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Monitoring GPCR-β-arrestin1/2 interactions in real time living systems to accelerate drug discovery

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COVER LETTER

Dear Editor,

We would be grateful if the final version of our manuscript entitled “Monitoring GPCR-b-arrestin1/2 interactions in real time living systems to accelerate drug discovery” could be reviewed one last time and considered for publication in Journal of Visualized Experiments.

Changes were made for this final version of our manuscript.

1. Based on comments from the reviewers we rearranged a few sentences within the text protocol and we created a new Fig. 2 where we show how our GPCR-β-arrestin assay works according to the request of reviewer # 2.

2. Based on your editorial requests we made minor modifications to the manuscript following each one of your suggestions (e.g. reducing the use of personal pronouns, you, your, we etc.) and in all the text and figures we included spaces between the numbers and units. In addition we included e-mail addresses from all the authors of this manuscript.

We really appreciate your editorial efforts and valuable time

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TITLE:

Monitoring GPCR-β-arrestin1/2 interactions in Real Time Living Systems to Accelerate Drug Discovery

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KEYWORDS:

β-arrestins, G-protein coupled receptors, drug discovery, living systems, real time, structural complementation assay, drug development.

SUMMARY:

GPCR-β-arrestin interactions are an emerging field in GPCR drug discovery. Accurate, precise and easy to set up methods are necessary to monitor such interactions in living systems. We show a structural complementation assay to monitor GPCR-β-arrestin interactions in real time living cells, and it can be extended to any GPCR.

ABSTRACT:

Interactions between G-protein coupled receptors (GPCRs) and β-arrestins are vital processes with physiological implications of great importance. Currently, the characterization of novel drugs towards their interactions with β-arrestins and other cytosolic proteins is extremely valuable in the field of GPCR drug discovery particularly during the study of GPCR biased agonism. Here, we show the application of a novel structural complementation assay to accurately monitor receptor-β-arrestin interactions in real time living systems. This method is simple, accurate and can be easily extended to any GPCR of interest and also it has the advantage that it overcomes unspecific interactions due to the presence of a low expression promoter present in each vector system. This structural complementation assay provides key features that allow an accurate and precise monitoring of receptor-β-arrestin interactions, making it suitable in the study of biased agonism of any GPCR system as well as GPCR c-terminus 'phosphorylation codes' written by different GPCR-kinases (GRKs) and post-translational modifications of arrestins that stabilize or destabilize the receptor-β-arrestin complex.

INTRODUCTION:

GPCRs represent the target of nearly 35% of current drugs in the market[1,2] and a clear understanding of their pharmacology is crucial in the development of novel therapeutic drugs[3]. One of the key aspects in GPCR drug discovery, particularly during the development of biased agonists is the characterization of novel ligands towards receptor-β-arrestin interactions[4] and β-arrestin interactions with other cytosolic proteins such as clathrin[5].

It has been documented that β-arrestin dependent signaling plays a key role in neurological disorders such as bipolar disorder, major depression, and schizophrenia[6] and also severe side effects in some medications such as morphine[7].

Current methods used to monitor these interactions usually do not represent actual endogenous levels of the proteins in study, in some cases they show weak signal, photobleaching and depending of the GPCR it might be technically challenging to set up[8]. This novel structural complementation assay uses low expression promoter vectors in order to mimic endogenous physiological levels and provides high sensitivity compared to current methods[9]. Using this approach, it was possible to easily characterize Galanin receptor-β-arrestin1/2 and also β-arrestin2-clathrin interactions[10]. This methodology can be widely used to any GPCR of particular interest where β-arrestins play a key physiological function or their signaling is relevant in some diseases.

PROTOCOL:

1. **Primer design strategy**

1.1. Design primers to introduce genes of interest into pBiT1.1-C [TK/LgBiT], pBiT2.1-C [TK/SmBiT], pBiT1.1-N [TK/LgBiT] and pBiT2.1-N [TK/SmBiT] Vectors.

1.2. Select at least one of these three sites as one of the two unique restriction enzymes needed for directional cloning due to the presence of an in-frame stop codon that divides the multicloning site as shown in **Figure 1**[11].

1.3. Incorporate nucleotide sequence into the primers as shown in **Table 1** to encode the linker residues shown in red in **Table 2**[11].

1.4. For pBiT1.1-C [TK/LgBiT] and pBiT2.1-C [TK/SmBiT] vectors, make sure that the 5′ primer contains an ATG codon and a potent Kozak consensus sequence (GCCGCCACC).

1.5. For pBiT1.1-N [TK/LgBiT] and pBiT2.1-N [TK/SmBiT] vectors, ensure that the 3′ primer contains a stop codon.

NOTE: Each vector contains the HSV-TK promoter to minimize nonspecific association and reduce experimental artifacts, also each vector has an expression cassette for ampicillin resistance in bacteria.

2. PCR

2.1. Set up and run PCR reactions to amplify the insert DNA of the gene of interest using the primers designed from step 1. It is important to use a high-fidelity DNA polymerase to minimize mutations.

2.2. Use exactly the following order to prepare 50 µL of PCR reaction. Add 35.5 µL of distilled water, 5 µL of 10x Polymerase buffer, 5 µL of dNTP mixture (2.5 mM each), 1 µL of plasmid template (200 ng/µL), 1.25 µL of forward and reverse primers (10 µM) and 1 µL of high-fidelity polymerase (5 U/µL).

2.3. Using a thermocycler set up the following DNA amplification program.

2.3.1. Denature at 95 °C for 5 min.

2.3.2. Repeat 25 times the following thermal cycle: 95 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min per 1 kbp to be amplified.

2.3.3. Run a final extension at 72 °C for 10 min.

2.3.4. Hold the samples at 4 °C within the thermocycler.

NOTE: It is highly recommended to use a high-fidelity polymerase in order to minimize point mutations particularly those occurring during the amplification of long sequences. As the amplicon becomes longer, the degree of accuracy in the replication of the DNA decreases. For the PCR reaction, choose the annealing temperature based on the melting temperature of the region where the oligos directly hybridize with the DNA template and not with all the sequence of the primer.

2.4. PCR product purification

2.4.1. Isolate the PCR product from the rest of the PCR reaction using a kit from a manufacturer of preference[12]. The PCR product is now ready for restriction digestion.

3. DNA digestion

3.1. For the PCR product digestion prepare 50 µL of digestion reaction as follows.

3.1.1. Using a 1.5 mL tube, add 12 µL of distilled water.

3.1.2. Add 5 µL of 10x buffer with the best compatibility with both restriction enzymes.

3.1.3. From step 2.4 add 30 µL of PCR product

3.1.4. Finally, add 1.5 µL of each restriction enzyme.

3.1.5. Briefly mix by vortex and incubate at 37.5 °C overnight.

3.2. For the recipient plasmid digestion prepare 50 µL of digestion reaction as follows:

3.2.1. Using a 1.5 mL tube, add 23 µL of distilled water.

3.2.2. Add 5 µL of 10x buffer with the best compatibility with both restriction enzymes.

3.2.3. Add 15 µL of recipient plasmid (200 ng/µL).

3.2.4. Add 1.5 µL of each restriction enzyme.

3.2.5. Briefly mix by vortex and incubate at 37.5 °C overnight.

NOTE: It is important to use 3 µg of recipient plasmid in order to obtain sufficient material after DNA agarose gel purification. It is also relevant to leave DNA digestions overnight using both enzymes to obtain high cloning efficiency.

4. DNA agarose gel purification and cloning

4.1. Prepare a 1% agarose gel to run the digested DNA plasmid and inserts and proceed to cut the corresponding bands. Once the corresponding vector and insert bands have been purified12, determine the DNA concentration using a spectrophotometer.

4.2. Perform DNA ligation to fuse the insert to the recipient plasmid.

4.3. Prepare ligation reactions of around 100 ng of total DNA including 50 ng of plasmid vector.

4.4. Set up recipient plasmid-insert ratio of approximately 1:3; it can be calculated using a vector-insert calculator[13].

4.5. Set up negative controls in parallel. For instance, a ligation of the recipient plasmid DNA without any insert will provide information about how much background of undigested or self-ligating recipient plasmid is present.

5. Transformation of clones

5.1. Place a tube of DH5α competent cells from the freezer at -80 °C and immediately transfer it on ice for 20 min.

5.2. After that time, take 55 µL of DH5α competent cells and add 4 µL of ligation reaction and mix by flicking the tube and store on ice for 45 min.

5.3. Place the tube in a water bath previously warmed at 42 °C for exactly 48 s and immediately get the tubes back on ice for another 3 min.

5.4. Add 600 µL of Luria Broth (LB) medium previously warmed at 37.5 °C and incubate with shaking for 1 hour at 200 rpm.

5.5. Transfer 200 µL into an agar plate containing ampicillin 100 µg/mL and gently spread over the surface with the liquid is mostly absorbed.

5.6. Incubate the plates overnight to see the colonies next morning. The recipient plasmid on the insert plate should have significantly more colonies than the recipient plasmid alone plate.

6. Isolation of the finished plasmid

6.1. Pick 3-10 individual bacterial colonies and transfer into 1 mL of LB medium containing ampicillin (100 µg/mL) and incubate for 6 h.

6.2. Take 200 µL of bacterial suspension and transfer to 5 mL of LB medium containing the same concentration of ampicillin as in step 6.1 and incubate overnight at 37.5 °C with shaking at 200 rpm.

6.3. Using a miniprep DNA kit purification, perform miniprep DNA purifications using 5 mL of LB grown overnight following the manufacturer instructions[14].

6.4. To identify successful ligations, set up PCR reactions in the same way as in section 2 using the DNA obtained from step 6.3 as a template with the same primers as in section 2 during the first PCR. Positive clones will produce PCR products with the corresponding size.

6.5. After large prep DNA purification of the positive clones, conduct a diagnostic restriction digestion of 500 ng of purified DNA with the enzymes used during the cloning step and run the digested products on an 1% agarose gel. There should be two bands: one the size of the vector and one the size of the new insert.

6.6. Verify the construct sequence by sequencing using the following primers: Forward 5'-aaggtgacgcgtgtggcctcgaac-3' and reverse 5'-gcatttttttcactgcattctagtt-3'.

NOTE: When DNA is replicated using PCR, there is always the possibility of errors during the amplification even when using a high-fidelity polymerase, therefore is very important to sequence the final constructs.

7. Transfection and protein expression

7.1. In a previously poly-L-lysine-coated white 96 well plate, perform cell seeding one day before transfection at 2.5 x 10^4 cells per well using Dulbecco's modified Eagle's medium supplemented with 10% of Fetal Bovine Serum, 100 U/mL penicillin G, and 100 µg/mL streptomycin.

7.2. Use only the 60 inner wells to minimize the potential for thermal gradients across the plate and edge effects from evaporation. Add 200 µL of sterile distilled water to the 36 outside wells and 150 µL in the spaces between wells and incubate overnight at 37.5 °C and 5 % of CO_2.

7.3. The following morning perform transfection using 100 ng of total DNA (50 ng each construct).

7.4. Set up four different plasmid combinations (receptor:β-arrestin) according to **Figure 2b**.

7.5. For each plasmid combination use 20 µL of modified Eagle's Minimum Essential Media buffered with HEPES using 0.3 µL of lipidic transfection reagent per well.

7.6. Add 20 µL of lipidic transfection reagent-DNA mixture to each well and mix the plate in circles for 10 s.

7.7. Change fresh medium after 6 hr incubation at 37.5 °C and 5% CO_2.

7.8. Incubate the plate for 24 h at 37.5 °C and 5 % CO_2.

8. Monitoring receptor-β-arrestin1/2 interactions in HEK293 cells

8.1. Aspirate medium and add 100 µL of modified Eagle's Minimum Essential Media buffered with HEPES to each well and let the plate stabilize at RT for 10 min.

8.2. Prepare the furimazine substrate by combining 1 volume of 100x substrate with 19 volumes of LCS Dilution Buffer (a 20-fold dilution)[11], creating a 5x stock to mix with cell culture medium.

8.3. Add 25 µL of 5x furimazine to each well and gently mix in circles for 10 s.

8.4. Measure luminescence for 10 min for signal stabilization at RT.

NOTE: By using this baseline signal to normalize the response of each well it will help to reduce variability caused by differences in the number of cells plated per well, also differences in transfection efficiency, etc. Once calculated, average the normalized response from replicate wells for a given drug treatment.

8.5. Prepare 13.5x ligand solution in modified Eagle's Minimum Essential Media buffered with

HEPES.

8.6. For experiments at room temperature with 10 µL of 13.5x ligand addition, add the compounds using injectors or a multichannel pipette and mix the plate by hand or using an orbital shaker (20 s at 200 rpm).

8.7. For experiments at 37.5 °C with 10 µL of 13.5x ligand addition, use injectors to dispense compounds and mix by using the instrument orbital shaker. In case of not using injectors, remove the plate from the luminometer, add the ligands and mix the plate by hand or using an orbital shaker (20 s at 200 rpm).

NOTE: Use injectors and a shaker within the detection instrument to minimize temperature fluctuations associated with removing the plate from the luminometer. Standard benchtop luminometers can be used for this assay. Use an integration time of 0.25–2 s.

REPRESENTATIVE RESULTS:

Using the procedure presented here, interactions between a prototypical GPCR and two β-arrestin isoforms were monitored. Glucagon like peptide receptor (GLP-1r) constructs were made using primers containing NheI and EcoRI enzyme restriction sites and cloned into the vectors pBiT1.1-C [TK/LgBiT] and pBiT2.1-C [TK/SmBiT] while in the case of β-arrestins, two additional vectors were used pBiT1.1-N [TK/LgBiT] and pBiT2.1-N [TK/SmBiT] using enzyme restriction sites BglII and EcoRI in the case of β-arrestin2 and NheI and XhoI in the case of β-arrestin1. HEK293 cells were transfected using 50 ng of GLP-1r-LgBiT/SmBiT and 50 ng of β-arrestin tagged with LgBiT or SmBiT at the N- or C-terminal. Four different plasmid combinations were screened (**Figure 3**) and the one with the highest luminescent signal was chosen for further experiments (**Figure 4**). In order to determine the EC50 values for each β-arrestin isoform recruitment, dose response curves were performed using 10 µM, 1 µM, 100 nM, 10 nM and 1 nM of GLP-1 ligand concentration (**Figure 4c**). Dose response curves were obtained from the maximum response of each concentration from the kinetic studies (**Figure 4a, 4b**).

FIGURE AND TABLE LEGENDS:

Figure 1. Nucleotide sequences of the multicloning sites of the vectors used in the design of GLP-1r-β-arrestin1/2 structural complementation assay. In order to develop the structural complementation assay for GLP-1r-β-arrestin1/2 system, it was necessary to tag at the C-terminal the GLP-1r with LgBiT and SmBiT using the enzyme restrictions NheI and EcoRI at the pBiT1.1-C [TK/LgBiT] and pBiT2.1-C [TK/SmBiT] vector. In the case of β-arrestin1/2 they also were tagged with the LgBiT and SmBiT at the C- and N-terminal using the enzyme restrictions BglII/EcoRI for β-arrestin2 and NheI/XhoI for β-arrestin1 at the four vectors. All vectors use the HSV-TK promoter to minimize nonspecific association and reduce experimental artifacts and each vector contains an expression cassette for ampicillin resistance in bacteria. Image adapted from reference 11.

Figure 2. Schematic representation of the GPCR:β-arrestin1/2 structural complementation assay. (**a**) How the GPCR:β-arrestin1/2 structural complementation assay works in the presence of ligand. (**b**) Structural representation of the different plasmid combinations for the GPCR and β-arrestin isoforms tagged with LgBiT or SmBiT.

Figure 3. GLP-1r/β-arrestins orientation screening. In order to obtain the highest sensitivity 4 different plasmid combinations were expressed during 24 h after transfection. Luminescent signals were detected in almost all different plasmid combinations (**a-c**) except for only one GLP-1r:β-arrestin orientation (**d**). The results are expressed as mean ± S.E.M. of two experiments performed in duplicate; each duplicate was averaged before calculating the S.E.M. The arrows indicate the time at which the cells were treated with GLP-1 at 10 μM final concentration.

Figure 4. GLP-1r-β-arrestin1/2 interactions are dose dependent manner. Dose dependent ligand relationship of β-arrestin2 (**a**) and β-arrestin1 recruitment (**b**). Dose response curves showing differential recruitment between β-arrestin1 and β-arrestin2 by GLP-1r (**c**). The results are expressed as mean ± S.E.M. of two experiments performed in duplicate; each duplicate was averaged before calculating the S.E.M. The arrows indicate the time at which the cells were treated with GLP-1 at the corresponding concentrations (10 μM, 1 μM, 100 nM, 10 nM and 1 nM, final concentrations).

Table 1. Sequences of primers for the different restriction enzyme sites in the coding sequence of the linker for the pBiT1.1 and pBiT2.1 Vectors. SI = Sequence of interest; Rev SI = reverse complementary of the sequence of interest. Table adapted from reference 11.

Table 2. Linker amino acid sequences related with SacI, EcoRI or XhoI restriction sites in the pBiT1.1 and pBiT2.1 Vectors. Red residues have to be encoded by PCR primers. Table adapted from reference 11.

DISCUSSION:

Using the method presented here, interactions between any GPCR and β-arrestin1/2 can be monitored in real time living systems using this GPCR-β-arrestin structural complementation assay. In this regard, we were able to observe differential β-arrestin recruitment between the two β-arrestin isoforms by the GLP-1r (A prototypical Class B GPCR), we also observed a dissociation of the receptor-β-arrestin complex a few minutes after reaching the maximum luminescent signal.

In order to have the best sensitivity in the structural complementation assay system, it was screened with four different spatial orientations between the receptor and each β-arrestin isoform and the one with highest luminescent signal was used for posterior studies such as dose response stimulation curves (**Figure 4**). Using this methodology it was possible to characterize receptor-β-arrestin interactions using a GPCR of high therapeutic value in endocrinological diseases such as Diabetes mellitus[15]. In the same way this strategy can be easily adapted to any GPCR by simple tagging the GPCR of interest with LgBiT or SmBiT at the C-terminal and using the

β-arrestin1/2 constructs described here and it emerges as a powerful alternative to current methodologies without the necessity of a complex set up where the overlapping between the donor and acceptor can be an obstacle as in some cases of BRET and FRET. Another significant advantage is that the vectors used in this system contain low expression promoters in an attempt to mimic endogenous expression levels. With this feature, we can rule out the possibility of non-specific associations due to high expression levels of the receptor and/or β-arrestin. In **Figure 3** and **Figure 4**, there is a clear difference in the receptor-β-arrestin complex between β-arrestin1 versus β-arrestin2 and also a higher efficacy and intensity towards β-arrestin1 over β-arrestin2. The screening into four different orientations was proposed to increase the sensitivity of the assay making the system highly sensitive even at endogenous expression levels.

This methodology is very straight forward to perform. Perhaps the most critical step within this protocol is the primer design to amplify the receptor of interest. The user must be very careful in selecting what restriction enzyme to use according to **Figure 1** and based on this to add the corresponding nucleotides to the primers (**Table 1**) to encode the red highlighted amino acids (**Table 2**).

One limitation of this methodology can be that the furimazine will degrade in an aqueous solution at or near physiological pH, leading to a gradual decrease in luminescence intensity independent of any change in GPCR-β-arrestin interactions[16]. To overcome this limitation the user should always include a normalization control (vehicle treated samples) when continuously monitoring luminescence for extended time periods. It is also important to use low levels of fetal bovine serum during the assay since its presence it might increase the rate of furimazine degradation[16]. One problem that may arise during the assay is that for luminescent values for a known GPCR-β-arrestin interaction can be no significant increase is registered compared to the base line values in all four different plasmid combinations. This can be due to the low expression from the HSV-TK promoter[17]. In that case, one alternative is to subclone the Open Reading Frames encoding LgBiT and SmBiT fusion proteins into expression vectors using the CMV promoter. When changing to a stronger promoter, optimization of the amount of transfected DNA should be done in order to obtain the best assay response.

Using this structural complementation assay we were able to observe with great accuracy the β-arrestin1/2 recruitment interactions by a prototypical class B GPCR. Using this method, it is possible to pharmacologically characterize novel drugs of particular interest targeting GPCRs.

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DISCLOSURES:
The authors declare no competing interests.

REFERENCES:

1. Sriram, K., Insel, P. A. GPCRs as targets for approved drugs: How many targets and how many drugs?. *Molecular Pharmacology.* **93** (4), 251-258, 10.1124/mol.117.111062 (2018).

2. Hauser, A. S., Attwood, M. M., Rask-Andersen M., Schiöth, H. B., Gloriam, D. E. Trends in GPCR drug discovery: new agents, targets and indications. *Nature Reviews Drug Discovery.* **16** (12), 829-842. 10.1038/nrd.2017.178 (2017).

3. Langmead, C. J., Summers, R. J. Molecular pharmacology of GPCRs. *British Journal of Pharmacology.* **175** (21), 1754005–4008. 10.1111/bph.14474 (2018).

4. Lohse, M. J., Hoffmann, C. Arrestin Interactions with G Protein-Coupled Receptors. *Handbook of Experimental Pharmacology.* **219**, 15-56. 10.1007/978-3-642-41199-1_2 (2014).

5. Kang, D. S. et al. Structure of an arrestin2-clathrin complex reveals a novel clathrin binding domain that modulates receptor trafficking. *Journal of Biological Chemistry.* **284**, 29860-29872. 10.1074/jbc.M109.023366 (2009).

6. Park, S. M. et al. Effects of β-Arrestin-Biased Dopamine D2 Receptor Ligands on Schizophrenia-Like Behavior in Hypoglutamatergic Mice. *Neuropsychopharmacology.* **41** (3), 704-15. 10.1038/npp.2015.196 (2016).

7. Zhu, L., Cui, Z., Zhu, Q., Zha, X., Xu, Y. Novel Opioid Receptor Agonists with Reduced Morphine-like Side Effects. *Mini-Reviews in Medicinal Chemistry.* **18** (19), 1603-1610. 10.2174/1389557518666180716124336 (2018).

8. Smith, J. S., Lefkowitz, R. J., Rajagopal, S. Biased signalling: from simple switches to allosteric microprocessors. *Nature Reviews Drug Discovery.* **17** (4), 243-260. 10.1038/nrd.2017.229 (2018).

9. Dixon, A. S. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chemical Biology.* **11** (2), 400–408. 10.1021/acschembio.5b00753 (2016).

10. Reyes-Alcaraz, A., Lee, Y. N., Yun, S., Hwang, J. I., Seong, J. Y. Conformational signatures in β-arrestin2 reveal natural biased agonism at a G-protein-coupled receptor. *Communications Biology.* **3** 1:128. 10.1038/s42003-018-0134-3 (2018).

11. Promega. Nanobit Protein Protein Interaction System Protocol. https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/nanobit-protein-protein-interaction-system-protocol.pdf?la=en (2019).

12. Life Biomedical. HiYield Gel/PCR Fragments Extraction Kit. https://www.lifebiomedical.com/uploads/2/4/7/2/24727678/gel_pcr_dna_fragments_extraction_kitydf_protocol_v3.0.pdf (2019).

13. New England BioLabs. Ligation Calculator. https://nebiocalculator.neb.com/#!/ligation (2019).

14._Cosmo Genetech. http://www.cosmogenetech.com/cosmo/productmngr/prm11.jsp?P_BR_CD=1&P_CTG_CD=1&P_PRODUCT_CD=1&P_N_PAGE=1&P_CAT_POS= (2019).

15. Baggio, L. L., Drucker, D. J. Biology of incretins: GLP-1 and GIP. *Gastroenterology.* **132,** 2131–2157. 10.1053/j.gastro.2007.03.054 (2007).

16. ProMega. NanoGLO Endurazine and Vivazine Live Cell Substrates Technical Manual. https://www.promega.com/-/media/files/resources/protocols/technical-manuals/500/nano-glo-endurazine-and-vivazine-live-cell-substrates-technical-manual.pdf?la=en (2019).

17. Ali R., Ramadurai S., Barry F., Nasheuer H. P. Optimizing fluorescent protein expression for quantitative fluorescence microscopy and spectroscopy using herpes simplex thymidine kinase promoter sequences. **8** (6), 1043–1060. 10.1002/2211-5463.12432 (2018).

Figure

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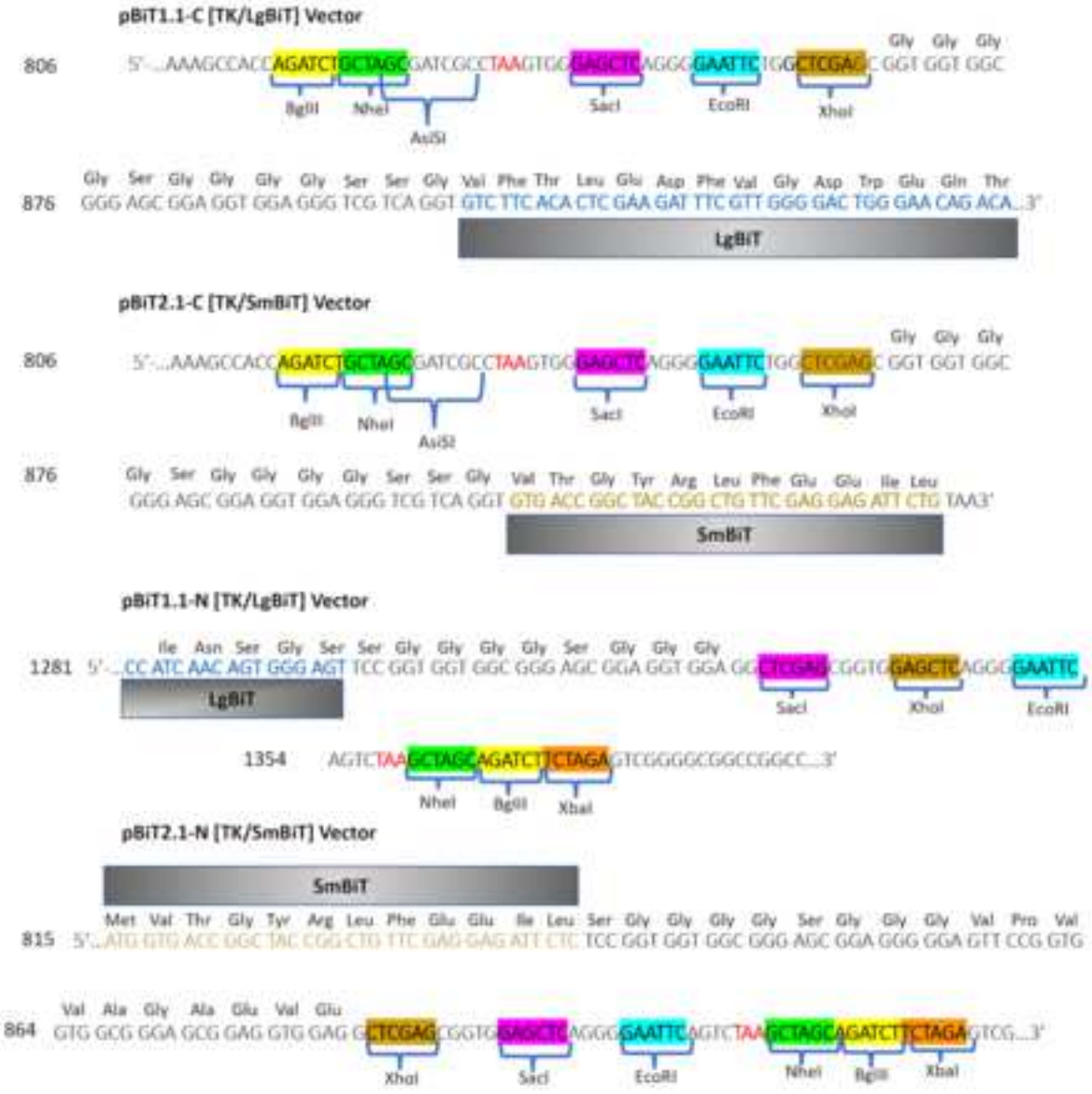

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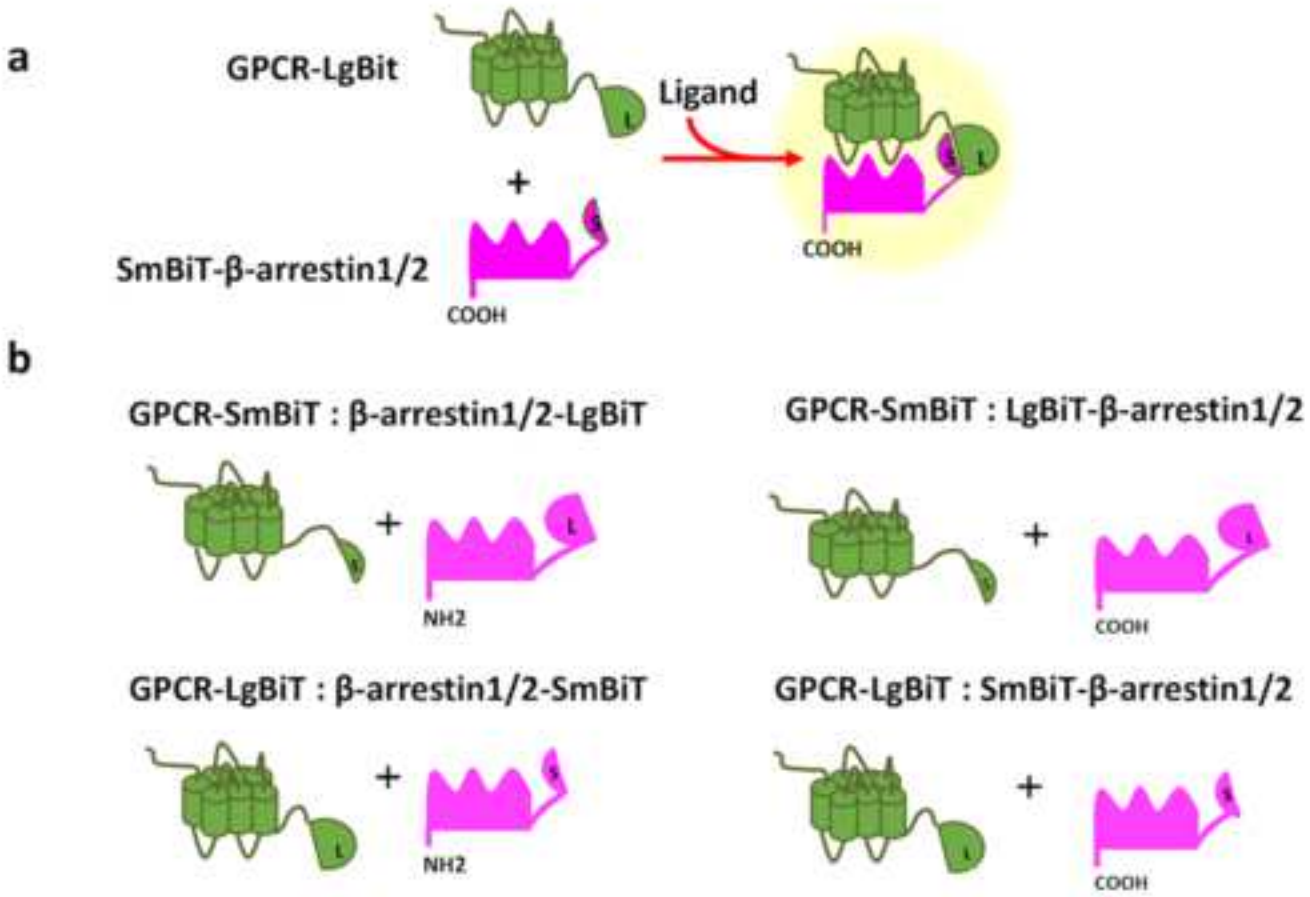

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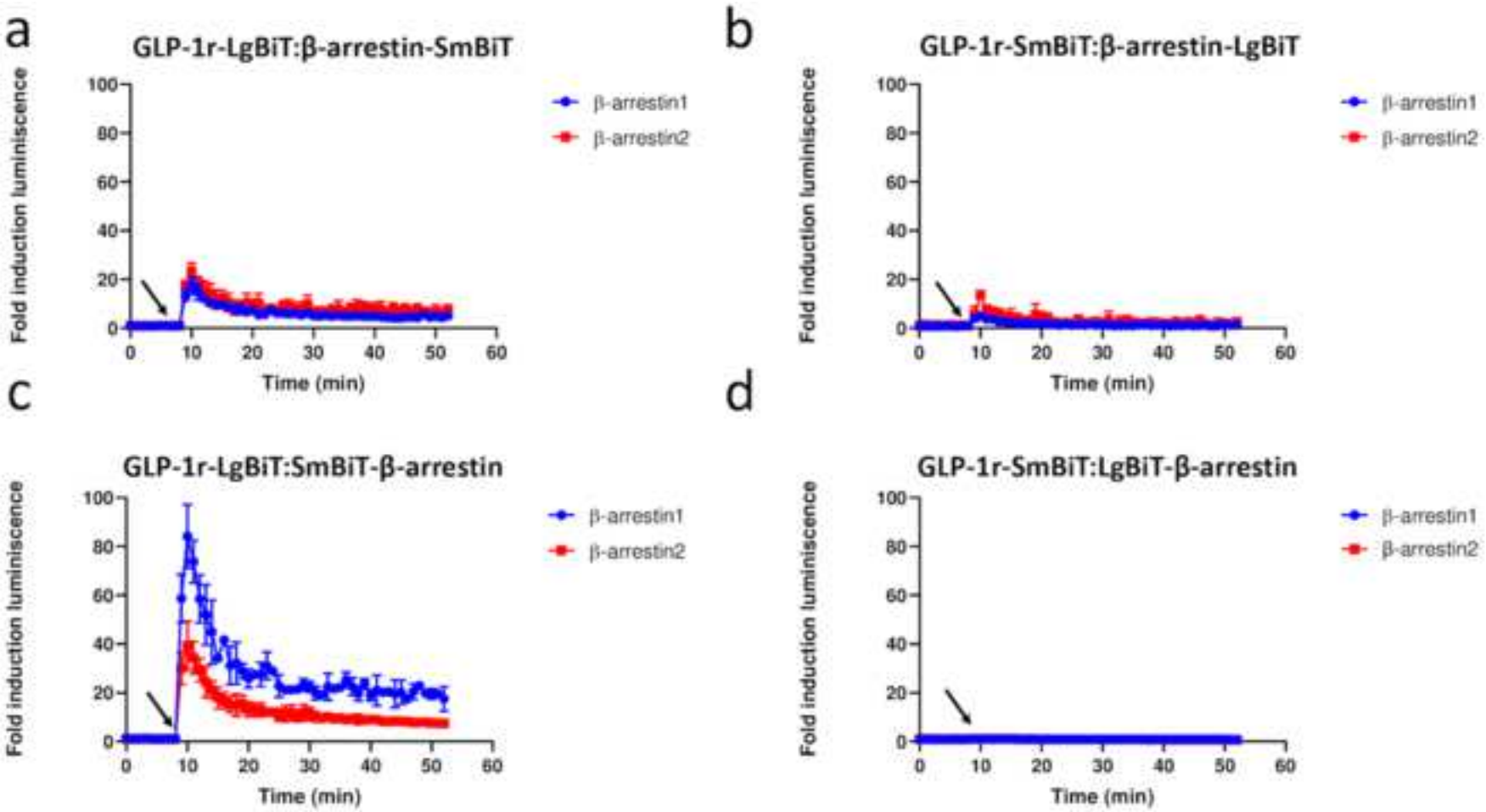

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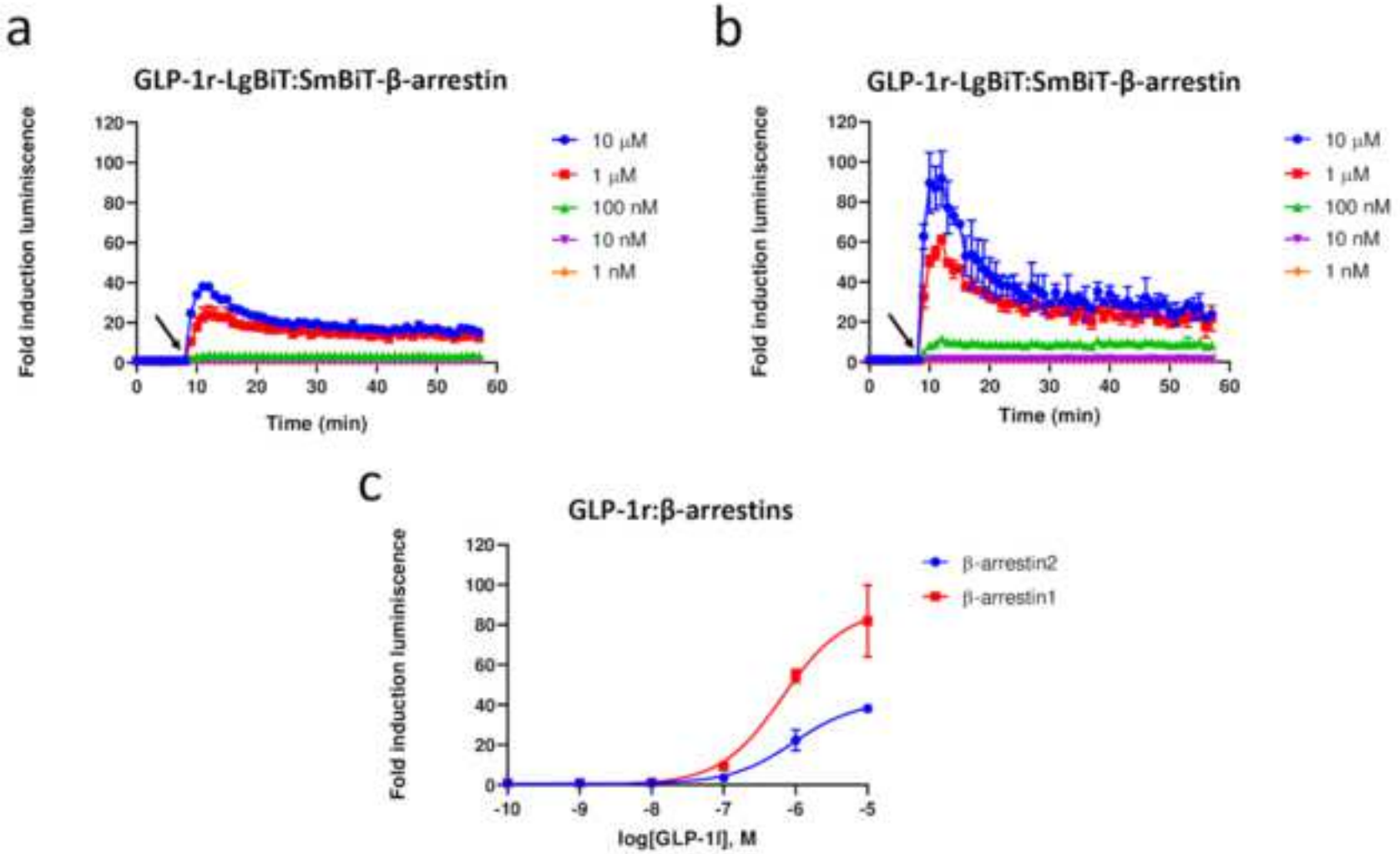

Table

Vector	Enzyme restriction used
pBiT1.1-C [TK/LgBiT] / pBiT2.1-C [TK/SmBiT]	SacI EcoRI XhoI Xho
pBiT1.1-N [TK/LgBiT] / pBiT2.1-N [TK/SmBiT]	SacI EcoRI

Primer sequence

5′-XXXXXXXXGAGCTC**C**(Rev SI)-3′

5′-XXXXXXXXGAATTC**CC**(Rev SI)-3′

5′-XXXXXXXXCTCGAG**CC**(Rev SI)-3′

5′-XXXXXXXXCTCGAG**CGGT** (SI)-3′

5′-XXXXXXXXGAGCTC**AG**(SI)-3′

5′-XXXXXXXXGAATTC**A**(SI)-3′

Table

Vector
pBiT1.1-C [TK/LgBiT] / pBiT2.1-C [TK/SmBiT]
pBiT1.1-N [TK/LgBiT] / pBiT2.1-N [TK/SmBiT]

Linker sequence

SI-**Gly**AlaGlnGlyAsnSerGlySerSerGlyGlyGlyGlySerGlyGlyGlyGlySerSerGly-(LgBiT/SmBiT)

SI-**Gly**AsnSerGlySerSerGlyGlyGlyGlySerGlyGlyGlyGlySerSerGly-(LgBiT/SmBiT)

SI-**Gly**SerSerGlyGlyGlyGlySerGlyGlyGlyGlySerSerGly-(LgBiT/SmBiT)

(LgBiT/SmBiT)-GlySerSerGlyGlyGlyGlySerGlyGlyGlyGlySer**SerGly**-SI

(LgBiT/SmBiT)-GlySerSerGlyGlyGlyGlySerGlyGlyGlyGlySerSerGlyGlyAla**Gln**-SI

(LgBiT/SmBiT)-GlySerSerGlyGlyGlyGlySerGlyGlyGlyGlySerSerGlyGlyAlaGlnGlyAsnSer-SI

Enzyme restriction used

SacI

EcoRI

XhoI

XhoI

SacI

EcoRI

Name of Material/ Equipment	Company
Antibiotics penicillin streptomycin	Welgene
Bacterial Incubator	JEIO Tech
Cell culture medium	Welgene
Cell culture transfection medium	Gibco
CO2 Incubator	NUAIRE
Digital water bath	Lab Tech
DNA Polymerase proof reading	ELPIS Biotech
DNA purification kit	Cosmogenetech
DNA Taq Polymerase	Enzynomics
Enzyme restriction BglII	New England Biolabs
Enzyme restriction buffer	New England Biolabs
Enzyme restriction EcoRI	New England Biolabs
Enzyme restriction NheI	New England Biolabs
Enzyme restriction XhoI	New England Biolabs
Fetal Bovine Serum	Gibco Canada
Gel/PCR DNA MiniKit	Real Biotech Corporation
Ligase	ELPIS Biotech
Light microscope	Olympus
lipid transfection reagent	Invitrogen
Luminometer	Biotek/Fisher Scientific
NanoBiT System	Promega

Nanoluciferase substrate	Promega
PCR Thermal cycler	Eppendorf
Poly-L-lysine	Sigma Aldrich
Trypsin EDTA	Gibco
White Cell culture 96 well plates	Corning

Catalog Number	Comments/Description
LS202-02	Penicillin/Streptomycin
IB-05G	Incubator (Air-Jacket), Basic
LM 001-05	DMEM Cell culture medium
31985-070	Optimem 1X cell culture medium
NU5720	Direct Heat CO2 Incubator
LWB-122D	Digital water bath lab tech
EBT-1011	PfU DNA polymerase
CMP0112	miniprepLaboPass Purificartion Kit Plasmid Mini
P750	nTaq DNA polymerase
R0144L	BglII
B72045	CutSmart 10X Buffer
R3101L	EcoRI-HF
R01315	NheI
R0146L	XhoI
12483020	Fetal Bovine Serum
KH23108	HiYield Gel/PCR DNA MiniKit
EBT-1025	T4 DNA Ligase
CKX53SF	CKX53 Microscope Olympus
11668-019	Lipofectamine 2000
12504386	Synergy 2 Multi-Mode Microplate Readers
N2014	NanoBiT PPI MCS Starter System

N2012	Nano-Glo Live Cell assay system
6336000015	Master cycler Nexus SX1
P4707-50ML	Poly-L-lysine solution
25200-056	Trysin EDTA 10X
3917	Assay Plate 96 well plate

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Title of Article:
Monitoring GPCR-B-arrestin1/2 interactions in real time living systems to accelerate drug discovery

Author(s):
Arfaxad Reyes-Alcaraz, Yoo-Na Lee, Seongsik Yun, Jong-Ik Hwang, & Jae Young Seog

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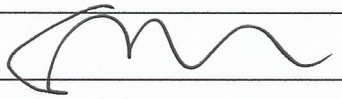
Signature:	
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Editorial comments:

First of all, many thanks for your comments to improve our manuscript. We carefully made changes following your observations as you will read in our manuscript using track changes on for a better identification of our modifications.

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Following your suggestion, we carefully revised all the manuscript there are no grammar nor spelling issues.

2. Please revise lines 89-91, 105-106, 111-113, and 170-172 to avoid overlap with previous publications.

According to your observation we made changes in the lines you mentioned above in order to avoid overlapping with previous publications. Please see lines 144-149, 271-273, 277-279 and 415-417.

3. Please reduce the use of personal pronouns (we, you, your).

Personal pronouns were reduced from all the manuscript according to your suggestion.

4. Please include email addresses for all authors in the manuscript.

All the e-mail addresses for all authors were included in the manuscript, please see lines 13-17.

5. Please include at least 6 key words or phrases.

More key words and phrases were added, please see lines 20-21.

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For example: NanoBiT, Lipofectamine, Nano-Glo, Optimem

All commercial terms were substituted by the corresponding generic names instead and all the commercial products are referenced in Table of Materials and Reagents.

Protocol:

1. Is the bold text intended to be highlighted for filming? Please use yellow highlighting instead, and ensure the total highlighted length is less than 2.75 pages.

We properly yellow highlighted the text for filming.

2. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. 2, 3, etc.: Even though PCR, digestion, etc., are common molecular biology procedures, please include detailed steps if you intend to film them.

We included details in the steps we intend to film particularly the steps 2 and 3 were written in detail. Please see lines (128-142, 156-269) and all the yellow highlighted text was also writen in better detail and referenced.

2. 8.4, 8.6: Please include more information on luminescence measuring (e.g., how to use the relevant instrument).

More information about this was added, particularly for the sub steps 8.4 and 8.6. Please see lines (506-509 and 626-636).

Figures:

1. Figure 3: Please include spaces between numbers and units (e.g., ‘100 nM’). Please also define the error bars in the legend.

In Figure 3 (Now Figure 4), spaces were included between the numbers and units and also error bars were defined in the corresponding legend.

Discussion:

1. Discussion: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique

Following your recommendation in the discussion section were explicitly covered with citations the following:

a) Critical steps in the protocol (Please see lines 887-891)
b) Troubleshooting of the technique (Lines 898-904)
c) Possible limitations of the technique (Lines 892-897)

References:
1. Please do not abbreviate journal titles.

In the reference section the name of the journals was modified according to your recommendation. Please see references section.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

The Table of Materials was carefully revised, and it has all materials and equipment used in the manuscript.

Reviewers' comments:

Reviewer #1:

The manuscript is well written and well described and may be acceptable for publication after the following review

Thank you so much for your comments towards our manuscript and we made some changes in our manuscript following your helpful comments as follows:

1. Figure 2 describes the testing of GLP-1r/b-arrestin orientation and in Figure 2 it shows that the orientation GLP-1r-SmBIT: LgBit-b-arrestin does not generate productive protein complementation and hence no light is generated from this construct upon stimulus. However, in Figure 3, the same orientation is used to generate ligand titration data. Is this correct?

Thanks to your observation. Thanks to you we realized about this mistake in the previous Figure 3 (now Figure 4) particularly in the description where is shown the orientation corresponding to the maximal luminescent response. We properly changed the orientation description in the Figure of the ligand titration data (Figure 4).

Reviewer #2:

Manuscript Summary:
The manuscript by Reyes-Alcaraz et al describes the experimental setup, plasmid design & functional validation of their luciferase fragment complementation assay based on the NanoBit platform marketed by Promega. Using the functional reconstitution of Nluc, the ligand-induced protein:protein interaction between GLP receptor and beta arrestin could be demonstrated in HEK2963 cells. As proposed by the authors, the approach can be used to study the association of beta arrestin with G-protein coupled receptors and with a wide application for pharmacological characterization of novel drugs for receptor activation. I trust that the protocol described will be of interest to the readers working on drug screening, receptor dimerization as well as post-receptor signaling.

We really appreciate your helpful comments to improve our manuscript. We carefully revised your suggestions that we addressed as follows:

Minor Concerns:
As a whole, the manuscript is well written with detailed descriptions on plasmid design & execution of the experiment. My comments are only minor and can be summarized as follows:

(i) A schematic diagram to illustrate the principle of the luciferase fragment complementation assay will be really useful for the authors who are new to the NanoBit technology.

We properly designed a schematic representation about how the GPCR-β-arrestin1/2 structural complementation assay works and also it shows the different plasmid combinations that were screened in order to select the one with the maximal response. Please see the new Figure 2.

(ii) Fig.1 and Table 1 & 2 are direct copy of the information from the manual of NanoBiT Protein:Protein Interaction System (N2014) from Promega. I suggest doing some "modifications/reformatting" with the figure/tables or with proper citation of the source of information from Promega to avoid the copy right issue.

Figure 1 and Tables 1 and 2 were substantially modified to avoid overlapping with the manual of NanoBiT from Promega. In addition, we also properly cited the NanoBiT manual to avoid copy right issues. Please see new Fig. 1 and Tables 1 & 2 as well as their respective legends (lines 655-661, 808-814).

(iii) Fig.3a & 3b, the meaning of the arrow in this figure is unclear. I suppose it is referring to the time of the introduction of ligand to activate the GLP receptor expressed in HEK293 cells. If so, please add the information on the dose & type of ligand (e.g., GLP ?) used in these experiments in the respective legends.

The meaning of the arrows in Figure 3 and 4 is when the cells were treated with the corresponding concentration of GLP-1. This is properly indicated in the respective figure legends. Please see lines 792-806.

(iv) Line 186-187, 192-19*4, 205 & 207, please insert a space between the number and the respective unit (e.g., 6 hr, 100 ul & 10 min). Make similar changes for other parts of the manuscript as well.

In all parts of the manuscript, were inserted spaces between the number and the respective unit.