avoided, and very small changes in BRET efficiency are detectable. From a practical standpoint, BRET experiments can be carried out on transfected cells with commonly-available luminometers and plate readers, allowing for relatively high throughput in a standard laboratory setting.

Activation of many GPCRs ultimately leads to the generation or depletion of second messenger molecules. For example, activation of $G_s$ family heterotrimers leads to accumulation of cyclic AMP (cAMP), whereas activation of $G_i$ heterotrimers leads to a decrease in cAMP. Similarly, activation of $G_q$ family heterotrimers leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate, and the production of inositol 1,4,5-triphosphate and diacylglycerol [3]. A variety of FRET-and BRET-based sensors exist for both of these second messenger systems [1]. In contrast, several heterotrimeric G proteins do not change second messenger levels, and therefore their activation is more difficult to monitor in living cells. Furthermore, monitoring changes in downstream second messenger cascades as a proxy for GPCR activation does not capture the G protein specificity of the observed effects due to extensive signaling cross-talk.

Here we provide the protocol for a BRET-based system that can be used to monitor activation of all four families of heterotrimeric G proteins ($G_s$, $G_q$, $G_{i/o}$ and $G_{12/13}$). The assay relies on the decrease in affinity that occurs between Gα subunits and Gβγ dimers when heterotrimers are activated (bind GTP), and the resulting release of free Gβγ. A C-terminal fragment of the G protein receptor kinase 3 (GRK3ct) that binds to free Gβγ dimers fused to a luciferase serves as a BRET donor. Originally developed using *Renilla* luciferase as a BRET donor [4], the assay has been improved by substitution of the engineered NanoLuc from *Oplophorus* [2, 5]. This sensor is coexpressed with an unlabeled Gα subunit and a Gβγ dimer labeled with a BRET acceptor, generally the fluorescent protein venus (assembled by bimolecular fluorescence complementation; BiFC) [6]. The free Gβγ-venus us that is released upon heterotrimer activation binds to a membrane-associated GRK3ct-luciferase (e.g. masGRK3ct-NanoLuc), leading to a detectable increase in BRET [4]. We have found that this system reports activation-induced dissociation of all four G protein families [7] (and I.M. and K.M., unpublished results). The affinity of free Gβγ-venus us for GRK3ct is such that it allows for rapidly-reversible binding, similar to many Gβγ binding effectors. As a consequence the assay is capable of reporting changes in upstream signaling events that are rate-limiting [7, 8].

While the procedures outlined in this protocol are what we use for this particular BRET assay, it should be noted that most of the steps apply equally well to any of the myriad BRET-based assays that monitor second messengers, G protein dissociation, coupling of receptors and G proteins, conformational changes in receptors and arrestins, recruitment of arrestins, etc… [1].

## 2. Materials

### 2.1. Cell Culture and Transfection

1. HEK293T/17 cells or other cell lines suitable for transient transfection.

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2. 100-mm tissue culture dishes.
3. 60-mm tissue culture dishes.
4. Culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), MEM non-essential amino acids, 1 mM sodium pyruvate, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).
5. Matrigel solution: approximately 10 μg/ml growth factor-reduced Matrigel (BD Biosciences) in culture medium without antibiotics.
6. Lipofectamine LTX and PLUS Reagents (Invitrogen).
7. BRET sensors: Venus 156-239-Gβ1, Venus1-155-Gγ2 (*see* Note 1), and masGRK3ct-NanoLuc (*see* Note 2).
8. GPCRs and Gα subunits in mammalian expression vectors: Many constructs encoding GPCRs and Gα subunits are available from Missouri S&T cDNA Resource Center (cdna.org).

### 2.2. BRET Measurement

1. LUMIstar, FLUOstar or POLAstar Omega (*see* Note 3).
2. PBS containing 5 mM EDTA (EDTA/PBS): Measure 100 ml of 10× PBS and 10 ml of 0.5 M EDTA pH 8.0 by pipettes and transfer to a 1 L graduated cylinder containing about 800 ml of type 1 ultrapure water. Make up to 1 L with type 1 ultrapure water and filter through a 0.22 μm filter. Store at room temperature.
3. PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose (BRET buffer): Weigh 1 g of glucose and transfer to 1 L graduated cylinder containing about 800 ml of type 1 ultrapure water. Add 100 ml of 10× PBS and 0.5 ml of 1 M $MgCl_2$. Make up to 1 L with type 1 ultrapure water and filter through a 0.22 μm filter. Store at room temperature.
4. White flat-bottom 96-well plates (Greiner bio-one, Cat. No. 655073) for luminescence and BRET measurement (*see* Note 4 and 5).
5. Black flat-bottom 96-well plates (Greiner bio-one, Cat. No.655900) for fluorescence measurement (*see* Note 5 and 6).

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[1]A pair of Venus156-239-Gβ1 and Venus1-155-Gγ2 is a bimolecular fluorescence complementation (BiFC)-based BRET acceptor, allowing contribution of functional Gβγ dimer as a BRET acceptor.

[2]masGRK3ct-NanoLuc construct was generated by replacement of Rluc8 in masGRK3ct-Rluc8 with NanoLuc (Promega) [9]. NanoLuc produces approximately 150-fold brighter luminescence than Rluc8, allowing measurement of changes in BRET signal at low expression level of GPCR signaling component. Furthermore, the blue-shifted and narrow excitation spectrum of NanoLuc based sensor allows better separation of excitation spectrums of NanoLuc and Venus [2], decreasing noise content in the BRET assay (I.M. unpublished data). Additionally, the long signal half-life (> 2 h) of NanoLuc offers an advantage for long-term BRET measurements.

[3]LUMIstar, FLUOstar and POLAstar Omega (BMG LABTECH) has a simultaneous dual emission detection system that allows the detection of two emitted wavelengths at the same time. The system enables highest possible time resolution of 20 ms per data point. These plate readers are equipped with two reagent injectors to apply two different reagents (*e.g.* luciferase substrate, agonist, or antagonist).

[4]Since white plates reflect light and maximize light output signal, white plates are used for luminescence and BRET measurements.

[5]The plates can be reused multiple times if washed with detergent and rinsed well with distilled water immediately after use.

[6]Since black plates absorb light and reduce background, black plates are used for fluorescence measurements.

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6. **6.** Furimazine (Nano-Glo™ Luciferase Assay Substrate): An imidazopyrazinone substrate (furimazine) is available from Promega. Store at −20°C.
7. **7.** 2× luciferase substrate: Combine one volume of Nano-Glo™ Luciferase Assay Substrate with 50 volumes of BRET buffer.

## 3. Methods

### 3.1.Cell Culture and Transfection

All cell culture and transfection procedures should be carried out in a biological hood using sterile conditions. Transfection (step 2–6) should be performed with culture medium without antibiotics.

1. **1.** Maintain HEK293T/17 cells in 100-mm tissue culture dishes containing 10 ml culture medium at 37°C with 5% $CO_2$ (*see* Note 7).
2. **2.** On the day of transfection, coat 60-mm culture dishes with 2.5 ml of Matrigel solution by incubation for 10 min at 37°C (*see* Note 8).
3. **3.** Seed cells into the 6-cm dishes containing Matrigel solution at a density of $4\times 10^6$ cells/dish.
4. **4.** Incubate the cells at 37°C with 5% $CO_2$ for 4 h (see Note 9).
5. **5.** Transfect the cells with expression constructs (total 7.5 μg/dish) using PLUS (7.5 μl/dish) and Lipofectamine LTX (12μl/dish) reagents (see Note 10).
6. **6.** Culture the cells at 37°C with 5% $CO_2$ for 16–24 h.

### 3.2. Preparation of the Reagent Injectors

1. **1.** On the day of BRET measurement, fill the injectors with a solution of 10% bleach or 70% EtOH.
2. **2.** Leave the solution in the injector for 30 min.
3. **3.** Rinse the injectors a minimum of ten times with type 1 ultrapure water to remove the bleach or EtOH.
4. **4.** Fill the injectors with solution (lucifarase substrate, agonist, or antagonist) prior to use.

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[7]Care should be taken to avoid culturing cells close to confluence. Refresh cells from low passage number stock approximately once every two months.

[8]Treatment of tissue culture dishes with 10 μg/ml growth factor-reduced Matrigel decreases toxicity of DNA-transfection reagent complex without decreasing transfection efficiency. This treatment allows high amounts of DNA and Lipofectamine LTX to obtain high transfection efficiency.

[9]In our hands, tight adhesion of cells to dishes significantly improves efficiency and reproducibility of transfections with multiple constructs. Therefore, it is necessary to make sure that cells are tightly-adhered by observing them under a microscope prior to transfection.

[10]We generally transfect cells with G protein-coupled receptor, Gα, Venus155-239-Gβ1, Venus1-155-Gγ2, masGRKct-Nanoluc constructs at a 1:2:1:1:1 ratio(ratio 1 = 0.42 μg of plasmid DNA). The exact amounts of GPCR and Gα used for transfection may need to be optimized individually (*see* Subhead 3.6). An empty vector (*e.g.* pcDNA3.1) can be used to normalize the amount of transfected DNA ensuring equivalent levels of total DNA across all transfection conditions.

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### 3.3. Cell Preparation for BRET assay

1. Aspirate culture medium and wash the cells with 2.5 ml of EDTA/PBS.
2. Aspirate EDTA/PBS and add 1 ml of EDTA/PBS.
3. Incubate 5 min at room temperature.
4. Detach cells by gently pipetting, and transfer to a 15-ml tube.
5. Harvest remaining cells on a tissue culture dish with 1 ml of BRET buffer.
6. Centrifuge cells for 5 min at 500 × *g*, room temperature.
7. Discard the supernatant and resuspend the cells in 3 ml of BRET buffer.

### 3.4. Assessment of Protein Expression Levels by Measuring Fluorescence and Luminescence Intensities

1. Add 25 μl of the cell suspension (containing $5 \times 10^4$ to $1 \times 10^5$ cells) to each well of a white flat-bottom 96-well plate. Add 25 μl of 2× luciferase substrate, wait at least 3 min and measure total luminescence with no filter selection.
2. For fluorescence measurement, add 100 μl of the cell suspension to each well of a black flat-bottom 96-well plate. Measure fluorescence levels by exciting at 480 nm and recording the emission at 530 nm (*see* Note 11).

### 3.5. BRET Measurement

1. Add 25 μl of the cell suspension to each well of a white flat-bottom 96-well plate.
2. Add 25 μl of freshly prepared 2× luciferase substrate and mix the solution by tapping the plate with a hand.
3. Measure the BRET ratio after substrate addition (*see* Note 12).
4. Add test compounds (such as a receptor agonist or antagonist) by the automated reagent injectors and incubate with the cells for the desired period of time (*see* Notes 13 and 14).

### 3.6. Optimization of Gα and Venus-Gβγ ratio

Gα and Venus-Gβγ stoichiometry can be optimized by titrating the amount of Gα with a constant amount of Venus-Gβγ for transfection.

1. Transfect cells with a fixed amount of Venus-Gβγ and increasing amounts of the Gα (*see* Subhead 3.1).

[11]Subtract mean fluorescence intensity of mock-transfected cells (autofluorescence) from that of BRET sensor-transfected cells.

[12]Calculate the BRET ratio as follows:

BRET ratio = (emission of Venus at 535 nm with a 30 nm band path width)/(emission of Rluc at 475 nm with a 30 nm band path width).

[13]We usually apply 50 μl of 2× agonist followed by 50 μl of 3× antagonist.

[14]Agonist-induced changes in BRET ratio are calculated by subtracting the BRET ratio obtained before agonist application from the value after agonist application.

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2. Measure BRET ratio before agonist application and agonist-induced BRET amplitude (*see* Subhead 3.5).

3. Chose the transfection condition which produces minimum BRET ratio before agonist application and the maximum agonist-induced BRET signal.

### 3.7. Control Experiments for Specificity

The specificity of agonist-induced BRET signals can be examined [7]. The absence of agonist-induced changes in the BRET signal in the absence of exogenous GPCR or Gα indicates that the response is specifically mediated by the exogenous GPCR and Gα cotransfected into cells.

1. Transfect cells with the three different conditions, Gα only, GPCR only, or GPCR plus Gα together with BRET sensor pair, Venus-Gβγ and masGRK3ct-Rluc8. (*see* Subhead 3.1).

2. Measure agonist-induced changes in BRET signal (*see* Subhead 3.5).

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