Published in final edited form as:

Methods Mol Biol. 2019 ; 1957: 309–322. doi:10.1007/978-1-4939-9158-7_19.

Probing arrestin function using intramolecular FIAsH-BRET biosensors

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Summary:

Information contained in the structure of extracellular ligands is transmitted across the cell membrane through allosterically induced changes in G protein-coupled receptor (GPCR) conformation that occur upon ligand binding. These changes, in turn, are imprinted upon intracellular effectors like arrestins and help determine which of its many functions are performed. Intramolecular fluorescent arsenical hairpin (FIAsH) bioluminescence resonance energy transfer (BRET), in which both the fluorescence donor and acceptor are contained within the same protein, can be used to report on activation induced changes in protein conformation. Here, we describe a method using a series of *Rluc*-arrestin3-FIAsH-BRET biosensors to measure stimulus-induced changes in arrestin conformation in live cells. Each *Rluc*-arrestin3-FIAsH-BRET construct contains an N-terminal *Renilla* luciferase fluorescence donor that excites a fluorescent arsenical targeted to a different position within the protein by mutational insertion of a tetracycline tag motif. Changes in net BRET upon GPCR stimulation can thus be viewed from multiple vantage points within the protein and used to develop an arrestin3 “conformational signature” that is receptor- and ligand-specific. This method can be used to determine how differences in GPCR and ligand structure influence information transfer across the plasma membrane and to classify GPCRs and/or ligands based on their capacity to induce different arrestin3 activation modes.

Keywords

Arrestin; bioluminescence resonance energy transfer; fluorescent arsenical; G protein-coupled receptor; ligand efficacy; signal transduction

1. Introduction:

Despite their relatively simple architecture, arrestins perform remarkably diverse roles in cells [1]. The non-visual arrestins, arrestin2 and 3 (β -arrestin1 and 2), for example, are able to recognize and bind hundreds of different activated GPCRs and are integral to the control of GPCR desensitization, sequestration and intracellular trafficking. At the same time, they can interact with and localize dozens of cargo proteins, including signaling pathway intermediates that influence the tonic level of pathway activity and in some cases confer

GPCR-dependent regulation. Importantly, these actions are influenced by multiple factors, among them cell background, the GPCR to which the arrestin has bound, and the structure of the ligand, suggesting that, like receptors, arrestins can adopt conformationally discrete activation modes that specify the functions(s) they will perform.

But how does it work? High-resolution x-ray crystallography has provided atomic level structural information on arrestins alone and in complex with rhodopsin [2-6]. While these “static” structures provide critical details about the conformational rearrangements that occur upon arrestin activation, they are unable to capture the kinetics of the process or explain how receptor and ligand structure impose their effects on arrestin function. A useful approach to addressing these questions involves fluorescence or bioluminescence resonance energy probes (FRET or BRET). BRET is a phenomenon that results from non-radiative energy transfer between an enzymatically-activated luminescent substrate donor and a fluorophore acceptor. BRET occurs only when the donor and acceptor are in extremely close proximity (typically <10 nM) and changes in observed BRET are generally interpreted as movement of the donor and acceptor relative to each other. BRET was initially employed to examine regulated protein-protein interactions, e.g. arrestin recruitment to a GPCR [7]. In this setting, the donor, e.g. *Renilla* luciferase (*Rluc*) and acceptor, e.g. yellow fluorescent protein (YFP), are appended to distinct proteins. Due to the proximity requirement for energy transfer to occur, increases in intermolecular BRET can be interpreted as a protein-protein interaction. Alternatively, conformational shifts within a protein can be observed using an adaptation known as intramolecular BRET, in which both donor and acceptor are appended to a single protein, e.g. *Rluc*-arrestin-YFP [8]. In this case, changes in BRET may reflect a number of things including, but not limited to, conformational shifts within the protein of interest, steric interference of the donor and acceptor by recruitment of binding partners, or changes in the subcellular environment of the reporter.

A limitation of intramolecular BRET or FRET using conventional donor-acceptor pairs is that relatively large donor and acceptor proteins must be either appended to the N- and C-terminus of the protein of interest or inserted within the protein itself, potentially interfering with its function [8,9]. A less disruptive approach is to employ a small membrane-permeant fluorescein derivative with two arsen-(III) substituents (fluorescein arsenical hairpin binder or FAsH) as the acceptor along with either cyan fluorescent protein or *Rluc* as the FRET or BRET donor, respectively. The FAsH acceptor can then be targeted to multiple sites within the protein by mutational insertion of a small tetracysteine tag motif [10]. Intramolecular FAsH FRET or BRET has been used to report on activation-dependent conformational changes in arrestin3 [11,12], as well as to characterize the temporal relationships between GPCR-arrestin binding (measured by intermolecular FRET) and the conformational rearrangements that accompany arrestin activation (measured by intramolecular FAsH-FRET) [12]. Here, we describe a method using a series of *Rluc* -arrestin3-FAsH-BRET biosensors to measure stimulus-induced changes in arrestin conformation in live cells. The observed changes in BRET observed from multiple vantage points within the protein can be used to develop an arrestin3 “conformational signature” that is receptor- and ligand-specific and to classify GPCRs and/or ligands based on their capacity to induce different arrestin3 activation modes [11].

2. Materials

2.1. Design and validation of arrestin3 FIAsh-BRET biosensors

1. The series of six *Rluc*-arrestin3-FIAsh expression plasmids employed here were constructed by inserting a cDNA sequence encoding the tetra-cysteine motif, C-C-P-G-C-C, immediately following amino acid residues 77, 140, 171, 225, 263, and 410 of arrestin3 (see Figure 1A) into the previously described pcDNA3.1-*Rluc*-arrestin3 plasmid [13]. Constructs were prepared using a modification of the precise gene fusion polymerase chain reaction (PCR) method of Yon and Fried [14]. For each construct, two PCR steps were performed using the primer sets shown in Table 1. The first step was to generate two PCR fragments using the primer pairs: *Rluc*HindF/FlashR and FlashF/*Rluc*ApalR. One PCR product contained a *HindIII* restriction site at the 5' end and the C-C-P-G-C-C FIAsh motif at the 3' end, while the other contained the complementary FIAsh sequence at the 5' end and an *Apal* restriction site at the 3' end. A second PCR step was used to fuse the two fragments using three primers: *Rluc*HindF, FlashR and *Rlu*ApalR, and the two PCR fragments as template DNA. The resultant full-length arrestin3 PCR product containing the FIAsh motif insert was digested with *HindIII* and *Apal* and cloned into the parent pcDNA3.1-*Rluc*-arrestin3 plasmid to generate the *Rluc*-arrestin3-FIAsh1-6 expression plasmids. All constructs were verified by dideoxynucleotide sequencing. As shown in Figure 1B, the resultant *Rluc*-arrestin3-FIAsh mutants enable monitoring BRET between the N-terminal *Rluc* donor and a fluorescent arsenical acceptor located at various positions within N- and C-terminal globular domains of arrestin3 (see Note 1).
2. Confirmation that insertion of the tetra-cysteine motif does not compromise arrestin3 function was obtained by measuring inter-molecular BRET between each *Rluc*-arrestin3-FIAsh construct and the GPCR of interest bearing a C-terminal YFP tag (see Note 2). These assays are performed in the absence of the fluorescent arsenical to permit measurement of BRET between the FIAsh mutant *Rluc*-arrestin3 and GPCR-YFP, and change in net BRET (Net BRET) is compared with that of the unmodified parent *Rluc*-arrestin3 construct. Figure 1C shows the comparison of the Net BRET between *Rluc*-arrestin3 and each of six *Rluc*-arrestin3-FIAsh constructs upon recruitment to the type 1 human parathyroid hormone receptor (hPTH₁R). Note that five of the FIAsh constructs (F1, F2, F4, F5 and F6) produce a BRET signal similar to the control, while one construct (F3) does not generate a significant signal (see Note 3).

¹-This technique is adaptable to other allosterically regulated proteins, e.g. GPCRs themselves [15]. In selecting the site for insertion of the tetra-cysteine motif it is helpful to take advantage of x-ray crystallographic structures if available (Figure 1B). Optimal sites would be in flexible regions of the protein that might be expected to move upon activation and away from key structural elements necessary for protein folding and stability. Since arrestin activation is associated with displacement of the C-terminus and an approximately 20° rotation of the N and C domains relative to one another [4,6], we chose to place tetra-cysteine motifs near the C-terminus and at sites predicted to be on the surface of the N and C domains away from known sites of GPCR interaction.

²-*Rluc* luminescence cannot be visualized with sufficient resolution using microscopy equipment available to most laboratories, and cells loaded with FIAsh-EDT₂ may fluoresce diffusely upon direct excitation, making direct visualization of their recruitment to an activated GPCR difficult. We chose to validate that FIAsh mutant arrestins are functional by quantifying the efficiency of intermolecular BRET between each *Rluc*-arrestin3-FIAsh construct and a YFP-tagged GPCR.

2.2. Cell culture and transfection

1. pcDNA3.1 β Luc-arrestin3-FIAshH1-6 expression plasmids (Addgene; Plasmids #74129, #74130, #74131, #74132, #74133, #74134)
2. cDNA expression plasmid encoding the GPCR of interest without a fluorescent tag
3. HEK293 cells (ATCC; CRL1573)
4. Minimum essential medium (MEM) without additives
5. MEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution
6. FuGENE® HD transfection reagent (Promega Corporation)
7. 10 cm sterile tissue culture plates or 75 cm² tissue culture flasks
8. 6-well sterile tissue culture plates
9. Sterile 1.5 mL clear polypropylene microfuge tubes with snap caps

2.3. Intramolecular FIAsh BRET

1. Dulbecco's Phosphate Buffered Saline (DPBS)
2. DPBS containing 2 mM EDTA
3. Hank's Balanced Salt Solution (HBSS) with calcium and magnesium and without phenol red
4. TC-FIAsh™ In-Cell Tetracysteine Tag Detection Kit (Life Technologies Corp.). Each kit contains sufficient reagents for approximately 360 96-well plate wells. The FIAsh-EDT₂ Labeling Reagent is supplied as an 800X solution. To avoid repeated freeze-thaw cycles, store the FIAsh-EDT₂ Labeling Reagent in 5-10 μ L aliquots in microfuge tubes at -20 °C in the dark (see Note 4). BAL Wash Buffer is supplied as a 100X concentrate and is stored at 4 °C. BAL wash buffer should be diluted to 1X in HBSS at room temperature just before use.
5. BRET buffer: 1mM CaCl₂, 140mM NaCl, 2.7mM KCl, 900 μ M MgCl₂, 370 μ M NaH₂PO₄, 5.5mM d-glucose, 12mM NaHCO₃, 25mM HEPES, pH 7.4
6. Coelenterazine (NanoLight Technology): Prepare 2.2 mM stock solution by dissolving 10 mg coelenterazine in a solution of 10 mL absolute ethanol and 200 μ L 5N HCl. Store 1.0 mL aliquots in black microfuge tubes at -80 °C.
7. GPCR ligand stocks as appropriate for the experiment
8. TempAssure PCR 8-tube strips or clear round bottom 96-well plates for dispensing ligands and coelenterazine

³.In our experiments, we chose to include the F3 construct, which is poorly recruited to GPCRs, as an internal negative control on the experimental plate.

⁴.The FIAsh-EDT₂ Labeling Reagent is a bi-arsenical compound and toxic. Wear appropriate personal protective equipment when handling and discard used solutions as specified by your Institutional guidelines for toxic waste.

9. Opaque white 96-well plates for BRET assay
10. 8-Channel multi-channel pipettes (1-20 μ L)
11. Berthold Technologies Tristar 3 LB 941, Molecular Devices i3x Multi-Mode Detection Platform, or similar instrument with 485nm excitation and 525-585 emission filters (*see* Note 5)
12. Microsoft Excel®, GraphPad Prism® or similar spreadsheet software for data calculation, graphing, and statistical analysis

3. Methods

3.1. Cell culture and transfection

1. Maintain HEK293 cells in 10 cm tissue culture plates or 75 cm² flasks using MEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (*see* Note 6).
2. On Day 1, passage 90% confluent HEK293 cells into 6-well tissue culture plates at a seeding density that will attain approximately 50% confluence within 24 hours. Each individual well of a 6-well plate will yield sufficient cells for six wells of a 96-well plate. For small experiments, one or two wells of 6-well plate can be transfected with each reporter. Transfecting two wells with each reporter allows identically transfected cells to be labeled with FIAsh-EDT₂ Labeling Reagent for intramolecular FIAsh BRET or left unlabeled for determining background BRET (*see* **Step 3.2.8**).
3. On Day 2, transfect 50% confluent cells with one *Rluc*-arrestin3-FIAsh expression plasmid along with the GPCR of interest.
4. For each transfection, combine 94 μ L of plain MEM with 6 μ L of FuGENE® HD transfection reagent in a sterile plastic 1.5 mL microfuge tube. Vortex to mix and pulse in a microfuge. Allow to stand for 5 min at room temperature.
5. Add 1.0-1.5 μ g of cDNA expression plasmid encoding the untagged GPCR of interest, e.g. pCMV6-hPTH₁R, and 100-300 ng of one *Rluc*-arrestin3-FIAsh expression plasmid to each tube (*see* Note 7). Flick tubes to mix and pulse in a microfuge. Allow to stand at room temperature for 15 min.

⁵The method described here is for BRET measurements performed using a Berthold Technologies Tristar 3 LB 941, but is readily adaptable to other instruments. Note that intramolecular Net BRET signals are often small, on the order of 1-5%, so the plate reader must have sufficient sensitivity and stability to perform reliable measurements.

⁶Any readily transfectable cell line and transient transfection protocol can be used once optimized. The method described here has been optimized for HEK293 cells transfected using FuGENE® HD.

⁷The optimal ratio of GPCR and *Rluc*-arrestin3-FIAsh expression plasmids needs to be determined empirically. Since the Net BRET observed upon stimulation reflects the “population average” change in arrestin conformation, the most consistent results are obtained when experiments are performed in relative receptor excess, such that at steady state the maximum attainable fraction of *Rluc*-arrestin3-FIAsh reporter is receptor-bound. We recommend performing two test transfections: 1) transfect a fixed amount of *Rluc*-arrestin3-FIAsh reporter plasmid (200 ng) and increasing amounts of GPCR plasmid (0.1-1.5 μ g) to determine the quantity of GPCR cDNA at which the Net BRET signal plateaus; 2) transfect a fixed amount GPCR plasmid (1.0-1.5 μ g) and increasing amounts of *Rluc*-arrestin3-FIAsh reporter plasmid (10-500 ng) to determine the quantity of reporter plasmid that gives the most reproducible Net BRET signal. In the latter case, as quantity of *Rluc*-arrestin3-FIAsh reporter plasmid is decreased the Net BRET will become greater but BRET signal intensity will decrease leading to more variability. In our hands, 100-300 ng of *Rluc*-arrestin3-FIAsh reporter plasmid per well produces the most consistent and robust Net BRET signals.

6. Add the transfection mixtures to each well of a 6-well plate and return cells to the tissue culture incubator. Allow cells to incubate at 37 °C overnight.
7. On Day 3, aspirate and discard medium from cells. Replace with 2 mL/well of MEM containing 10% FBS and antibiotic/antimycotic and return cells to incubator. Allow cells to incubate at 37 °C overnight. Cells will be labeled on Day 4 after attaining 90% confluence.

3.2. FIAsH labeling the tetracysteine tag

1. On Day 4, aspirate and discard growth medium from cells. Wash monolayers 1X with DPBS to remove serum.
2. Detach cells by adding 1 mL of DPBS containing 2 mM EDTA to each well. Gently pipette cells and transfer to 1.5 mL microfuge tubes.
3. Centrifuge at 3,000 x g for 3 minutes to pellet cells. Aspirate and discard the supernatant. Resuspend the cell pellet in 500 µL of HBSS. Gently triturate several times to disperse cells.
4. Add 0.67 µL (2.5 µM final concentration) of FIAsH-EDT₂ Labeling Reagent from the TC-FIAsH™ In-Cell Tetracysteine Tag Detection Kit to each microfuge tube. Gently invert tubes 4-5 times to mix. Incubate at room temperature in the dark for 30 min (*see* Note 8).
5. Centrifuge at 3,000 x g for 3 minutes to pellet cells. Aspirate and discard the labeling medium. Resuspend cells by gentle trituration in 1 mL of room temperature 1X BAL Wash Buffer diluted from the 100X stock with HBSS.
6. Centrifuge at 3,000 x g for 3 minutes to pellet cells. Aspirate and discard the wash buffer. Resuspend cells in 650 µL of BRET buffer, gently triturating several times to ensure cells are evenly dispersed.
7. Transfer 100 µL of cells suspended in BRET buffer per well of an opaque white 96-well plate (approximately 1.5 x 10⁵ cells/well) as needed for the experiment (*see* Notes 9 and 10). After preparing the experimental plate, wrap it in aluminum foil and maintain it in the dark at room temperature until ready to perform the experiment.

⁸The optimum final concentration of FIAsH-EDT₂ Labeling Reagent may vary from 1-10 µM and labeling time from 30-60 min. For labeling *Rluc*-arrestin3-FIAsH proteins in detached HEK293 cells we find that using 2.5 µM final concentration FIAsH-EDT₂ Labeling Reagent and labeling for 30 min gives consistent results.

⁹The optimal cell number per well will vary somewhat between different plate readers and should be determined empirically in pilot assays.

¹⁰For a simple experiment, e.g. generating an *Rluc*-arrestin3-FIAsH BRET signature in response to agonist exposure, we might transfect two wells of a 6-well plate with each pcDNA3.1*Rluc*-arrestin3-FIAsH1-6 expression plasmid along with the receptor of interest. Following the protocol above each transfected well provides enough transfected cells for six wells of a 96-well plate. Transfecting two wells with each reporter permits cells isolated from one well to be labeled with FIAsH-EDT₂ Labeling Reagent to measure intramolecular FIAsH-BRET, while cells from the second well are processed identically except for omitting the labeling reagent to measure background BRET. Cells expressing the reporters would be arrayed in a 6 row X 8 column grid on a single 96-well plate, with each row representing a different *Rluc*-arrestin3-FIAsH construct and each column representing a ligand or vehicle treatment. The first two columns are for unlabeled vehicle-treated cells (background BRET), and the next six columns are for labeled vehicle- and agonist-treated cells (FIAsH-BRET). We recommend using at least two adjacent wells for each ligand-reporter condition (technical replicates) per plate so that CV can be determined for quality control.

8. To determine background BRET, it is necessary to prepare cells from identically transfected wells following steps 3.2.1-7, except for omitting the FIAsh-EDT₂ Labeling Reagent from step 3.2.4. The background BRET ratio is that measured in the absence of the fluorescence acceptor, which in this case is achieved by omitting the fluorescent bi-arsenical (*see Step 3.5.2*).

3.3. Preparing the drug plate

1. Prepare vehicle and ligand solutions during the 30 min FIAsh labeling period.
2. Dilute ligand stocks to 12X desired final concentration in BRET buffer (*see Note 11*). Prepare nonstimulated controls using the same dilution of ligand vehicle in BRET buffer.
3. Aliquot ~15 μ L of 12X ligand per experimental well into PCR tube strips or round bottom 96-well drug plates, whichever is more convenient. Stimulations will be carried out using a multi-channel pipettor to transfer ligand one row at a time so the drug plate must be laid out in a grid corresponding to the layout of the experimental plate (*see Note 12*).

3.4. Intramolecular FIAsh BRET assay

1. Just before use, dilute the 2.2 mM stock coelentraine solution coelentraine to 12X desired final concentration (60 μ M) in BRET buffer. Once added to the cell plate the final coelentraine concentration will be 5 μ M. Transfer the 12X solution into PCR tube strips or round bottom 96-well drug plates for dispensing with a multi-channel pipettor.
2. Using an appropriate multi-mode plate reader, verify *Rluc-arrestin3*-FIAsh expression and tetracycline tag labeling by measuring TC-FIAsh fluorescence with an excitation wavelength of 485 nm and emission wavelength of 535 nm.
3. Using a multi-channel pipettor transfer 10 μ L of 12X ligand from the PCR tube strips or round bottom 96-well drug plate to the corresponding wells in the experimental plate. Pipette up and down several times to ensure mixing. Change pipette tips between rows to avoid mixing ligands or cells expressing different reporters.
4. Allow cells to pre-incubate with ligand for 0-10 min at room temperature before adding coelentraine (*see Notes 13 and 14*).

¹¹Since the most consistent *Rluc-arrestin3*-FIAsh BRET profiles are obtained when the maximum fraction of reporter is GPCR-bound, stimulations are usually carried out at saturating ligand concentration. It may be helpful to perform an initial ligand concentration-response curve to determine the ligand concentration that gives the maximum Net BRET signal. In the example shown here, hPTH(1-34) stock (0.1 mM in sterile distilled water) was diluted to 1.2 μ M in BRET Buffer (12X final concentration) to yield a final ligand concentration of 0.1 μ M in the assay well.

¹²Depending on the experiment, e.g. performing concentration response curves, it may be more practical to prepare ligands in a 96-well drug plate. If using a microplate reader equipped with auto-injectors, it will be necessary to prepare both ligands and coelentraine in 96-well plates.

¹³Both the time and temperature of ligand stimulation are relevant variables and should be determined empirically. Since coelentraine should be added just prior to reading, when performing a time course experiment, stagger the addition of drug to the wells and add coelentraine to all wells simultaneously. In our hands, the Net BRET ratios produced by *Rluc-arrestin3*-FIAsh reporters are stable from 2 and 10 min after ligand stimulation and we routinely pre-incubate for 3 min with ligand before adding coelentraine (5 min ligand exposure before reading).

5. Using a multi-channel pipettor transfer 10 μ L of coelentrastine solution from the PCR tube strips or round bottom 96-well drug plate to the corresponding wells in the experimental plate. Pipette up and down several times to ensure mixing. Change pipette tips between rows to avoid mixing ligands or cells expressing different reporters.
6. Return the experimental plate to the plate reader and allow 2 min for the *Rluc* emission to stabilize, then record 6 consecutive 1 sec readings of *Rluc* (485 nm) luminescence and TC FAsH fluorescence (530 nm; YFP setting) at 1 min intervals. Total read time in 5 min, beginning 2 min after the addition of coelentrastine.

3.5. Data analysis and interpretation

1. The BRET ratio is the TC FAsH fluorescence signal (530 nm) over the *Rluc* signal (485 nm) measured simultaneously. This ratio is automatically calculated by the software of multi-mode plate readers and exported as a Microsoft Excel spreadsheet.
2. Determine the Net BRET for each ligand-stimulated condition relative to vehicle-stimulated in the same experiment. For each well, the mean of the 6 consecutive reads is calculated to obtain the average BRET ratio. The Net BRET ratio for each well is then calculated by subtracting the background BRET ratio measured in cells in the same experiment that lack the BRET acceptor, i.e. were not labeled with FAsH-EDT₂, from the mean BRET ratio observed in FAsH-EDT₂ labeled cells. The Net BRET is then obtained by dividing the Net BRET ratio obtained in stimulated cells by the Net BRET ratio measured in cells exposed to vehicle only. This ratio represents the fractional change in BRET occurring upon ligand stimulation, and is typically $\pm$ 0.01-0.05 using our *Rluc*-arrestin3-FAsH reporters. If duplicate or triplicate wells are used, a coefficient of variance (CV) can be determined and the mean Net BRET technical replicates on the plate can be used as a datum for determining the mean Net BRET across multiple biological replicates of the experiment (*see* Note 15).
3. The GPCR-specific “conformational signature” of activated arrestin3 can be displayed graphically plotting the Net BRET observed upon ligand stimulation for each of the six *Rluc*-arrestin3-FAsH reporters as in Figure 1D (*see* Note 16).

¹⁴-We have also performed ligand stimulations at 37 °C to determine whether incubation temperature affected the *Rluc*-arrestin3-FAsH BRET profile. For stable arrestin-binding GPCRs [16], e.g. the PTH₁R, temperature has no significant effect on the profile, even though incubation at 37 °C allows the GPCR-arrestin complexes to exit the plasma membrane and transit to early endosomes.

¹⁵-In our hands the observed Net BRET for GPCR-stimulated change in *Rluc*-arrestin3-FAsH BRET is reproducible enough that five to six biological replicates are sufficient to discern effects of $\pm$ 0.01 with $p < 0.05$.

¹⁶-It should be evident that an *Rluc*-arrestin3-FAsH1-6 BRET profile is not a “conformational signature” *per se* in that changes in intramolecular BRET reflect both changes in the distance/orientation of the donor and acceptor fluorophores due to conformational rearrangement, and steric effects generated by arrestin interaction with its receptor and non-receptor binding partners. Thus, it is not possible to ascribe the *Rluc*-arrestin3-FAsH BRET signal at a given position to specific conformational shifts or engagement of binding partners. The utility of the method is in comparing/classifying the effects of different ligand/GPCR complexes on the arrestin3 signature. For example, we have found that features of the signature are conserved between receptors with similar arrestin binding/signaling characteristics, and that the Net BRET at selected positions correlates with downstream arrestin function, e.g. ‘class A’ versus ‘class B’ trafficking [16] and arrestin-dependent ERK1/2 activation, suggesting that *Rluc*-arrestin3-FAsH BRET probes can predict arrestin function based on the ligand-induced conformational signature [11].

Acknowledgements:

This work was supported by National Institutes of Health Grants R01 DK055524 (LML), R01 GM095497 (LML), Department of Veterans Affairs Merit Review Grant I01 BX003188 (LML), and the Research Service of the Charleston, SC Veterans Affairs Medical Center. The contents of this article do not represent the views of the Department of Veterans Affairs or the United States Government.

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Figure 1:

Construction, validation and use of arrestin3 FIAsH-BRET biosensors. **A.** Linear representation of a panel of six *RLuc*-arrestin3-FIAsH BRET reporters (F1-F6) constructed by inserting the tetracycysteine motif, C-C-P-G-C-C, following amino acid residues 77, 140, 171, 225, 263, and 410 of *RLuc*-arrestin3. The location of each FIAsH motif is shown in relation to the globular N and C domains of arrestin3, as well as the clathrin and adapter protein 2 (AP2) binding sites and reported phosphorylation sites (Ser³⁶¹ and Thr³⁸³) in the arrestin3 C-terminal regulatory (R2) domain. **B.** Three-dimensional computational model of *RLuc*-arrestin3-FIAsH1-6 showing the spatial orientation of the six tetracycysteine motifs as yellow space-filling molecules relative to the *RLuc* moiety. The FIAsH molecule is depicted as bound to the F1 site. The structure of the N-terminal *Renilla* luciferase is shown in blue. The position of the *RLuc* is highly flexible in this model and was manually placed for ease of representation. **C.** Typical intermolecular BRET results demonstrating the efficiency of ligand-dependent recruitment of *RLuc*-arrestin3 FIAsH1-6 to the hPTH₁R. The bar graph depicts Mean $\pm$ SEM of independent biological replicates (n=3). **D.** Representative intramolecular *RLuc*-arrestin3 FIAsH1-6 BRET signature produced by interrogating the hPTH(1-34) stimulated hPTH₁R with each of the six biosensors shown in panel A. The bar graph depicts Mean $\pm$ SEM of independent biological replicates (n=5). In panels C and D; * p<0.05, # p<0.005 greater or less than vehicle stimulated control.

TABLE 1:Primer sequences used to generate *Rluc-arrestin3-FlAsH1-6*

Primer	Sequence
RlucHindF	ATCAAGCTTGCGTTACCGGATCCATGGGTGAA
RlucApaIR	AACGGGCCCTCTAGACTAGCAGAACTGGTCA
FlAsH1F	GGATCCTGTCGATGGTTGTTGTCCTGGTTGTTGTGTGGTGCTTGTGGATC
FlAsH1R	GATCCACAAGCACACACAACAACCAGGACAACAACCATCGACAGGATCC
FlAsH2F	GAGGACACAGGGAAGTGTGTCCTGGTTGTTGTGCCTGTGGAGTAGAC
FlAsH2R	GTCTACTCCACAGGCACAACAACCAGGACAACACTTCCCTGTGTCCTC
FlAsH3F	GCTTATCATCAGAAAGTGTGTCCTGGTTGTTGTGTACAGTTTGCTCCTG
FlAsH3R	CAGGAGCAAAGTGTACACAACAACCAGGACAACACTTTCTGATGATAAGC
FlAsH4F	CCACGTCACCAACAATTGTTGTCCTGGTTGTTGTTCTGCCAAGACCGTCA
FlAsH4R	TGACGGTCTTGGCAGAACACAACCAGGACAACAATTGTTGGTGACGTGG
FlAsH5F	AGCTTGAACAAGATGACCAGTGTGTCCTGGTTGTTGTGTGTCTCCAGTCCACATT
FlAsH5R	AATGTGGAAGTGGGAGACACACAACAACCAGGACAACACTGGTCATCTTGTTCAAGCT
FlAsH6R	ACGGGCCCTCTAGACTAACAACAACCAGGACAACAGCAGAACTGGTCATC