ACS MEASUREMENT SCIENCE Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Open Access
This article is licensed under CC-BY-NC-ND 4.0

pubs.acs.org/measureau
Letter

Accessible and Generalizable in Vitro Luminescence Assay for Detecting GPCR Activation

Published as part of the ACS Measurement Science Au virtual special issue "2023 Rising Stars".

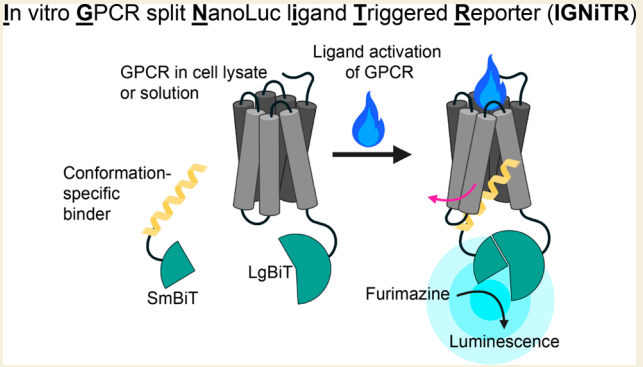
Ruby M. Miller,[#] Jennifer Sescil,[#] Marina C. Sarcinella, Ryan C. Bailey, and Wenjing Wang*

Cite This: *ACS Meas. Sci. Au* 2023, 3, 337−343

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: G protein-coupled receptors (GPCRs) serve critical physiological roles as the most abundant family of receptors. Here, we describe the design of a generalizable and cell lysate-based method that leverages the interaction between an agonist-activated GPCR and a conformation-specific binder to reconstitute split nanoluciferase (NanoLuc) in vitro. This tool, **I**n vitro **G**PCR split **N**anoLuc ligand **T**riggered **R**eporter (IGNiTR), has broad applications. We have demonstrated IGNiTR's use with three G_s-coupled GPCRs, two G_i-coupled GPCRs and three classes of conformation-specific binders: nanobodies, miniG proteins, and G protein peptidomimetics. As an in vitro method, IGNiTR enables the use of synthetic G protein peptidomimetics and provides easily scalable and portable reagents for characterizing GPCRs and ligands. We tested three diverse applications of IGNiTR: (1) proof-of-concept GPCR ligand screening using dopamine receptor D1 IGNiTR; (2) detection of opioids for point-of-care testing; and (3) characterizing GPCR functionality during Nanodisc-based reconstitution processes. Due to IGNiTR's unique advantages and the convenience of its cell lysate-based format, this tool will find extensive applications in GPCR ligand detection, screening, and GPCR characterization.



KEYWORDS: *In vitro, Luminesce assay, GPCR, Split NanoLuc, High-throughput screening, Nanodisc*

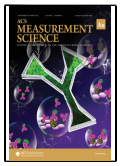
INTRODUCTION

G protein-coupled receptors (GPCRs) play essential roles in many physiological processes.[1,2] Hence, GPCRs remain crucial targets for therapeutic development, with approximately 30% of U.S. Food and Drug Administration-approved therapeutics targeting GPCRs.[3] Live cell-based assays have been instrumental for GPCR drug screening, as well as GPCR signaling and mechanistic studies.[4−13] However, there is still a lack of accessible and generalizable in vitro methods for detecting GPCR activation, which will complement live cell assays for broad applications. For example, it is infeasible with live cell-based assays to validate the functionality of extracted GPCRs for biochemical and structural studies. Most of the existing in vitro assays, including radioligand binding assays, monitor GPCR-ligand binding but do not measure ligand efficacy for inducing the active conformation that couples to G proteins.[4,14−19] Therefore, there is still a need for an in vitro assay that measures the GPCR-G protein interaction induced by the ligands.

Split nanoluciferase (NanoLuc),[20] which reconstitutes and gains its activity only when brought into proximity, has been widely applied to detect protein−protein interactions (PPIs) in live cells.[11,21−29] Notably, NanoLuc generates a luminescence signal that can be quantifiable in complex biological environments. Therefore, we harnessed the split NanoLuc to track ligand-induced GPCR-G protein binding in a cell lysate-based system.

Here, we designed a highly adaptable GPCR luminescent assay for use in cell lysates and in solution. IGNiTR (**I**n vitro **G**PCR split **N**anoLuc ligand **T**riggered **R**eporter) utilizes the agonist-dependent GPCR conformational change and subsequent recruitment of G proteins and other conformation-specific binders[30,31] to reconstitute split NanoLuc[24,26,32] (Figure 1A). Unlike in live cell-based assays, IGNiTR components are easily stored frozen and portable as cell pellets that express GPCRs preserved in their native lipid environment. Additionally, the in vitro environment enables

Received: May 9, 2023
Revised: June 11, 2023
Accepted: June 20, 2023
Published: July 7, 2023



© 2023 The Authors. Published by American Chemical Society

https://doi.org/10.1021/acsmeasuresciau.3c00021
ACS Meas. Sci. Au 2023, 3, 337−343

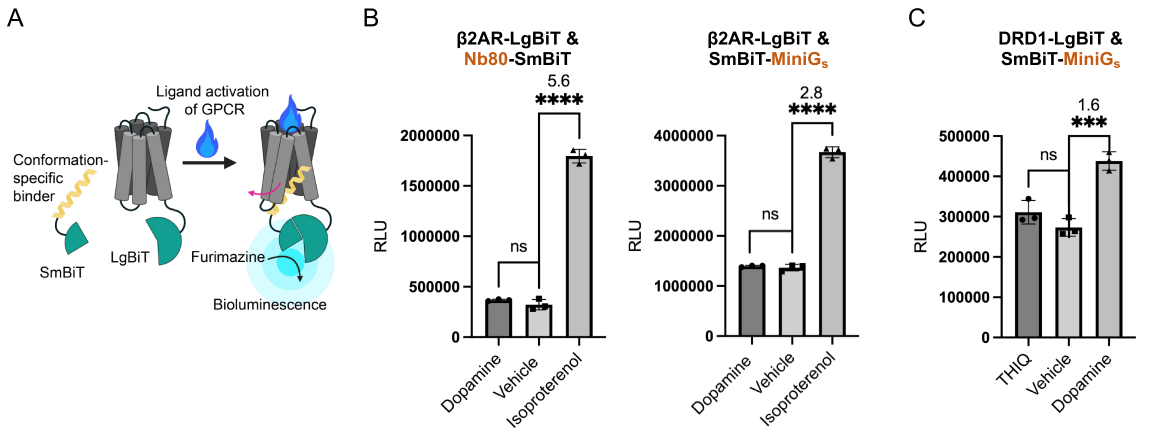


Figure 1. Characterization of IGNiTR with miniG$_s$ and a conformation-specific nanobody. (A) Schematics of the IGNiTR assay: LgBiT attached to a GPCR and SmBiT attached to a conformation-specific binder. Ligand activation of a GPCR results in a GPCR-conformation-specific binder interaction, triggering split NanoLuc reconstitution. (B) Characterization of β2AR-based IGNiTR with Nb80 or miniG$_s$ as the conformation-specific binder. (C) Characterization of DRD1-based IGNiTR with miniG$_s$ as the conformation specific binder. Drug, 10 μM. RLU: Relative luminescent units. $n = 3$ technical replicates. Values above the bars represent the DDR. Asterisks indicate significance after performing an unpaired Student's t test. ***$P < 0.001$, ****$P < 0.0001$. "ns" indicates no significant difference.

the use of peptidomimetics as conformation-specific binders, expanding the range of assay applications.

We have demonstrated the versatility of IGNiTR through three applications. First, IGNiTR demonstrated a robust Z′ value in a proof-of-concept high-throughput screening of ligands using dopamine receptor D1 (DRD1) IGNiTR. Second, IGNiTR detected the mu-opioid receptor (MOR) agonist fentanyl in the nanomolar range in an easy and portable setup for potential point-of-care implementation. Finally, IGNiTR was used to characterize the GPCR functionality of samples at different stages of the Nanodisc-based GPCR extraction and reconstitution process. IGNiTR's adaptability enables unique applications that are complementary to existing live cell-based and biochemical assays.

RESULTS AND DISCUSSION

Developing the IGNiTR Assay

The IGNiTR assay is inspired by live cell-based split NanoLuc assays but has the potential to bring more versatility and accessibility to detect GPCR activation outside of the live cell context.[21] As shown in Figure 1A, IGNiTR is composed of two parts: the GPCR fused to one fragment of the split NanoLuc and a conformation-specific binder fused to the other fragment of the split enzyme. We tested the IGNiTR assay using β2-adrenergic receptor (β2AR) with two conformation-specific binders: nanobody 80 (Nb80) which binds specifically to the activated β2AR[33−36] and miniG$_s$ which can bind to a number of activated G$_s$-coupled receptors.[34,37] Using β2AR-LgBiT and SmBiT-miniG$_s$, we tested cell lysis conditions with and without detergent that contains 1% (w/v) DDM (*n*-dodecyl β-D-maltoside and 0.1% (w/v) CHS (cholesteryl hydrogen succinate). We characterized the ratio of the IGNiTR luminescence with drug to that without drug, which is referred to as the drug-dependent ratio (DDR) in this paper. Both protocols produced significant DDRs, indicating that miniG$_s$ could selectively bind to the active conformation of β2AR in cell lysate (see Figure S1). We found that the detergent-containing lysis condition generates a higher DDR due to lower luminescence in the agonist-free condition. However, sonication-based cell lysis without detergent generates a higher luminescence signal than the condition with detergent (Figure S1), presumably because the β2AR function can be better preserved in its native lipid environment without detergent.[38] To facilitate the preservation in a native lipid environment, we used detergent-free sonication for cell lysate-based IGNiTR reagent preparation for the remaining studies in this paper.

We then further characterized the IGNiTR assay composed of β2AR-LgBiT with Nb80-SmBiT or SmBiT-miniG$_s$ with drug, vehicle, and a nonagonist small molecule as a control (Figure 1B). The agonist (isoproterenol) produced significantly increased luminescence compared to the vehicle and a DDR of 5.6, but the nonagonist control molecule dopamine did not produce a significant increase. This significant difference between the agonist and vehicle was also demonstrated in an independent biological replicate (Figure S2). However, we found the absolute value of the DDRs of the IGNiTR assay varies in different experiments, which could result from different IGNiTR component expression levels. Similar variations have been observed in multicomponent live cell-based reporter systems.[39] We also titrated isoproterenol for β2AR-LgBiT with SmBiT-miniG$_s$ using both IGNiTR and a live cell-based split NanoLuc assay (Figure S3). IGNiTR was less sensitive, producing a higher EC$_{50}$ value compared to the live cell-based split NanoLuc assay.

Next, the generalizability of IGNiTR was evaluated by testing miniG$_s$ with another G$_s$-coupled GPCR, DRD1. The DRD1 agonist dopamine produced a DDR of 1.6, while a nonagonist small molecule (THIQ) did not produce a statistically significant difference from the vehicle condition (Figure 1C).

Overall, these studies show that IGNiTR can detect a GPCR's agonist-dependent conformational change in the cell lysate. Furthermore, these results suggest that miniG$_s$ is generally applicable for G$_s$-coupled GPCRs. Even though IGNiTR is less sensitive than the live cell-based assay, IGNiTR has its own advantages as a convenient and accessible in vitro assay, including the capability to store components frozen and achieve consistency across experiments by pooling large batches of components together.

https://doi.org/10.1021/acsmeasuresciau.3c00021
ACS Meas. Sci. Au 2023, 3, 337−343

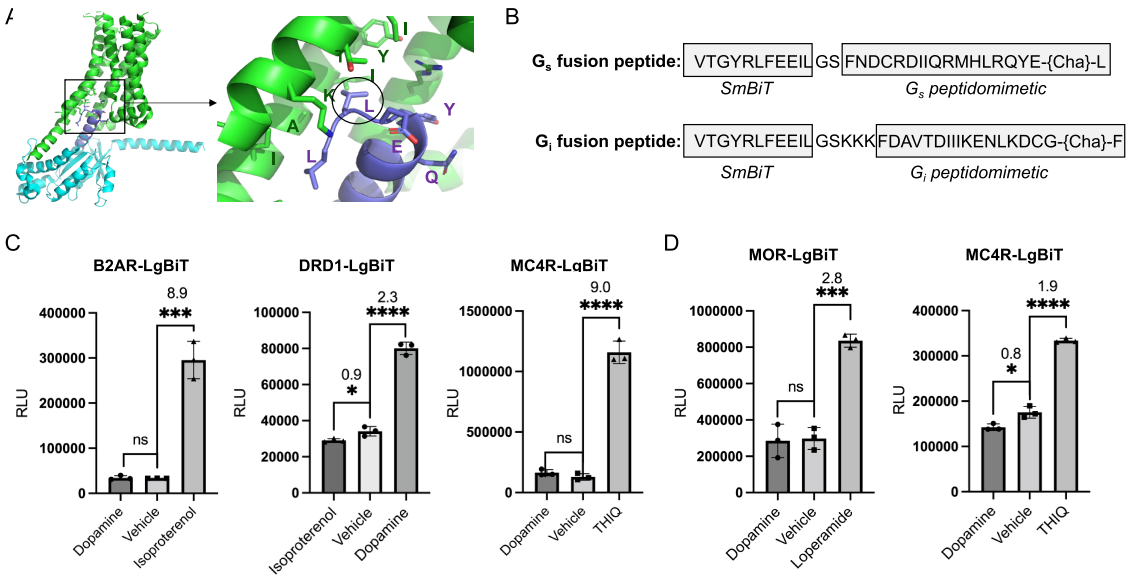


Figure 2. Characterization of the IGNiTR assay with peptidomimetics as the conformation-specific binder. (A) Crystal structure of LY3154207-bound dopamine receptor D1 (DRD1) (PDB: 7X2F). DRD1, green. G_s protein's α-5-helix is highlighted in purple blue to illustrate the interaction between the α-5-helix and the hydrophobic binding pocket of the activated DRD1. The penultimate position is enclosed inside the circle. (B) Amino acid sequences for the G_s and G_i fusion peptides. (C) Characterization of IGNiTR with the G_s fusion peptide for the GPCRs indicated. (D) Characterization of IGNiTR assay with the G_i fusion peptide for the GPCRs indicated. $n = 3$ technical replicates. G_s fusion peptide, 2 μM. G_i fusion peptide, 2 μM. Drug, 10 μM. Values above the bars represent the DDR. Asterisks indicate significance after performing an unpaired Student's t test. ***$P < 0.001$, ****$P < 0.0001$. "ns" indicates no significant difference.

Using G Protein Peptidomimetics in IGNiTR

To expand the versatility of IGNiTR, we used a G protein peptidomimetic as the conformation-specific binder in IGNiTR. Peptidomimetics enable the use of unnatural amino acids to increase binding affinity for the GPCR target.[40,41] Additionally, peptide synthesis facilitates the standardization of the peptidomimetic concentration across large batches of well plates.

Our design was inspired by a reported G_s peptidomimetic[40] (SI, Figure 2A and B) which was based on the α-5-helix of Gα_s in the crystal structure of the G_s protein complex bound with β2AR.[42] The G_s peptidomimetic (FNDCRDIIQRMHLRQYE-{Cha}-L) conserves the Gα_s protein α-5-helix amino acid sequence while adding a cyclohexylalanine (Cha) residue to increase hydrophobic interactions with the large hydrophobic pocket of the activated β2AR.[40,43]

Our design fused the SmBiT (11 amino acids) to the G_s peptidomimetic to create a SmBiT-G_s peptidomimetic fusion peptide. To test the peptidomimetic version of IGNiTR, the β2AR-LgBiT cell pellet was mixed with the G_s fusion peptide and NanoLuc substrate. Then, agonist, nonagonist small molecule control, or vehicle was added to evaluate the DDR (Figure 2C and Figure S4). β2AR IGNiTR with the G_s fusion peptide produced a DDR of 8.9 (Figure 2C). We further tested the G_s fusion peptide with the G_s-coupled GPCR DRD1 and the promiscuous GPCR melanocortin 4 receptor (MC4R), producing DDR values of 2.3 and 9.0, respectively (Figure 2C). These results validated the G_s peptidomimetic's selectivity for the active conformation of the G_s-coupled GPCRs.

We next designed a G_i peptidomimetic using a similar strategy by incorporating the unnatural amino acid Cha at the penultimate position (Figure 2B), since the α-5-helix of the Gα_i protein also interacts with a highly hydrophobic binding pocket based on the Gα_i protein structure.[44] The G_i fusion peptide is composed of SmBiT fused to the G_i peptidomimetic. We tested IGNiTR with the G_i fusion peptide and two G_i-coupled GPCRs, MOR, and MC4R, which couples to both G_s and G_i proteins.[45] DDR values of 2.8 and 1.9 were observed for MOR and MC4R IGNiTR, respectively (Figure 2D). This study validates the G_i peptide's selective binding to the agonist-activated MOR and MC4R and establishes the use of a G_i fusion peptide in IGNiTR for G_i-coupled GPCRs.

The IGNiTR Assay Can Characterize a GPCR Ligand's Efficacy and Potency

To further establish IGNiTR's ability to characterize the various conformational states of a GPCR induced by different ligands, we applied the technique to DRD1 IGNiTR with full agonists, partial agonists, and antagonists. As shown in Figure 3A, the full agonist dopamine produced higher DDR than the partial agonist fenoldopam at saturated concentrations, with both producing a DDR > 1. The result validates that both full and partial agonists induce the active conformational state[46,47] and that IGNiTR can differentiate ligand efficacies. DRD1 antagonist, SCH 23390, does not increase luminescence compared to the no drug condition.[46] These results further validate the G_s peptidomimetic's selective binding to the active conformation of DRD1. Consistent with reports that fenoldopam, while showing decreased maximum efficacy as a partial antagonist, is more potent than dopamine, DRD1 titration with dopamine and fenoldopam produced EC_{50} values of 2.6 μM and 145 nM, respectively (Figure 3B).[46,47] To show IGNiTR can also be used for antagonist titration, we performed DRD1 titration with antagonist SCH 23390 in the presence of 10 μM agonist dopamine and yielded an IC_{50} of 26 nM.[48] Overall, these characterizations demonstrate that the GPCR in IGNiTR maintains a function similar to in live cell-based assays, and that IGNiTR can differentiate among various ligand efficacies.

https://doi.org/10.1021/acsmeasuresciau.3c00021
ACS Meas. Sci. Au 2023, 3, 337−343

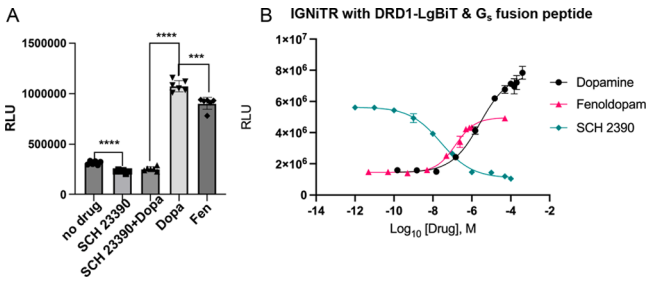


Figure 3. Characterization of the DRD1-IGNiTR assay with G_s fusion peptide. (A) Comparison of DRD1-IGNiTR signal with a panel of drugs at saturated concentrations. SCH23390, 50 μM; SCH23390+Dopa (dopamine), 50 and 10 μM; Dopa, 10 μM; Fen (fenoldopam), 10 μM. Luminescence values were taken at 30 min post drug incubation. (B) Dose−response curve of DRD1 IGNiTR with dopamine, fenoldopam, and SCH 23390. For dopamine, the EC_{50} range within 95% confidence is 1.8−4.1 μM. For fenoldopam, the EC_{50} range within 95% confidence is 114−181 nM. For SCH 23390, the IC_{50} range within 95% confidence is 20−33 nM. n = 4 technical replicates. Asterisks indicate significance after performing an unpaired Student's t test. ***P < 0.001, ****P < 0.0001.

IGNiTR Application: Proof-of-Principle High-Throughput Screening (HTS) with Robust Z′ Values

IGNiTR could provide a valuable alternative for preliminary HTS of GPCR ligands, especially since IGNiTR components can be mixed in a batch, increasing consistency across different batches of screens. As a proof-of-concept, we used DRD1 IGNiTR to demonstrate IGNiTR optimization for potential HTS applications. We first estimated the concentration of DRD1-LgBiT in the cell lysate. A standard curve of LgBiT was plotted using purified maltose binding protein (MBP) fused with LgBiT (MBP-LgBiT). Different concentrations of MBP-LgBiT were mixed with the HiBiT peptide, which has high affinity for LgBiT and reconstitutes NanoLuc activity, generating luminescence that is positively correlated with the reconstituted NanoLuc (Figure S5). The DRD1-LgBiT in cell lysate was diluted to be within the range of the standard curve. After obtaining an estimated concentration of DRD1-LgBiT of 15.8 nM, we varied the DRD1-LgBiT and G_s fusion peptide concentrations to find the optimal condition (SI and Figures S6 and S7). We used the optimized DRD1 IGNiTR condition to determine its Z' value, which is an important parameter to indicate the feasibility of an HTS platform. The Z' value was consistent across the plates with an average of 0.79 (Figure S8) within the range of optimal Z' values for HTS ($1 > Z' > 0.5$).[49] The proof-of-principle study demonstrated the feasibility of IGNiTR for HTS.

IGNiTR Application: Portable Assays for Rapid Detection of Opioids

IGNiTR could be packaged as a kit for detecting GPCR agonists, such as opioids, to be used for portable point-of-care testing. The ongoing opioid crisis is fueled by the emergence of additional synthetic opioids.[50,51] There is a need for accessible methods to detect opioid derivatives, which often are highly potent and can cause lethal overdoses.[51] To address this need, we used MOR IGNiTR to detect the synthetic opioid, fentanyl.

First, we optimized the concentrations of MOR and G_i fusion peptide (Figure S7). To increase the accessibility of IGNiTR for detection, we measured IGNiTR luminescence with a less sophisticated gel-imaging camera rather than a plate reader. As shown in Figure 4A and B, higher concentrations of fentanyl result in increased luminescence intensity, with a signal plateau around 500 nM. Notably, MOR IGNiTR could detect 10 nM fentanyl.

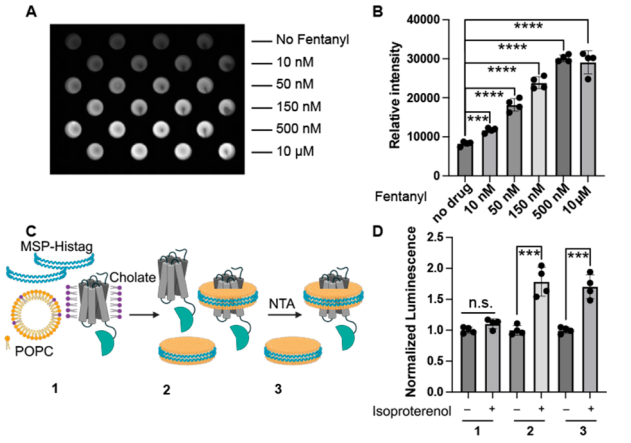


Figure 4. Applications of IGNiTR. (A) Imaging and (B) quantification of the IGNiTR assay were performed with MOR LgBiT and the G_i fusion peptide to detect varied concentrations of fentanyl. n = 4 technical replicates. (C) Workflow for the incorporation of β2AR-LgBiT into POPC-based Nanodiscs. "NTA" represents Ni-NTA column purification. (D) Analysis of the β2AR-LgBiT samples in C used IGNiTR with the G_s fusion peptide. Asterisks indicate significance after performing an unpaired Student's t test. ****P < 0.0001, ***P < 0.001, "ns" indicates no significant difference.

Our results demonstrate that IGNiTR can successfully detect various levels of opioids. Notably, IGNiTR reports on the general presence of many types of opioids, which complements existing assays for detecting specific synthetic opioid molecules.[52,53] Because the IGNiTR reagents can be stored frozen, we envision the components of IGNiTR being packaged into a kit for the detection of MOR agonists in a variety of settings. Furthermore, IGNiTR can potentially be adapted to detect other GPCR agonists, enabling the development of biosensors for a wide range of molecules.

IGNiTR Application: Characterizing GPCR Functionality during Nanodisc-Based GPCR Extraction and Reconstitution

Nanodiscs have been widely applied for GPCR reconstitution by embedding the GPCR in a lipid bilayer, forming stable GPCR-lipid complexes.[54] It is particularly important to track GPCR structural integrity and functionality throughout the Nanodisc assembly process, but this remains challenging.[55] Therefore, we tested IGNiTR's ability to characterize β2AR functionality during the three crucial steps of POPC-based Nanodisc formation[56] as indicated in Figure 4C. Higher DDR suggests greater content of functional β2AR that can undergo agonist-dependent conformational changes and reconstitute the split NanoLuc.

As shown in Figure 4D, β2AR reconstituted in Nanodisc with detergent cholate removed (sample 2) and its subsequent Ni-NTA purified sample (sample 3) produced significant DDRs, while the β2AR mixed with Nanodisc components as well as cholate (sample 1) did not yield a significant DDR. The result validates the importance of removing cholate to maintain the correct folding and functionality of β2AR during its incorporation into the Nanodisc. The study establishes that

https://doi.org/10.1021/acsmeasuresciau.3c00021
ACS Meas. Sci. Au 2023, 3, 337−343

IGNiTR could be used to monitor GPCR activity throughout the protein extraction and reconstitution process, which is useful for optimizing these protocols.

CONCLUSION

We have developed a generalizable in vitro GPCR assay, IGNiTR, that can characterize a GPCR's structural integrity and activity by detecting the agonist-induced interaction of the GPCR with a conformation-specific binder. We have demonstrated IGNiTR's usage with three G_s-coupled GPCRs and two G_i-coupled GPCRs. In this paper, we used lysis conditions without detergent to preserve the GPCR structure in its native lipid environment. However, one could also use cell lysis conditions with the detergent mix used in Figure S1, which may result in higher DDRs.

Even though IGNiTR is less sensitive than the live cell-based version of the split NanoLuc assay, IGNiTR has other advantages that can be harnessed under conditions where live cell-based assays are infeasible. First, IGNiTR components, including the GPCR and the conformation-specific binder, can be prepared in advance and stored frozen until usage. Second, IGNiTR can potentially be packaged to perform as a portable testing kit without the restrictions of working with live human mammalian cells following biosafety level 2 regulations. Third, the preparation of IGNiTR in a cell lysate solution allows the use of a synthetic fusion G protein peptidomimetic, whose concentration can be well-controlled for assay fine-tuning, including the optimization of DDR.

IGNiTR has advantages over existing in vitro assays. IGNiTR's bioluminescent readout is quantifiable in a single step and therefore can be easily scaled up and performed under less complex laboratory conditions, while existing in vitro GPCR assays, such as radioligand binding, may require complicated setups.[18,19] We demonstrated diverse applications under a variety of conditions in: 1) proof-of-concept HTS using DRD1 IGNiTR; 2) characterization and detection of opioids using a basic imaging system; and 3) verifying GPCR structural integrity for in vitro GPCR characterizations. In future work, IGNiTR can be expanded for more GPCRs and used in broad applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmeasuresciau.3c00021.

Additional experimental details, workflow, materials, and methods; characterization of IGNiTR in detergent and detergent-free conditions; independent replicates of Figure 1; comparison of live cell split NanoLuc vs IGNiTR; standard curve quantifying concentration of DRD1-LgBiT from HTS; optimization of peptidomimetic and GPCR concentrations for HTS; Z′ values from HTS (PDF)

AUTHOR INFORMATION

Corresponding Author

Wenjing Wang − *Life Sciences Institute and Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States;* orcid.org/0000-0001-6025-9848; Email: wenjwang@umich.edu

Authors

Ruby M. Miller − *Life Sciences Institute and Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States*

Jennifer Sescil − *Life Sciences Institute and Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States*

Marina C. Sarcinella − *Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States*

Ryan C. Bailey − *Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States;* orcid.org/0000-0003-1021-4267

Complete contact information is available at: https://pubs.acs.org/10.1021/acsmeasuresciau.3c00021

Author Contributions

#R.M.M. and J.S. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Ruby M. Miller** conceptualization (lead), data curation (lead), formal analysis (lead), funding acquisition (supporting), investigation (lead), methodology (lead), project administration (equal), validation (lead), writing-original draft (lead), writing-review & editing (lead); **Jennifer Sescil** conceptualization (equal), data curation (lead), formal analysis (lead), funding acquisition (supporting), investigation (equal), methodology (lead), validation (lead), writing-original draft (lead), writing-review & editing (lead); **Marina C. Sarcinella** conceptualization (supporting), data curation (supporting), formal analysis (supporting), methodology (supporting), writing-original draft (supporting), writing-review & editing (supporting); **Ryan C. Bailey** conceptualization (supporting), formal analysis (supporting), project administration (supporting), supervision (supporting), writing-original draft (supporting), writing-review & editing (supporting); **Wenjing Wang** conceptualization (lead), formal analysis (lead), funding acquisition (lead), investigation (lead), project administration (lead), supervision (lead), writing-original draft (lead), writing-review & editing (lead).

Notes

The authors declare the following competing financial interest(s): A patent application has been submitted: Miller, R., Wang, W. (2022) G-PROTEIN COUPLED RECEPTOR ASSAY. U.S. Patent Application No. 63/311,216. Washington, DC: U.S. Patent and Trademark Office.

ACKNOWLEDGMENTS

The research of the manuscript was funded by the University of Michigan, NIH MH132939. R.M.M. is supported by NIH 1F31DA056192-01. J.S. is supported by NSF GRFP DGE 1841052. We thank Emma Fu for helping to prepare reagents for IGNiTR. We thank Dr. Andy Alt and Dr. Aaron Robida for their help with high-throughput screening set up. We thank, Dr. Shoji Maeda, Dr. Peter Toogood, Joseph Loomis, and Wang lab members Guanwei Zhou and Aubrey Putansu for proofreading the manuscript and providing edits. Figures created with BioRender.com.

REFERENCES

(1) Santos, R.; et al. A comprehensive map of molecular drug targets. *Nat Rev Drug Discov* **2017**, *16*, 19−34.

(2) Congreve, M.; de Graaf, C.; Swain, N. A.; Tate, C. G. Impact of GPCR Structures on Drug Discovery. *Cell* **2020**, *181*, 81−91.
(3) Wacker, D.; Stevens, R. C.; Roth, B. L. How Ligands Illuminate GPCR Molecular Pharmacology. *Cell* **2017**, *170*, 414−427.
(4) Yasi, E. A.; Kruyer, N. S.; Peralta-Yahya, P. Advances in G protein-coupled receptor high-throughput screening. *Current Opinion in Biotechnology* **2020**, *64*, 210−217.
(5) Siehler, S. Cell-based assays in GPCR drug discovery. *Biotechnol. J.* **2008**, *3*, 471−483.
(6) Martins, S. A. M.; et al. Towards the miniaturization of GPCR-based live-cell screening assays. *Trends in Biotechnology* **2012**, *30*, 566−574.
(7) Haider, R. S.; Godbole, A.; Hoffmann, C. To sense or not to sense—new insights from GPCR-based and arrestin-based biosensors. *Current Opinion in Cell Biology* **2019**, *57*, 16−24.
(8) Sakamoto, S.; Kiyonaka, S.; Hamachi, I. Construction of ligand assay systems by protein-based semisynthetic biosensors. *Current Opinion in Chemical Biology* **2019**, *50*, 10−18.
(9) Olsen, R. H. J.; et al. TRUPATH, an open-source biosensor platform for interrogating the GPCR transducerome. *Nat Chem Biol* **2020**, *16*, 841−849.
(10) Olsen, R. H. J.; English, J. G. Advancements in G protein-scoupled receptor biosensors to study GPCR-G protein coupling. *Br. J. Pharmacol.* **2023**, *180*, 1433−1443.
(11) Höring, C.; et al. A Dynamic, Split-Luciferase-Based Mini-G Protein Sensor to Functionally Characterize Ligands at All Four Histamine Receptor Subtypes. *IJMS* **2020**, *21*, 8440.
(12) Hauge Pedersen, M.; et al. A novel luminescence-based β-arrestin recruitment assay for unmodified receptors. *J. Biol. Chem.* **2021**, *296*, No. 100503.
(13) Inoue, A.; et al. Illuminating G-Protein-Coupling Selectivity of GPCRs. *Cell* **2019**, *177*, 1933−1947.e1925.
(14) Wiseman, D. N.; et al. Expression and purification of recombinant G protein-coupled receptors: A review. *Protein Expression and Purification* **2020**, *167*, No. 105524.
(15) Enrico Rovati, G. Ligand-binding studies: old beliefs and new strategies. *Trends Pharmacol. Sci.* **1998**, *19*, 365−369.
(16) Masureel, M.; et al. Structural insights into binding specificity, efficacy and bias of a β2AR partial agonist. *Nat Chem Biol* **2018**, *14*, 1059−1066.
(17) Keen, M. In *Signal Transduction Protocols*; Humana Press, 1995; Vol. *41*, pp 1−16.
(18) Saumell-Esnaola, M.; et al. Design and validation of recombinant protein standards for quantitative Western blot analysis of cannabinoid CB1 receptor density in cell membranes: an alternative to radioligand binding methods. *Microb Cell Fact* **2022**, *21*, 192.
(19) Volz, M. R.; Moosmann, B. Development of a non-radioactive mass spectrometry-based binding assay at the μ-opioid receptor and its application for the determination of the binding affinities of 17 opiates/opioids as well as of the designer opioid isotonitazene and five further 2-benzylbenzimidazoles. *Anal. Chim. Acta* **2022**, *1219*, No. 339978.
(20) Dixon, A. S.; et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem Biol* **2016**, *11*, 400−408.
(21) Laschet, C.; Dupuis, N.; Hanson, J. A dynamic and screening-compatible nanoluciferase-based complementation assay enables profiling of individual GPCR−G protein interactions. *J. Biol. Chem.* **2019**, *294*, 4079−4090.
(22) Vasudevan, L.; et al. Assessment of structure-activity relationships and biased agonism at the Mu opioid receptor of novel synthetic opioids using a novel, stable bio-assay platform. *Biochem. Pharmacol.* **2020**, *177*, No. 113910.
(23) Reyes-Alcaraz, A.; et al. A NanoBiT assay to monitor membrane proteins trafficking for drug discovery and drug development. *Communications Biology* **2022**, *5*, 212.
(24) Soave, M.; et al. Monitoring Allosteric Interactions with CXCR4 Using NanoBiT Conjugated Nanobodies. *Cell Chemical Biology* **2020**, *27*, 1250−1261.e1255.
(25) Azad, T.; et al. SARS-CoV-2 S1 NanoBiT: A nanoluciferase complementation-based biosensor to rapidly probe SARS-CoV-2 receptor recognition. *Biosensors and Bioelectronics* **2021**, *180*, No. 113122.
(26) Dixon, A. S.; et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol.* **2016**, *11*, 400−408.
(27) Guo, S.; Zhao, T.; Yun, Y.; Xie, X. Recent progress in assays for GPCR drug discovery. *American Journal of Physiology-Cell Physiology* **2022**, *323*, C583−C594.
(28) Cannaert, A.; et al. Activity-Based Concept to Screen Biological Matrices for Opiates and (Synthetic) Opioids. *Clin Chem* **2018**, *64*, 1221−1229.
(29) England, C. G.; Ehlerding, E. B.; Cai, W. NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconjugate Chemistry* **2016**, *27*, 1175−1187.
(30) Roth, B. L.; Irwin, J. J.; Shoichet, B. K. Discovery of new GPCR ligands to illuminate new biology. *Nat Chem Biol* **2017**, *13*, 1143−1151.
(31) Weis, W. I.; Kobilka, B. K. The Molecular Basis of G Protein−Coupled Receptor Activation. *Annu. Rev. Biochem.* **2018**, *87*, 897−919.
(32) Ni, Y.; Arts, R.; Merkx, M. Ratiometric Bioluminescent Sensor Proteins Based on Intramolecular Split Luciferase Complementation. *ACS Sens.* **2019**, *4*, 20−25.
(33) Manglik, A.; Kobilka, B. K.; Steyaert, J. Nanobodies to Study G Protein−Coupled Receptor Structure and Function. *Annu. Rev. Pharmacol. Toxicol.* **2017**, *57*, 19−37.
(34) Nehmé, R.; et al. Mini-G proteins: Novel tools for studying GPCRs in their active conformation. *PLoS ONE* **2017**, *12*, No. e0175642.
(35) Rasmussen, S. G. F.; et al. Crystal structure of the β2 adrenergic receptor−Gs protein complex. *Nature* **2011**, *477*, 549−555.
(36) Ring, A. M.; et al. Adrenaline-activated structure of β2-adrenoceptor stabilized by an engineered nanobody. *Nature* **2013**, *502*, 575−579.
(37) Wan, Q.; et al. Mini G protein probes for active G protein−coupled receptors (GPCRs) in live cells. *J. Biol. Chem.* **2018**, *293*, 7466−7473.
(38) Staus, D. P.; Wingler, L. M.; Pichugin, D.; Prosser, R. S.; Lefkowitz, R. J. Detergent- and phospholipid-based reconstitution systems have differential effects on constitutive activity of G-protein–coupled receptors. *J. Biol. Chem.* **2019**, *294*, 13218−13223.
(39) Kim, M. W.; et al. Time-gated detection of protein-protein interactions with transcriptional readout. *eLife* **2017**, *6*, No. e30233.
(40) Mannes, M.; et al. Development of Generic G Protein Peptidomimetics Able to Stabilize Active State G s Protein-Coupled Receptors for Application in Drug Discovery. *Angew. Chem., Int. Ed.* **2021**, *60*, 10247−10254.
(41) Boyhus, L.-E.; et al. G s protein peptidomimetics as allosteric modulators of the β 2 -adrenergic receptor. *RSC Adv.* **2018**, *8*, 2219−2228.
(42) Carpenter, B.; Nehmé, R.; Warne, T.; Leslie, A. G. W.; Tate, C. G. Structure of the adenosine A2A receptor bound to an engineered G protein. *Nature* **2016**, *536*, 104−107.
(43) Gumpper, R. H.; Roth, B. L. G-Protein Peptidomimetics Stabilize GPCR Active State Conformations. *Trends Pharmacol. Sci.* **2021**, *42*, 429−430.
(44) Koehl, A.; et al. Structure of the μ-opioid receptor−Gi protein complex. *Nature* **2018**, *558*, 547−552.
(45) Heyder, N. A.; et al. Structures of active melanocortin-4 receptor−Gs-protein complexes with NDP-α-MSH and setmelanotide. *Cell Research* **2021**, *31*, 1176−1189.
(46) Teng, X.; et al. Ligand recognition and biased agonism of the D1 dopamine receptor. *Nat Commun* **2022**, *13*, 3186.

https://doi.org/10.1021/acsmeasuresciau.3c00021
ACS Meas. Sci. Au 2023, 3, 337−343

(47) Teng, X.; et al. Structural insights into G protein activation by D1 dopamine receptor. *Sci. Adv.* **2022**, *8*, No. eabo4158.
(48) Kumar, M.; Hsiao, K.; Vidugiriene, J.; Goueli, S. A. A Bioluminescent-Based, HTS-Compatible Assay to Monitor G-Protein-Coupled Receptor Modulation of Cellular Cyclic AMP. *ASSAY and Drug Development Technologies* **2007**, *5*, 237−246.
(49) Zhang, J.-H.; Chung, T. D. Y.; Oldenburg, K. R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *SLAS Discovery* **1999**, *4*, 67−73.
(50) Cuitavi, J.; Hipólito, L.; Canals, M. The Life Cycle of the Mu-Opioid Receptor. *Trends Biochem. Sci.* **2021**, *46*, 315−328.
(51) Mattson, C. L.; et al. Trends and Geographic Patterns in Drug and Synthetic Opioid Overdose Deaths - United States, 2013−2019. *MMWR Morb Mortal Wkly Rep* **2021**, *70*, 202−207.
(52) Glasscott, M. W.; et al. Electrochemical sensors for the detection of fentanyl and its analogs: Foundations and recent advances. *TrAC Trends in Analytical Chemistry* **2020**, *132*, No. 116037.
(53) Razlansari, M.; et al. Nanobiosensors for detection of opioids: A review of latest advancements. *European Journal of Pharmaceutics and Biopharmaceutics* **2022**, *179*, 79−94.
(54) Rouck, J. E.; Krapf, J. E.; Roy, J.; Huff, H. C.; Das, A. Recent advances in nanodisc technology for membrane protein studies (2012−2017). *FEBS Lett.* **2017**, *591*, 2057−2088.
(55) Serebryany, E.; Zhu, G. A.; Yan, E. C. Y. Artificial membrane-like environments for in vitro studies of purified G-protein coupled receptors. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2012**, *1818*, 225−233.
(56) Mitra, N.; et al. Calcium-Dependent Ligand Binding and G-protein Signaling of Family B GPCR Parathyroid Hormone 1 Receptor Purified in Nanodiscs. *ACS Chem. Biol.* **2013**, *8*, 617−625.

https://doi.org/10.1021/acsmeasuresciau.3c00021
ACS Meas. Sci. Au 2023, 3, 337−343