An Accessible and Generalizable In Vitro Luminescence Assay for Detecting GPCR Activation

by

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Dedication

To Mike, for always believing in me

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Table of Contents

List of Abbreviations

AA	amino acid
APEX	engineered ascorbate peroxidase
AS-MS	affinity selection-mass spectrometry
AuNP	Gold nanoparticles
BCA	bicinchoninic acid
BRET	bioluminescence resonance energy transfer
Ca2+	calcium ions
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CDR	complementary determining region
Cha	cyclohexylalanine
CHS	cholesteryl hydrogen succinate
cpGFP	circularly permutated green fluorescent protein
CSB	conformation specific binder
DCyFIR	Dynamic Cyan Induction by Functional Integrated Receptors
DDM	n-dodecyl β-D-maltoside
DDR	drug-dependent ratio
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
Dopa	Dopamine
DPBS	Dulbecco's Phosphate Buffered Saline
DRD1	dopamine receptor D1
EC50	Half maximal effective concentration
ELISA	enzyme-linked immunosorbent assay
FA	fluorescence anisotropy
FBDD	Fragment-Based Drug Design
FBS	Fetal Bovine serum

FDA	Food and Drug Administration
Fen	Fenaldopam
FLINT	fluorescence intensity
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
FRET	Förster resonance energy transfer
GCaMP	high-affinity Ca(2+) probe composed of a single GFP
GDP	guanosine diphosphate
GECI	genetically encoded calcium indicator
GPCR	G-protein coupled receptor
GRABDA	GPCR activation based dopamine sensor
GRK	GPCR kinase
GTP	guanosine triphosphate
HEK293T	Human Embryonic Kidney 293T cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HiBiT	High affinity BiT half of split NanoLuc
HT-SPR	High Throughput-Surface plasmon resonance
HTS	high throughput screening
IC50	half maximal inhibitory concentration
ICL3	instracellular loop 3
IGNiTR	In vitro GPCR split NanoLuc ligand Triggered Reporter
Iso	Isoproterenol
kd	Dissociation constant
kDa	kilo Daltons
Leu	leucine
LFA	lateral flow assay
LgBiT	Large BiT half of split NanoLuc
LOD	limit of detection
MBP	Maltose-binding protein
MC4R	melanocortin 4 receptor
MEM	Eagle's Minimal Essential Medium
miniG	smaller engineered form of a G protein

MRB	membrane resuspension buffer
MSP	membrane scaffold protein
NanoLuc	Nanoluciferase
Nb	Nanobody
Nb39	Nanobody 39
Nb80	Nanobody 80
NEB	New England Biolabs
Ni-NTA	nickel^{2+} nitriloacetic acid
NMR	nuclear magnetic resonance
NR	Nociceptor or Nociceptin Receptor
NS	not significant
PCR	polymerase chain reaction
PD	Parkinson's Disease
PEI	polyethlenimine
PKA	protein kinase A
POPC	phosphatidylcholine
PPI	protein-protein interaction
RLU	relative luminescence units
SCH 23390	halobenzazepine
SD	standard deviation
SEC	size exclusion chromatography
SmBiT	Small BiT half of split NanoLuc
SPASM	systematic protein affinity strength modulation
TEV	tobacco etch virus
TM5	transmembrane helix 5
TM6	Transmembrane helix 6
β2AR	β2-adrenergic Receptor
μ-OR	mu- opioid receptor

List of Figures

Abstract

G-protein coupled receptors (GPCRs) serve critical physiological roles as the most abundant family of receptors. The receptors are responsible for the regulation of signaling passing between different cells and their activation triggers cellular responses in the form of signaling cascades. It is this regulatory role that places GPCRs at the center of many disease mechanisms and creates incentive for better controlling their activation or inhibition. It follows that GPCRs are the targets of a significant percentage of clinically approved drugs. While there are many assays useful for studying the effects of GPCR activation, both live cell and biochemical, most rely on indirect tracking via downstream products or only report whether the drug is bound but not whether structural activation has occurred. None of them provide a direct readout of the activation mechanism or can be performed as a cell free method. This thesis describes the design of a generalizable and accessible **I**n vitro **G**PCR split **N**anoLuc l**i**gand **T**riggered **R**eporter (**IGNiTR**), having broad and diverse applications.

IGNiTR leverages the interaction between a conformation-specific binder and agonist-activated GPCR to reconstitute a split NanoLuciferase (NanoLuc) which can produce a bioluminescent signal in the presence of substrate. I describe the development of protocols for harvesting components expressed in live mammalian cells and for lysing the pellets (stored frozen) before mixing with other components of the assay. This thesis demonstrates IGNiTR with three G_s-coupled GPCRs and a G_i-coupled GPCR with three classes of conformation-specific binders: nanobodies, miniG proteins, and G-protein peptidomimetics. IGNiTR and the

lysate conditions also allows the use of a synthetic G-protein peptidomimetics, providing easily standardized reagents for characterizing GPCRs and ligands. Described here is the development of two novel peptidomimetics, a G_s peptidomimetic fused to the SmBiT component of split NanoLuc, as well as a G_i peptidomimetic version. These peptides feature the use of unnatural amino acids that enhance the selectivity of the peptidomimetic for the active conformation of the GPCR.

As an in vitro assay, IGNiTR components can be prepared in advance and stored frozen, and mixed, providing ready-to-go reagents and high consistency across reactions. The thesis describes three applications of IGNiTR: 1) a proof of concept high-throughput screening of ligands against DRD1; 2) detection of opioids using less sophisticated imaging instrumentation; and 3) characterizing GPCR functionality during Nanodisc-based reconstitution process. Due to its convenience, accessibility, and consistency, IGNiTR will find extensive applications in GPCR ligand detection, screening, and GPCR characterization.

Chapter 1
Introduction of Biosensors for Studying GPCR Activation

1.1 GPCRs as drug targets

G-Protein Coupled Receptors (GPCR) are a class of proteins responsible for regulation of a wide range of chemical processes throughout the body.[1] Signal transduction is the main process where such regulation plays a key role in the passing of signals (either chemical or light) through a series of molecular steps known as a biochemical cascade.[2] For example, GPCRs respond to the molecules released into a synapse between a signaling neuron and the receiving neuron, with each chemical triggering specific effects, (e.g. either an inhibitory or stimulatory) and consequentially effecting the downstream cellular response.[1] It is through the direct contact of the binding chemical with the GPCR that determines how the signal will be transduced and thus regulated. All GPCRs have a standard set of components and interaction partners that aid in this process.[2]

The first is a physical attribute which lends itself to the role in signal receiving and transducing. GPCRs are proteins composed of 7 transmembrane domains, meaning that they span the cellular membrane and have both an extracellular and intracellular facing side.[3] The extracellular face features a binding pocket to receive the signaling molecules while the intracellular side has another interface which is structurally modulated by the ligand interacting with the extracellular pocket (see **Figure 1**).[2] Based on the effected conformational change in the receptor, the intracellular pocket can adopt different configurations, “on” or “off” or “intermediate” states, to enable coupling to a heterotrimeric G-protein.[1] This coupling is another

key feature of GPCRs and is tied to the final hallmark of the GPCR system: downstream effector proteins and a desensitizing system.[4] All these features combine to make GPCRs effective regulators of signal transduction (**Figure 1**).

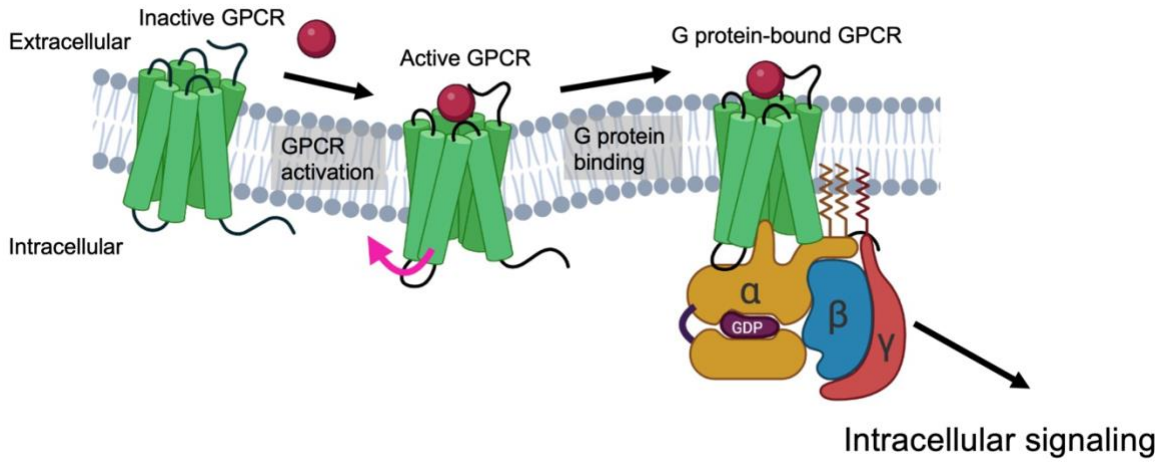


Figure 1 Overview of the G-protein Coupled Receptor activation by drug, conformational change, and subsequent coupling to the G-protein complex. If the G-protein is of the stimulatory class (G_s coupled), then the downstream intracellular signaling events result in an increased production of cyclic-AMP and increased concentration of calcium ions. If the G-protein is of the inhibitory class (G_i coupled) the downstream signaling effects are the opposite.

An example of a relevant GPCR (discussed in **Chapter 2**) is the Dopamine receptor D1 (DRD1) involved in memory, addiction, cognition, and fine motor control, among other neuronal circuits.[5] The receptor is implicated in several diseases, most notably Parkinson's Disease (PD), as the symptoms are directly connected to and dramatically affect memory and fine motor control.[6] Dopaminergic neurons in the substantia nigra degrade and fail to produce healthy levels of dopamine to be released as part of normal signaling pathways, affecting voluntary movement production.[7] Dopamine analogs are used for treatment of PD, in an effort to replace the lack of naturally occurring neurotransmitter.[8] GPCRs are ideal targets for drugs and treatment because they are such key regulators of the biochemical signaling cascades but also for that same reason they can be challenging to target.[9] The GPCR can affect several different downstream processes depending on the binding of the agonist and thus trigger some beneficial reactions while also

affecting other similar albeit unfavorable processes.[10] The implications become widespread depending on which neuronal pathway the GPCR is found in.

As demonstrated by the DRD1 example, GPCRs are ideal targets for therapeutics and roughly 35% of drugs on the market are designed for that purpose.[11] DRD1 also presents a prime model where several different types of agonists may be needed for treatment, since dopamine deficiencies require more effective ways to stimulate the neurotransmitter release. However, effective drugs must be functionally selective, due to the possibility of off target effects. For example cocaine and methamphetamine are similar stimulators which can trigger one signaling cascade (i.e. rapid increase of dopamine) but can also activate other cascaded with unwanted side effects, like addiction.[12] GPCRs have been shown to respond in a variety of ways depending on the various forms of modulation e.g., whether orthosteric or allosteric.[13] In this chapter I will be discussing several strategies used in the drug discovery process, including structural characterization studies as well as the development of biosensor systems. But first I will be introducing background on the known mechanisms of GPCR function and action.

1.2 GPCR activation mechanism

When an agonist docks in the GPCR binding pocket, the first mode of activation that is triggered is a conformational shift within the protein structure.[14] The GPCR consists of 7 transmembrane domains (helices) which each move differently in response to the chemical structure of the small molecule that binds. These triggered movements determine how the signal is transmitted via the various structural changes.[2] Transmembrane helix 5 (TM5) is connected to TM6 through intracellular loop 3 (ICL3).[15] The movement of ICL3 is affected by the outward shifting of TM6 and is characteristic of specific agonist activation (**Figure 2**).[16] It is through the

re-positioning of ICL3 that the GPCR confers selectivity for the subsequent protein binding interactions and frees the intercellular pocket for coupling.[16] Either a β-arrestin or heterotrimeric G-protein complex (made up of subunits Gα, Gβ, and Gγ) can dock at the site, initiating an exchange of guanosine diphosphate (GDP) for guanosine-5'-triphosphate (GTP) and the subsequent dissociation of Gβ and Gγ subunits.[17] The Gα subunit is free to bind to further downstream signal transducers, such as Adenylyl Cyclase and initiate the production of cyclic AMP (cAMP).[4] Importantly, the activation cycle is transient and includes regulation checkpoints, such as receptor internalization, for resetting and returning to the starting configuration.[4]

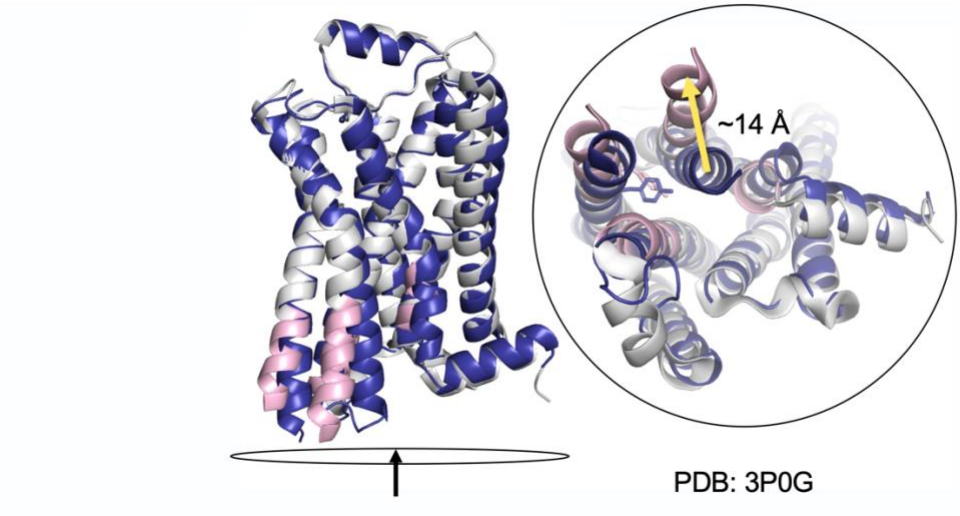


Figure 2 Crystal structure of β2-adrenergic Receptor (β2AR) intracellular pocket demonstrating the conformational shift that occurs upon activation by an agonist. The dark purple structure represents the inactive form transposed over the lighter-colored white and pink active form of the GPCR. Transmembrane helix 6 (TM6) shifts outward by approximately 14 angstroms (Å) shown by the yellow arrow. Inactive PDB: 2RH1; Active PDB: 3P0G.

One main factor that determines whether the G-protein complex or β-arrestin will bind to the GPCR is dependent upon the properties of the activating ligand binding to the GPCR and the type of signal propagation they induce.[18] The type of signaling that ensues is dependent upon the category of GPCR and to which classification of G-protein it couples.[19] The downstream effects

have become the way classes of ligands are categorized, including biased agonists, partial agonist, antagonists, inverse agonists, among others.[20] Both allosteric and orthosteric modulators can affect the ligand binding pocket, which in turn influences which ligands can bind.[19] There are examples of GPCRs which do not conform to a binary G-protein complex or β-arrestin, such as the promiscuous receptor Melanocortin-4 Receptor (MC4R) which can couple to Gs-, Gi-, and Gq- proteins, depending on which agonist binds to the receptor.[21,22] One of the reasons there are so many "orphan" GPCRs is that there is not a known endogenous agonist, or the mechanism of activation is unknown, including to which G-protein complex it couples.[15] All these factors compound to make untangling the signaling system extra complicated and are a major challenge when it comes to searching for new drugs or ways to target the receptors.[23]

The downstream results are specific to the type of signaling induced. When the GPCR is activated by agonists, the downstream signaling cascade can trigger increases in the concentration of secondary messengers such as cyclic adenosine monophosphate (cAMP) or Ca^{2+} concentration for further signal transduction.[19] There have been many assays designed to track receptor activation via these downstream effectors: i.e., GPCR activation can be detected indirectly by using sensors to visualize the increased Ca^{2+} or cAMP levels.[24]

1.3 Biosensors for studying downstream effects of GPCR activation

GPCRs continue to be one of the most actively studied class of proteins and yet so much is unknown about the complex ways that they stimulate various signaling pathways. Part of the challenge is finding ways to untangle what happens during each of the intermediate states of receptor activation. For example, a particularly difficult task has been finding ways to characterize structural changes effected by ligand binding or track unique coupling mechanisms

induced by agonists. The current options for addressing these endeavors have been limited to highly specialized methods, custom designed for the GPCR or system of interest. Here I describe examples of biosensors which have been engineered to study specific stages throughout the course of activation. Each of the strategies either requires extensive tailoring to the proteins of interest or are not direct readouts of activation but rather rely heavily on tracking products of the signaling.

Genetically encoded fluorescent indicator tools

Tracing GPCR activation events (especially in neurons) with high spatiotemporal resolution is crucial for a better understanding of GPCR signaling.[25] A wide variety of genetically encoded sensors have been designed to detect GPCR activation, either based on a GPCR's structural change itself or on downstream signaling events, and readout a signal (fluorescence, luminescence, etc).[24] The signal output of these sensors can be categorized into two main groups: a permanent integrator marker or a real-time responsive yet transient index.[26] The advantage of using a downstream genetic transcription is found in amplification and permanentizing of an otherwise transient interaction. However, these integrator sensors differ from real-time sensors as they are not capable of producing a change in signal to reflect temporal dynamics.[26] There are pros and cons to both approaches, which are highlighted in the following examples.

Genetically encoded fluorescent sensors for detecting secondary messengers in GPCR signaling

A real-time approach to tracking signal transduction downstream of GPCR activation involves building sensors that trigger immediate readouts.[27] Because cellular signal modulation relies so heavily on the flux of calcium ions, a lot of time and attention has been given to the

development of methods of tracking dynamic changes in cellular [Ca^{2+}].[28] One of the most broadly used has been fluorescent imaging of a genetically encoded sensor GCaMP, made of calcium-sensing domain calmodulin (CaM) and circularly permutated green fluorescent protein (cpGFP). GCaMP has been optimized to have the following strengths: rapid kinetic response to fluctuations in Ca^{2+} and high performing signal strength for sub-cellular resolution.[29] The sensor is useful for tracking signal activation by monitoring products of the indirect downstream cascade, yet does not provide a readout of the structural activation of GPCR directly(**Figure 3**).

Other real-time sensors have been built for detecting fluctuations in cAMP levels.[9] The luciferase based GloSensor relies on the engineering of a circularly permutated luciferase with the insertion of the human RIIβB subunit into the luciferase domains.[30] Upon binding to cAMP, the human RIIβB subunit undergoes a conformational change, which affects the luciferase structure and activity. Consequently, this sensor produces luminescence signal that is positively correlated to the cAMP concentration.[30] Similarly to the Ca^{2+} sensor, the cAMP sensor relies on a product of the cascade to provide indirect information about the activated state of a GPCR without revealing direct insight about the structural activation mechanism.

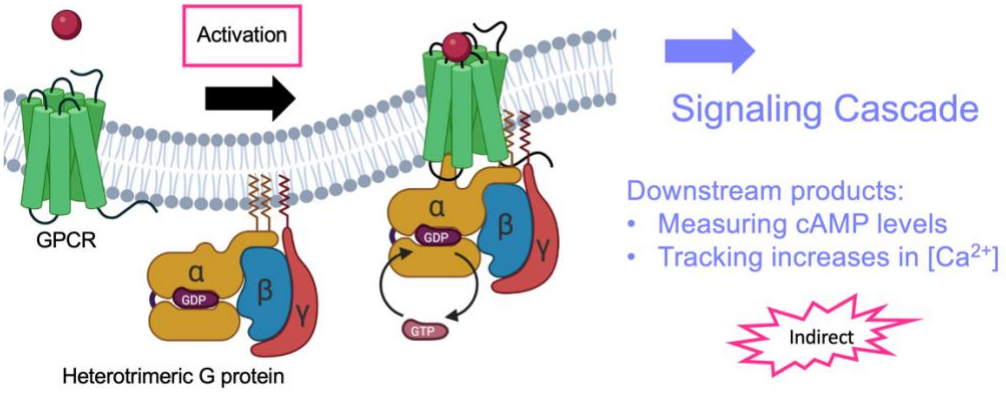


Figure 3 Overview of indirect methods of tracking GPCR activation by monitoring downstream cascade products.

Internalization assays (proximity labeling)

Finally, another option for tracking GPCR activation involves a later stage further downstream in the signal transduction cycle: receptor internalization. GPCR kinases (GRK) phosphorylate the receptor to recruit β-arrestins to bind and initiate a chain of events silencing the G-protein mediated effects.[31] Proximity dependent labeling is ideal for tracking and studying the internalization process since the technique can report on the components involved in physical translocation of the receptor. A peroxidase-catalyzed method using the engineered ascorbate peroxidase (APEX) enzyme was harnessed for mapping of GPCR sequestration away from G-proteins during the process of endocytosis.[32] To accomplish spatiotemporally-resolved monitoring of GPCR internalization process, APEX (attached to the GPCR) produced biotin-phenoxy radicals with a limited labeling radius (∼20 nm) that are short-lived (< 1 ms) and facilitated marking a significant portion of the proteins that interact with the receptor with biotin (provided as biotin-phenol during the labeling period). [33] The biotinylated proteins could be enriched using streptavidin beads and analyzed using mass spectrometry.

Directly tracking protein-protein interactions

BRET coupling with the TRUPATH method

Within the context of mapping the GPCR transducerome (16 G-protein signal transducers), one standard approach has been using either bioluminescence resonance energy transfer (BRET) or fluorescence resonance energy transfer (FRET) to link the activated GPCR to the class of G-protein which it has coupled to.[9] These sensors work by attaching a donor protein to one component of the protein interaction pair, which when activated can transfer energy to the acceptor protein when it is brought in proximity by the other interaction partner.[34] The TRUPATH system has developed BRET biosensors based on 16 sets of G-proteins by

engineering the Gα-RLuc8 donor and Gβγ-GFP2 acceptor BRET2 pairs. The TRUPATH system is unique because while most BRET sensors rely on an *increase* in signal initiated by protein-protein association, placing the donor and acceptor pairs on Gα and Gβγ results in a *decrease* in signal upon dissociation of the proteins post exchange of GTP/GDP due to the dissociation of Gα from Gβγ.[35] The TRUPATH system has joined other similar live-cell high throughput screening (HTS) approaches in facilitating discovery of coupling partners, particularly in orphan GPCR cases.[35]

PRESTO-tango

Another standard method for tracing the direct protein-protein interactions (PPIs) involved in GPCR activation has been engineered around β-arrestin coupling. The rationale behind tracking this interaction is that it is universal for most GPCRs. No matter what G-protein the receptor couples to, β-arrestin translocation has been found to occur.[36] The PRESTO-tango can detect β-arrestin interaction and provide a transcriptional readout. The GPCR is attached to a tobacco etch virus (TEV) protease cleavage site followed by a transcription factor.[37] When the β-arrestin brings a protease into proximity through association with the receptor, the TEV protease cleavage site is cut and the transcription factor will be released and can translocate to the nucleus to activate the reporter gene expression.[38] The benefit of this approach is its generalizability in that it can be applied to most GPCRs, and it provides signal amplification with a clear readout through the expression of a fluorescent protein.[38] All these features make it ideal for studying biased agonism which is widely known to be triggered by some ligands and studies have shown it can result in favoring one downstream pathway over another (i.e. G-protein coupling vs β-arrestin coupling).[39] The PRESTO-tango method is also useful for deorphanizing GPCRs.[38]

Split NanoLuciferase

Useful for tracking protein-protein interactions is a class of enzymes that have been split into two components so that they will only become functional once they are brought together and reconstitute. The split Nanoluciferase (NanoLuc) enzyme is an example which has proven its utility through tracking association of G-protein complexes with GPCRs.[9] The components of the split enzyme are stable and compact making them ideal for expression in a variety of configurations and geometries, allowing for simple tracking of PPIs.[40] One particularly useful split NanoLuc feature is that the interaction is proximity dependent giving the added benefit of very low background activity.[41] The luminescent signal that is a result of enzymatic activity can be temporally controlled by the addition of substrate. The split NanoLuc enzyme has demonstrated functional versatility in a variety of cellular environments and the resulting luminescent signal has proven robust and beneficial as a readout format.[42]

1.4 Tools for tracking changes in GPCRs

So far, I have presented more indirect methods for tracking the results of the GPCR activation. However, it would be ideal to characterize the functional activation mechanism of the GPCR to generate a direct readout. Function modulation has been one key solution to the need for monitoring adoption of certain confirmations.[43] Nanobodies or other engineered proteins have also been used as conformation-specific binders (CSB) (discussed in **Chapter 2**) to track and bind to only a specific form of the GPCR.[43] The types of conformational states GPCRs can adopt is diverse and dependent on the type of agonist, lipid, or interacting ligand.[44] Therefore, it is necessary to have multiple methods for detecting which conformation a GPCR takes on in response to ligand binding, especially when testing a protein for which very little is known about an activator or method of activation.[45,46] Conformation-specific nanobodies have been used to

detect activation tied to a BRET signal and activation specific methods like this have been championed in the discovery of biased ligands—where a GPCR demonstrates preferential adoption of one form over another depending on the ligand.[47]

Building a detection system into the GPCR

The most well-established strategy for biosensor development that involves tracking the conformational change of a GPCR is the insertion of a circularly permutated GFP (cpGFP) into the structure.[48] The engineering of cpGFP has joined the original N- and C- termini and generated new termini in the beta barrel surrounding the chromophore.[48] The insertion site of the cpGFP within the GPCR must be at a point affected by the activation conformational change so that the termini will be moved to close the barrel around the chromophore and enhance fluorescence.[48] For example, the GPCR-activation-based-DA ($GRAB_{DA}$) and dLight sensors feature insertion of cpGFP into the third intracellular loop (ICL3) of the dopamine receptor.[49] As a result of the activation conformational change, the fluorescence produced by cpGFP will increase due to a change in the environment surrounding the chromophore.[49]

Structural characterization methods

Building on strategies to track GPCR conformational changes, there are approaches to characterize GPCRs in various conditions and in the many ways these conditions can be modulated that yield different information.[50] This includes observing the GPCRs in a live cell vs purified protein solution or in a glycosylated vs un-glycosylated form.[51] Structural techniques such as nuclear magnetic resonance (NMR) have requirements for the proteins to maintain their form or preserve stability in various soluble environments whereas certain functional characterization techniques require high concentrations of the protein uniformly maintaining a specific conformation.[44] Each of these unique cases relies on a method to preserve the protein or

verify the stability (or the tertiary structure) of the protein under the desired conditions.[52] Those preparing the proteins will often adjust the method of isolation to ensure properly functioning end product, but working on a large scale for high throughput renders especially challenging.[52]

An example during the process of isolation and purification is the challenge of avoiding denaturation or improper folding of the protein. Some examples of the causes can be as simple as removing the native lipids from the protein during solubilization and size exclusion chromatography (SEC).[53] Denaturation can cause aggregation of the proteins and there are methods to detect this, but it does not tell you anything about the stability of the remaining folded proteins.[54] A similar logic applies for the use of thermostability analysis to detect changes in fluorescence when using a labeled and bound ligand: while the technique determines how resistant to denaturation the protein is under increasing temperatures,[55] it fails to report which conformation the protein has adopted to be thus resistant and must rely on further characterization.[56]

Radioligand tracers for biochemical characterization

The most popular starting point in GPCR characterization is the binding interaction, with assays employed for tracking the first step in the process.[57] Readouts are mostly binary (has the molecule interacted with the GPCR or not?) and the use of competition is conventional for measuring kinetics to answer the question: how much of the molecule is needed to displace a known ligand? Radioligand assays are an example of a decades-long standard practice for determining molecule-receptor binding strength.[58] There are three categories of radioligand binding assay: saturation, indirect, and kinetic.[59] For a saturation binding experiment, the concentration of the receptor is held constant and incubated with separate fixed concentrations of radiolabeled ligand until reaching equilibrium.[58]

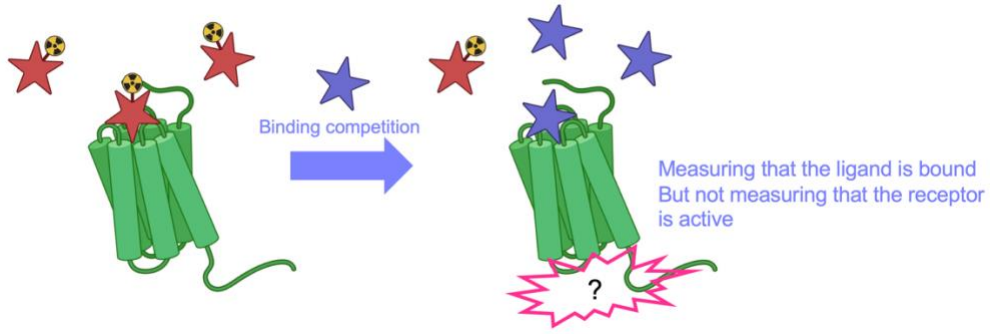


Figure 4 Overview of an example of a biochemical assay, the radioligand binding assay, which reports on the binding affinity of the drug for the GPCR but does not reveal any information about the active state of the receptor.

The biggest challenge for radioligand binding assays is accounting for non-specific binding interactions.[59] When measuring the output signal, it is a sum of all the radio signal from the ligand bound to the receptor plus the radioligand signal from bound to anything else and therefore needs to be corrected for accurate "bound" ligand measurement. Accounting for this discrepancy requires signal subtraction as well as an excess of unlabeled competitive ligand to ensure complete displacement of the known high-affinity radiolabeled ligand.[59] This step requires a follow-up where all unbound ligand is removed without disturbing established interactions, where washing out the ligand could be particularly disruptive of any weakly bound interactors.[60] Final downsides include that the assay requires GPCR purification, a step that is not always feasible for more delicate structures and the readout only reports on bound vs unbound interactions without a quantification of ligand efficacy.[61] This becomes particularly challenging when working on a large scale where using radioligands can be infeasible and dangerous.[52]

Radioligand binding assays are not the only methods that can be performed in a biochemical context for characterization. Others include mass-spectrometry techniques and fluorescent ligands, among others, which are discussed at length in **Chapter 3**. I will point out however, that most of the assays specific to direct measurement of conformational changes are

designed for a live-cell environment (which does provide certain advantages given their functional readouts) and aren't accessible for a biochemical application. There is still a need for functionality-detecting methods adapted to be used outside of a living cell to characterize GPCRs in solution (or lysate).

1.5 Engineering an alternative characterization assay

The strategies summarized here have been for tracking various products triggered through the signaling cascade by the GPCR activation or characterizing the interactions between the drugs and the GPCR. These methods do not report on the internal activation mechanism, a conformational change, that the GPCR undergoes after the drug binds. While some of them do readout the coupling interaction which ensues post activation and is dependent upon the GPCR assuming the activated conformation for the binding pocket, these methods have not been adapted for use outside of living cells. I envisioned avoiding using approximations of triggered activation (tracing consequential/downstream effects) and incorporating the best of both live cell and biochemical experimental elements: both a binding readout as well as an activation readout.

The focus of this thesis is whether it is possible to provide a simplified, streamlined functionality measurement for GPCRs outside of a live-cell environment. Working with these proteins in solution is common for a variety of experiments including kinetic isolation for structural and biophysical characterization studies.[44] Taking inspiration from a variety of existing methods, I will create a new assay and apply it in a variety of formats. From the live cell-based methods, harnessing conformation specific binding interactions would directly mark changes in structural activation and efficacy while from the biochemical side, readouts on ligand binding kinetics would enhance pharmacological characterization. Based on these established premises,

here I present my development and application of an **I**n vitro **G**PCR split **N**anoLuc l**i**gand **T**riggered **R**eporter (IGNiTR).

Chapter 2
Development of an In Vitro GPCR Characterization Method

Some of the work presented in this chapter was previously published in BioRxiv[62]

2.1 Introduction

G-protein coupled receptors (GPCRs) are a class of seven transmembrane proteins that function as essential intracellular signal transducers.[3] Approximately 30% of FDA approved therapeutics target GPCRs.[63,64] GPCRs remain crucial targets for new therapeutic development yet are difficult to study due to the complexity of their signaling pathways and high modularity.[18] Live cell-based assays have been instrumental for GPCR drug screening, as well as GPCR signaling and mechanistic studies.[24,35,65-72] However, there is still a lack of accessible and generalizable in vitro methods to detect GPCR activation for biochemical applications. Strategies for detecting activation have been limited to tracking the products of the activated signal cascade or measuring the strength of interactions between the GPCR and activating drug. But these existing assays do not readout the structural changes undergone in activation, and they also tend to be limited to either living cell environments or to purified extracted proteins, with no overlap in methodology. Neither condition is particularly convenient or accessible for experimentation and therefore a method that can report on the direct functional activation of the GPCR in a variety of conditions will be useful.

We envision a biochemical assay, adapted from live cell approaches and featuring GPCR protein in cell lysate, would provide a simple and easily adaptable format for broad applications. Existing in vitro assays, including radioligand binding, monitor GPCR-ligand binding, but do not

measure ligand efficacy for signal transduction.[60,61,65,73-76] One live-cell approach, reconstitution of split bioluminescent enzymes, has been used to report on protein-protein interactions.[77] Here we adapt and harness this robust luminescent signal that is quantifiable in a complex biological environment to track a ligand-induced coupling interaction in vitro.[40,78,79]

We report the design of a highly adaptable GPCR luminescent assay for use in cell lysates and in vitro. **IGNiTR** utilizes the agonist-dependent GPCR conformational change and subsequent recruitment of G-proteins mimics and other CSBs[1,13] to reconstitute split nanoluciferase (NanoLuc)[41,77,78] (**Figure 5**). Adding increased versatility over live cell assays, IGNiTR components are easily stored with cell pellets expressing GPCR components frozen to preserve the integral, native lipid environment. Additionally, IGNiTR allows the use of peptidomimetics as CSBs, broadening assay applications.

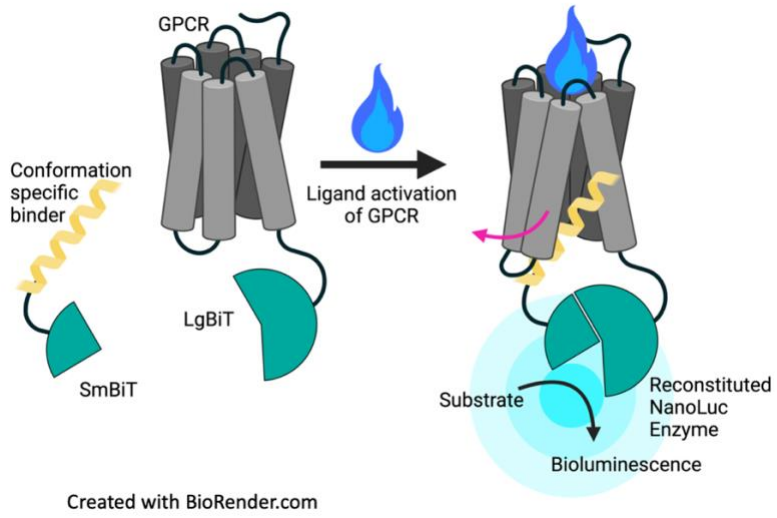


Figure 5 Schematics of the IGNiTR assay: LgBiT attached to a GPCR and SmBiT attached to a conformation-specific binder. Ligand activation of a GPCR results in a conformational change (pink arrow), followed by a GPCR-conformation specific binder interaction, which triggers split NanoLuc reconstitution. In the presence of substrate the enzyme can turnover and produce bioluminescence.

2.2 Orientation of the design components and configurations geometry

Figure 5 demonstrates the main components and their configuration. The general design features a GPCR and the corresponding conformation specific binder (CSB) expressed separately. Attached to the GPCR is one fragment(18kDa) of the split enzyme NanoLuc and the other fragment (1.3kDa, 11 amino acids) is attached to the CSB. NanoLuc is ideal for this application because it is compact (19kDa compared to other luminescent enzymes i.e., *Renilla* Luciferase = 36kDa) and it has been engineered with the capacity to generate up to 150-fold increase in luminescent signal.[42] Another very useful feature of the design is that low intrinsic affinity (kd = 190 μM) enables reversibility of the split enzyme association to monitor inhibitory effects.[41] Careful attention was given to the geometry of these interactions, for example, ensuring that an interaction between the GPCR and the CSB would successfully reconstitute the split NanoLuc. The geometry will be discussed in a later section. In addition to orientation, both effective expression of the protein components and selection of various CSB options were considered.

Selection of CSB components: nanobodies

Nanobodies were one of the first options considered as a CSB candidate. Nanobodies have occupied a unique space in the overlap between structural and cellular biology.[43] They have played crucial roles in binding to GPCRs for a variety of applications.[80] The versatility of these small proteins is demonstrated through the roles they play in probing GPCRs' activation mechanism as well as interactions with other proteins. Their compact structure features a paratope that protrudes, known as complementary determining region 3 (CDR3), capable of fitting within and recognizing the crevices of unique pocket epitopes.[81] Nanobodies are significantly smaller than antibodies (15kDa vs 150kDa) and stable enough for cellular expression, something that antibodies cannot accomplish because they cannot be expressed in cytosolic conditions.[82]

Nanobodies have been generated for the purpose of binding GPCRs in various states to lock them in that conformation for crystallization and solving their structure.[81] They are used in tandem with a ligand or allosteric modulators as a source of stability for proteins that are very unstable in their purified form (outside of the plasma membrane).[83] Nanobodies have also been used to tether or extend a transient interaction, such as the GPCR coupling to G-protein. Because of their ability to bind and perpetuate a transient state, this makes them strong candidates to for studying aspects of activation mechanisms, freezing the frame for analysis of stages of the dynamic process.[83] As just one example, to facilitate co-crystallization of the β2-adrenergic Receptor (β2AR) receptor with Gs protein, Nb35 was developed to bind both proteins and prevent the exchange of GTP to preclude dissociation.[84]

Conformation-specific nanobodies have also been extensively used in the development of biosensors as they can be controlled through spatial and temporal mechanisms to monitor dynamics of GPCRs inside living cells.[85] For example, nanobodies designed to have affinity for β2AR have been used to monitor and regulate association with G-proteins and β-arrestins.[43] These nanobodies can be expressed coupled to green fluorescent protein (GFP) for tracking internalization or trafficking to the plasma membrane.[86] The spatial control enables tracing of GPCR activation within the cell while activation can be controlled temporally by time-dependent addition of the activating agonist. Two standard examples of target-specific biosensor strategies that have been applied to map neural activation *in vivo* are conformational biosensors for β2AR to track internalization of the receptor in endosomes[87] and for μ-opioid receptor (μ-OR) to track opioid signaling[88]. The β2AR/Nb pairing incorporated a GFP attached to the Nb to track receptor activation and localization.[87]

Selection of CSB components: engineered "MiniG" proteins

Looking at options for tracking CSB begs the question of why not simply harness the full version of existing G-proteins to studying coupling. These proteins are unwieldy due to their size, instability, and multiple domains.[89] However, modified, smaller versions of the G-protein complex (termed "miniG") have been developed to facilitate ease of expression (only one domain), stability, and increased coupling versatility (meaning that a class of G-proteins could be imitated by one mini version). The GTPase, Ras-like domain of the Gα protein has been engineered into a smaller version in a way that prevents the dissociation of GDP and therefore perpetuate the interaction between the miniG and the receptor.[90] The result is that the miniG protein will be able to bind to the activated receptor but will not exchange GDP for GTP and therefore will remain bound as long as the receptor maintains the conformation.[89] Further engineering ensures that the miniG is stable enough to be expressed and harvested from *e.coli*.[91]

An example of the contributions of this engineered protein has been the enhanced stabilization (not always possible with full size G-proteins) which make it possible to crystalized Adenosine A_{2A} Receptor in complex with the miniGs. The crystal structure models interactions between the α-5 helix of miniGs interacting with the hydrophobic binding pocket of the receptor, contact points that will become crucially relevant in the next section and information that was not available without the engineered miniG protein.[92] MiniG proteins have been used previously in BRET-based biosensors, where the miniG protein is attached to either the receptor of the donor fluorescent protein and can be used to track coupling with labeled GPCRs at the membrane.[93] The miniG has also been used with split NanoLuc components to trace coupling in live cells.[70] Overall these biosensor applications demonstrate the versatility of miniG as a CSB and candidate for a component of our design.

Selection of CSB components: peptidomimetics

Peptidomimetics are peptide chains designed to mimic structural aspects of specific protein structures and therefore can be applied as CSBs. The interaction between the α-5 helix of the Gα protein (C-terminus) and the hydrophobic binding pocket of the GPCR (**Figure 6**) has been demonstrated to determine differentiation among the various coupling interactions.[94] A peptidomimetic CSB strategy was applied in designing a peptide version of the α-5 helix of the Gα protein to stabilize a GPCR in a specific conformation, acting as an allosteric modulator.[95] An example of a study to examine such allosteric interactions featured C-terminus G-protein peptides (G-peptides) tethered to GPCRs which were used to modulate downstream activity.[96] The study also provided insight into the use of these peptides for both positive and negative allosteric modulation.

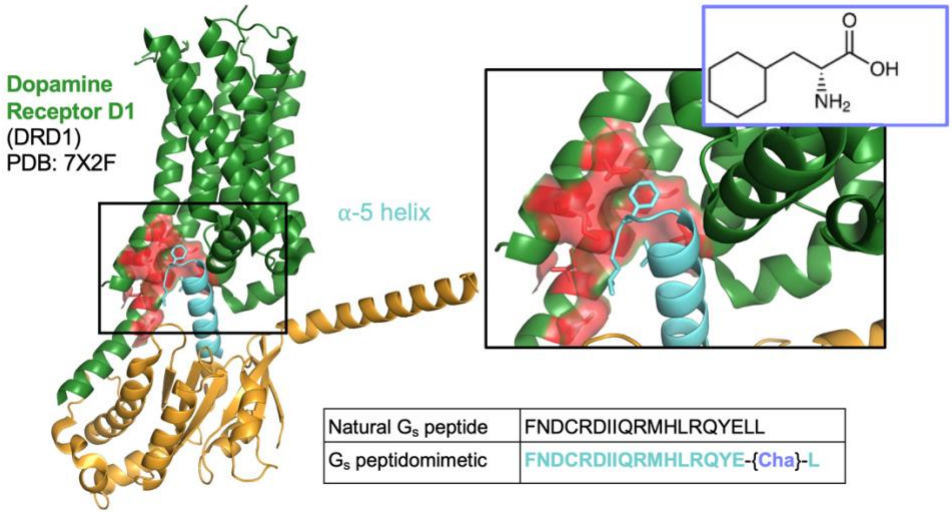


Figure 6 Model structure based on LY3154207-bound DRD1 (PDB: 7X2F). Mutation phenylalanine is introduced in the penultimate position of the G_s protein's α-5-helix to illustrate the interaction between the α-5-helix with the hydrophobic binding pocket of the activated DRD1 (represented by the red surface).

Another demonstration of peptidomimetics as CSBs within biosensor designs contributed to determine structure-based selectivity using systematic protein affinity strength modulation

(SPASM) sensors which incorporate a Gαpeptide attached by a long linker to the GPCR.[97] FRET pairs expressed as part of that linker region were configured to detect changes in the binding of the Gαpeptide to the GPCR.[98] The same strategy has also been employed to determine the efficacy of various ligands while also observing how an allosteric interaction (such as the peptide binding) can affect the affinity of the receptor for various ligands and stabilize various intermediate conformations.[99]

An example of both the specificity and adaptability of peptides as selective CSBs is the Dynamic Cyan Induction by Functional Integrated Receptors (DCyFIR) system. This approach developed Gα chimeras using the strategy of varying the α-5 helix to build the full library of possible coupling partners.[94] The strategy established that the residues responsible for determining specificity of G-protein coupling with GPCR were found in the α-5 helix sequence. And by extension, the approach suggested the use of the α-5 helix alone to differentiate coupling interactions could be enough, thereby replacing the need for making an entire G-protein chimera for imitating the behavior of the domain's interaction with the GPCR.[94]

As demonstrated by DCyFIR, the amino acids in the C-terminus of the Gα protein are crucial for the selectivity of the coupling interaction, a fact that can also be used to distinguish among helices of different G-proteins.[94] The interaction between C-terminus amino acids and the binding pocket have been mapped for the purpose of a) demonstrating the selectivity of Gαs vs Gαi coupling and b) determining which amino acid (AA) sites drive the selectivity of various activated conformations.[92] For the purposes of the data laid out in the following chapters, it is crucial to note that each of the peptides take on a specific orientation when interacting with the cytosolic cavity. Sandhu et al. compared how the G_s, G_i, and G_q helices interact with the binding

cavity (in terms of orientation) and highlight sequence comparison to show residues in energetically favorable hotspots.[100] All five AAs at the C-terminus of the helix have been shown to actively interface with the GPCR directly, which we aim to capitalize on when designing peptidomimetics for distinct G-protein helices. The contact sites on the peptide allow for thermodynamically favorable interactions with the shifting cavity, clamping it into place and stabilizing the conformation.[100]

It was against this backdrop that a group of scientists lead by Morgane Mannes developed a peptidomimetic designed to selectively bind the activated intracellular cavity of Gs coupled GPCRs.[101] The main idea behind this development was the Gα 5 helix c-termini accounts for ~70% of the interaction[72] making it an ideal starting point for G-protein peptidomimetics (expanded upon further in the subsequent chapters). Since peptidomimetics are generated synthetically, the affinity of the Gαs peptide for the pocket was increased by substituting an unnatural amino acid at that penultimate position.[101] Altogether, these advances, including compact, stable design, increased specificity, and further engineering potential, position peptidomimetics as ideal candidates for GPCR allosteric CSBs.

Addressing the pros and cons of the CSBs candidates

Well established CSBs have been either modified endogenous binders or based on mimicking endogenous interactions (**Figure 7**), yet there remain challenges associated with these strategies.[102] Nanobodies are a preferred alternative over antibodies since they are not as bulky or incapable of functioning inside living cellular environments. The CDR of nanobodies have been exploited as their binding can be tuned through immunization to produce target-specific binders, especially for GPCRs.[80] The challenge of establishing a selective CSB interaction is that it must either begin with natural affinity and specificity or be engineered from endogenous affinity levels

to increase selectivity. Some of these nanobodies have been used as scaffolding for the modification of their CDRs to tune them for intermediate conformations or other niche binding requirements.[86] In terms of generalizability, these nanobodies are not the best option however, because they require engineering for the target of choice and fine-tuning to ensure specificity.[102] Another consideration is the expression of the nanobodies within a cellular context: while engineered for cytosolic stability, a lack of expression level control could affect binding stoichiometry.

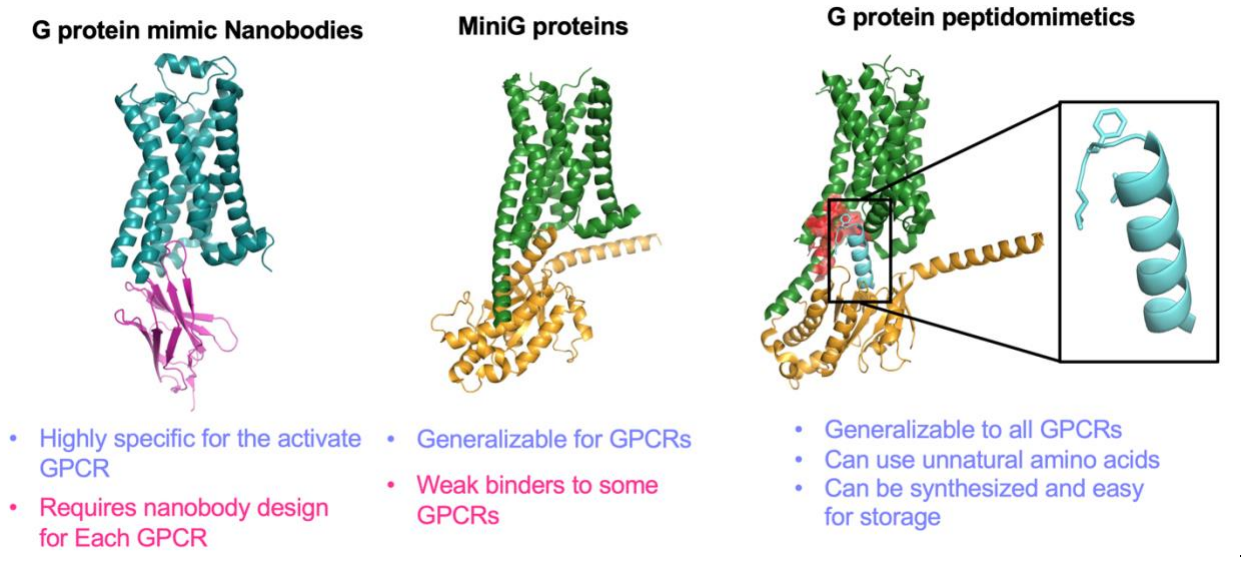


Figure 7 Crystal structures and comparison summaries of the pros and cons of various conformation specific binders. Nanobody PDB 3P0G; MiniG protein PDB 7X2F; peptidomimetic PDB modeled from 7X2F.

Many of the same objections raised for nanobodies apply (i.e., lack of generalizability) for the engineered Gα protein (miniG) including the fact that a full suite of these truncated proteins has yet to be developed.[24] Researchers have preferred the use of tagged full length G-protein heterotrimers for mapping constitutive activity via BRET in live cells.[103] However there have been successful libraries built of Gα c-termini chimeras using the Gαq backbone and swapping out 6 c-terminal amino acid sequence for differentiating the specificity.[72] These chimeras have been used

for determining coupling interactions.[94] However, among the GPCR community, there are mixed reviews on the usefulness of these approaches.[35]

Despite the specificity of nanobodies and their ability to modulate GPCR activity, there is a market for developing even smaller (1-2kDa) peptidomimetics to replace these biologics.[104] Attempts have been made to develop mimetics that capture the crucial interactions between CDR regions of CSBs and the GPCRS. For example, the penultimate amino acid of the Gα protein c-termini is crucial for defining affinity of the α-5 helix for the GPCR hydrophobic binding pocket. The goal would be to generate a modulator based on these essential interactions that is even smaller, more stable, cheaper and easier to produce.[105] Peptidomimetics solve these challenges.

2.3 Materials and methods

DNA constructs and cloning

Standard cloning procedures, including NEB restriction enzyme digest, Q5 polymerase PCR amplification, T4 ligation and Gibson assembly, were used. Oligonucleotide primers were purchased from Sigma Aldrich. The plasmid DNA encoding MC4R was a gift from the Roger Cone Lab. The plasmid DNA encoding miniGs and Nanobody 80 was purchased from Twist Biosciences.

Plasmid constructs were transformed into *Eschericha coli* cells via heat shock. XL1-Blue competent cells were used for all constructs. Sequences were confirmed by Sanger Sequencing (Eurofins, GeneWiz)

Cell culture and transfections

HEK 293T/17 cell lines (ATCC, cat#: CRL-11268) used in these experiments were cultured at 37 °C and 5% CO_2. Cells were grown in complete growth media (1:1 MEM (Eagle's Minimal Essential Medium): DMEM (Dulbecco's Modified Eagle Medium, Gibco): with 50 mM HEPES (Gibco), 10% Fetal Bovine Serum (Sigma), and 1% Penicillin-Streptomycin (Gibco).

Cells at 80-90% confluence were plated into a flask that had been pre-incubated with human fibronectin for 10 minutes at 37 °C. An hour after seeding, these cells were transfected using FBS-free MEM and polyethlenimine (PEI, 1 mg/ml, Polysciences) with a ratio of 1:10 between µL of PEI and µg of plasmid DNA. The cells were then incubated at 37 °C for 20 – 24 hours.

The cell pellet was harvested by aspirating the media and resuspending the cells with a cell scraper in Dulbecco's Phosphate Buffered Saline (DPBS) buffer. 18 mL of DPBS was used to resuspend the cells within a T-75 flask, with this ratio kept constant for other flask sizes. 1.5 mL of resuspended cells were placed into an Eppendorf tube, which was centrifuged at 6,010 g for 3 minutes. The supernatant was aspirated, and the pellet was resuspended in DPBS, then centrifuged again under the same conditions. The supernatant was aspirated again, and the cell pellet was flash frozen in liquid N_2 and stored at -80 °C until ready for use.

Preparation of cell pellet

The cell pellets were prepared immediately before the assay, by first placing the cell pellet onto ice. For cell pellets containing GPCR constructs, the pellet was treated with 210 µL of Membrane Resuspension Buffer (MRB). MRB is comprised of incomplete membrane

resuspension buffer (resuspension buffer comprising 20 mM HEPES, pH 7.5 and 2 mM $MgCl_2$) and benzoase (EMD Millipore, 70746) in a 4.5 mL to 1.8 µL ratio. For cell pellets containing cytosolic proteins (Nanobody 80 or miniGs), the pellet was treated with Mammalian Protein Extraction Reagent buffer. After this, 2 µL of 100X protease inhibitor (Sigma Aldrich, P1860#) was added to a final concentration of 1X and the pellet was resuspended by pipetting up and down. The solution was then sonicated using Model 50 *Sonic* Dismembrator (Fischer Brand) at a 20% amplitude (3 x 1 second pulse) and returned to ice.

IGNiTR assay

A master mix containing Nano-Glo Buffer and substrate (Promega, N2012), GPCR construct, and G-protein mimic was prepared. For one well, the ratio was 9.75 µL of Nano-Glo Buffer, 0.25 µL of Nano-Glo substrate, 5 µL of GPCR pellet and 5 µL of the CSB. The CSB may be either peptidomimetic (LifeTein), miniGs pellet or Nanobody 80 pellet. The GPCR construct cell pellet was prepared separately and added to the master mix immediately prior to adding the master mix to the well. The concentrations were varied for the optimization assays, but the volumes remained constant. Concentrations for each experiment are indicated in the figure legends. The 384 well cell culture plates were preloaded with 10 µL of drug per well, followed by loading of the 20 µL of the master mix per well. Times reported on figure captions were recorded from the addition of the master mix to the first well of the plate. The luminescence values for all the conditions were measured using an EnVision 2104 Multilabel Reader (Perkin Elmer).

2.4 Results and discussion

Preparation of components: method development

We chose to express the GPCRs in mammalian cells to facilitate the correct folding, membrane trafficking and post-translational modification of these complex proteins.[73] Cell lysis converts *in vivo* into biochemical conditions, enabling components outside the cells to access the intracellular loops of the GPCR and ensuring homogeneity of the protein components. Various methods have been developed for breaking up the cell membranes, as well as for solubilizing and stabilizing the GPCR protein, including the use of specific detergent mixes.[106] To maximally preserve GPCR protein folding and function, we tested two cell lysis conditions. We began by testing sonication of the cells in detergent-free solutions, since the native plasma membrane lipid environment provides crucial support for the structural integrity of membrane-bound GPCRs.[44,107] We also tested sonication in solutions containing a detergent mix frequently used to solubilized GPCR proteins (**Figure 8**).

The lysed solution was mixed with the cytosolic protein component solution. Substrate for the reconstituted NanoLuc was mixed in a buffer solution. To this solution, the protein lysate was added and finally, before using the plate reader to detect and quantify the intensity of subsequent bioluminescence, the drug was added directly to the wells with replicates for the +drug condition and mixed thoroughly. If the well contained properly folded and functional membrane protein, the cytosolic protein (nanobody attached to LgBit) bound in the presence of the agonist, allowing the reconstitution of the split NanoLuc.

We tested Nanobody 80 (Nb80), which specifically binds activated β2AR.[80,84,89,108] For a more generalizable application, we also tested miniG_s protein which has been shown to bind effectively to a range of the activated G_s-coupled GPCRs.[89,93]We tested two different fusion geometries with

β2AR fused to either the large portion of the split NanoLuc, LgBiT, or the small portion, SmBit, and the G-protein mimic fused to the other half of the split NanoLuc. As shown in **Figure 8**, significant drug-dependent luminescence increase (abbreviated as drug dependent ratio (DDR) in this paper) was observed for both Nb80 and miniG_s in the two geometries in the lysis condition without detergent.

The data in **Figure 8** also indicates that different fusion geometries could affect IGNiTR performance. For example, when using Nb80 as the CSB, the DDR was much higher as detected when GPCR was fused to SmBiT and Nb80 to LgBit (compared to the reverse geometry). However, when using miniG_s as the CSB, the opposite fusion geometry with GPCR fused to LgBiT and miniG_s fused to SmBiT yielded a greater DDR (compared to the miniG_s-LgBiT/GPCR-SmBiT fusions).

Figure 8 also shows that lysis with solutions containing detergent significantly diminished the luminescence in all conditions tested, suggesting the detergent disrupts the GPCR's functionality. The outcome was expected since it has been shown that detergents can cause perturbations that affect the ability of the protein to be activated.[80] This experiment highlights the importance of keeping the GPCRs in their native lipid environment. Therefore, for optimal IGNiTR assay performance, the cell pellet will be lysed by sonication without detergent.

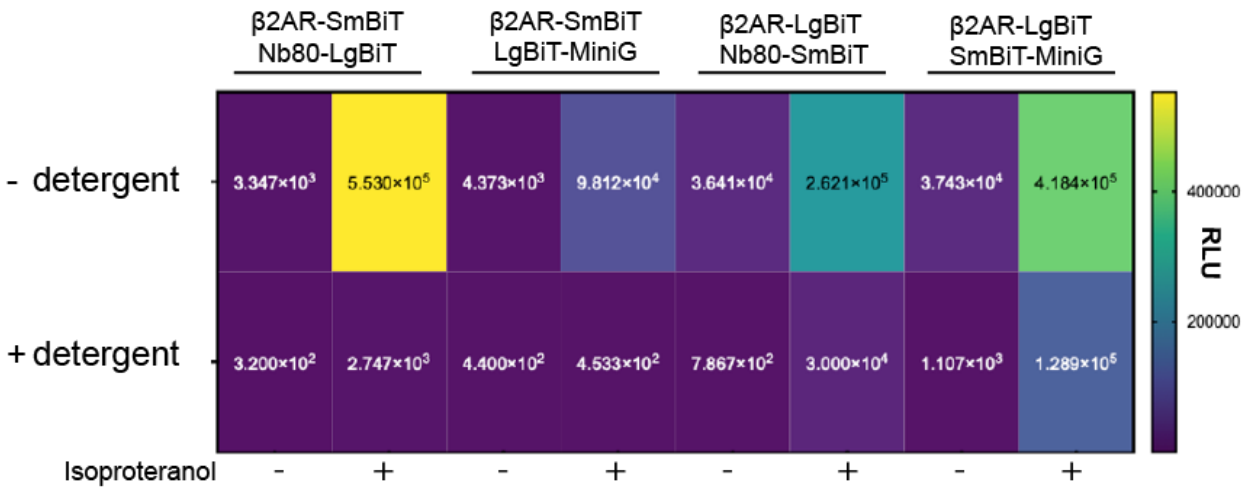


Figure 8 Comparison of IGNiTR component fusion geometry prepared in no detergent vs detergent conditions. Four versions of IGNiTR with different fusion geometries and Nb80 or miniG as conformation-specific binder were tested. The cell pellet expressing these IGNiTR components were lysed by sonication in solutions with or without the detergent mix (1% DDM (n-dodecyl β-D-maltoside) and 0.1% CHS (cholesteryl hydrogen succinate). Addition of detergent significantly decreases luminescence in all conditions. Therefore, we used sonication without detergent in the remaining work. Numbers inside the grids are relative luminescence signal.

Testing IGNiTR with cellularly expressed protein components

We expanded our testing to include three total $G\alpha_s$-coupled GPCRs as models to develop IGNiTR: in addition to β2AR used in **Figure 8**, we added DRD1 and MC4R.[79] All three of these GPCRs are well studied and have solved crystal structures.[109] Both β2AR and DRD1 have been standard subjects of similar coupling experiments and therefore are reliable for comparison with known activities and agonists.[110] MC4R is a more niche target known for its often promiscuous and difficult to untangle coupling interactions, and therefore presents a unique test subject without any engineered specific binders.[22] For the conformation-specific binding components, we first explored the use of nanobodies and $miniG_s$ which have been used in live cell-based split NanoLuc assays.[40,78,93]

All IGNiTR constructs (prepared via sonication, see **Figure 8**) produced significant ligand-dependent luminescence increase, hence a value > 1 for the ratio of the IGNiTR luminescence with drug to that without drug (DDR). IGNiTR composed of β2AR fused to LgBiT and SmBiT fused

to Nb80 or miniGs each yielded significant DDRs (**Figure 9**), indicating Nb80 and miniGs can both selectively bind to the active conformation of β2AR in cell lysate. As expected, Nb80 did not show significant DDR with either DRD1 or MC4R. While when using miniGs, both DRD1 and MC4R produced significant DDRs (**Figure 9**). The results validate that IGNiTR can detect a GPCR's agonist-dependence.

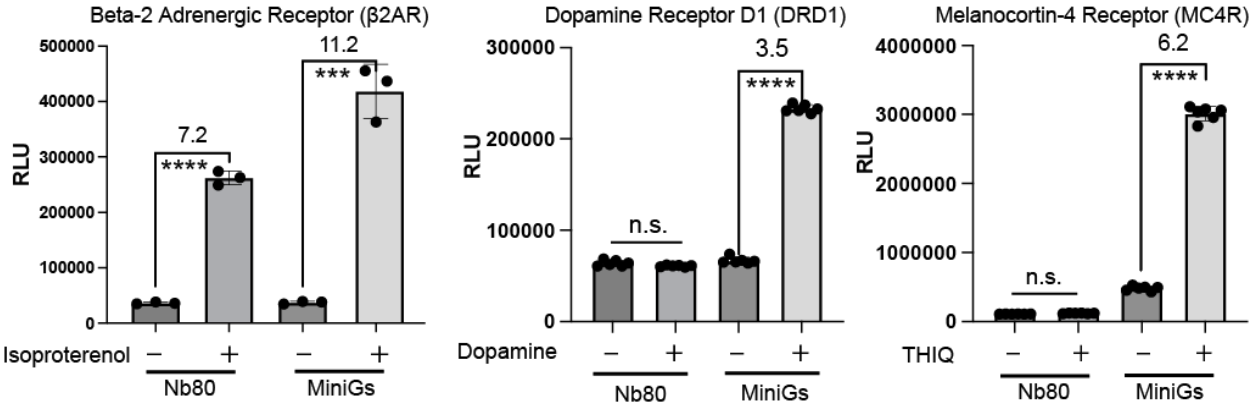


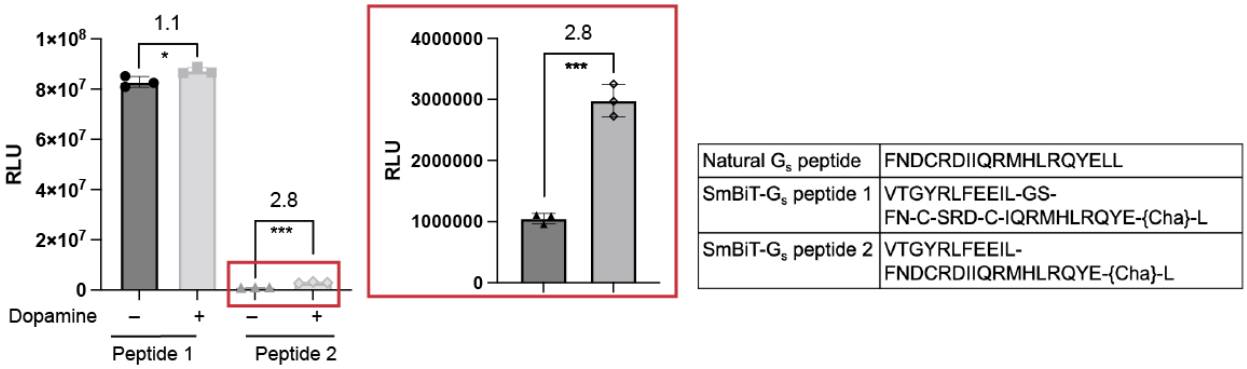
Figure 9 Characterization of IGNiTR with β2AR, DRD1, and MC4R with Nanobody 80 (Nb80) and miniGs as the conformation specific binder. Drug, 10 μM. RLU: Relative Luminescent Units. n=3 for β2AR and n=6 for MC4R and DRD1. Values above the bars represent the DDR. Stars indicate significance after performing an unpaired Student's t-test. ***P ≤ 0.001, ****P ≤ 0.0001. "n.s." indicates no significant difference between the two conditions.

Applying a synthetic Gs fusion peptide component to IGNiTR

Our design was inspired by a reported Gs peptidomimetic[101] (**Figure 10**), which was based on the α-5-helix of Gαs in the crystal structure of the Gs protein complex bound with β2AR.[92] The Gs peptidomimetic (FNDCRDIIQRMHLRQYE-[Cha]-L) conserves the Gαs 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.[101,111]

Our design fused the SmBiT (11 amino acids) to the Gs peptidomimetic to create a SmBiT Gs peptidomimetic fusion peptide (**Figure 10**). One version of the reported Gs peptidomimetic incorporated two unnatural amino acids in the helical backbone away from the GPCR contact sites

that form covalent bonds to generate stapled peptides to stabilize the helical structure.[101] In an attempt to mimic this stapled peptidomimetics strategy, we tested using cysteine residues instead to facilitate a di-sulfide bridge formation in the α-helix. We decided to test both our non-stapled and this stapled G_s peptidomimetic with DRD1-LgBiT (**Figure 10**). While the disulfide-bond containing peptide 1 did not demonstrate agonist-dependence, G_s peptidomimetic peptide 2 showed agonist-dependent DDR. The high background signal of G_s peptide 1 can be attributed to the cysteine-based disulfide bond failing to connect the helical structure of the α-5-helix.



Natural G_s peptide	FNDCRDIIQRMHLRQYELL
SmBiT-G_s peptide 1	VTGYRLFEEIL-GS-FN-C-SRD-C-IQRMHLRQYE-{Cha}-L
SmBiT-G_s peptide 2	VTGYRLFEEIL-FNDCRDIIQRMHLRQYE-{Cha}-L

Figure 10 Comparison of drug dependent activation (DDR) between two sequence versions of the G_s fusion peptide with DRD1. Peptide 1 incorporated cysteine residues in an attempt to facilitate backbone stabilization of the α helix. Stars indicate significance after performing an unpaired Student's t-test. ****P ≤ 0.0001. "n.s." indicates no significant difference between the two conditions.

To expand testing of the peptidomimetic version of IGNiTR, β2AR-LgBiT protein in sonicated cell lysate was mixed with the G_s fusion peptide and NanoLuc substrate. Then, agonist or vehicle was added to evaluate the DDR. β2AR IGNiTR with the G_s fusion peptide produced a significant DDR (**Figure 11**). We further tested the G_s fusion peptide with the other two G_s-coupled GPCRs, MC4R and DRD1, each producing significant DDRs (**Figure 7**). These results validated the G_s peptidomimetic's selectivity for the active conformation of the G_s-coupled GPCRs.

Using IGNiTR to 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 various ligands, we applied the technique to DRD1 IGNiTR with full agonists, partial agonists, and antagonists. The full agonist dopamine produced higher DDR than the partial agonist fenoldopam at saturated concentrations, with both producing a DDR > 1 **(Figure 11B)**. The result validates that both full and partial agonists induce the active conformational state[110,112] and that IGNiTR can differentiate ligand efficacies. DRD1 antagonist, SCH 23390, does not increase luminescence compared to the no drug condition.[110] These results further validate the G_s peptidomimetic's selective binding to the active conformation of DRD1. Lastly, DRD1 titration with dopamine and fenoldopam produced EC50 values of 2.6 μM and 145 nM, respectively (**Figure 11C**) that correspond well with the reported EC50 values.[110,112] DRD1 titration with antagonist SCH 23390 in the presence of 10 μM agonist dopamine yielded an IC50 of 26 nM, which is similar to other values reported in the literature.[113] Overall, these characterizations demonstrate that the GPCR in IGNiTR maintains function comparable to live cell assays and IGNiTR can detect various ligand efficacies.

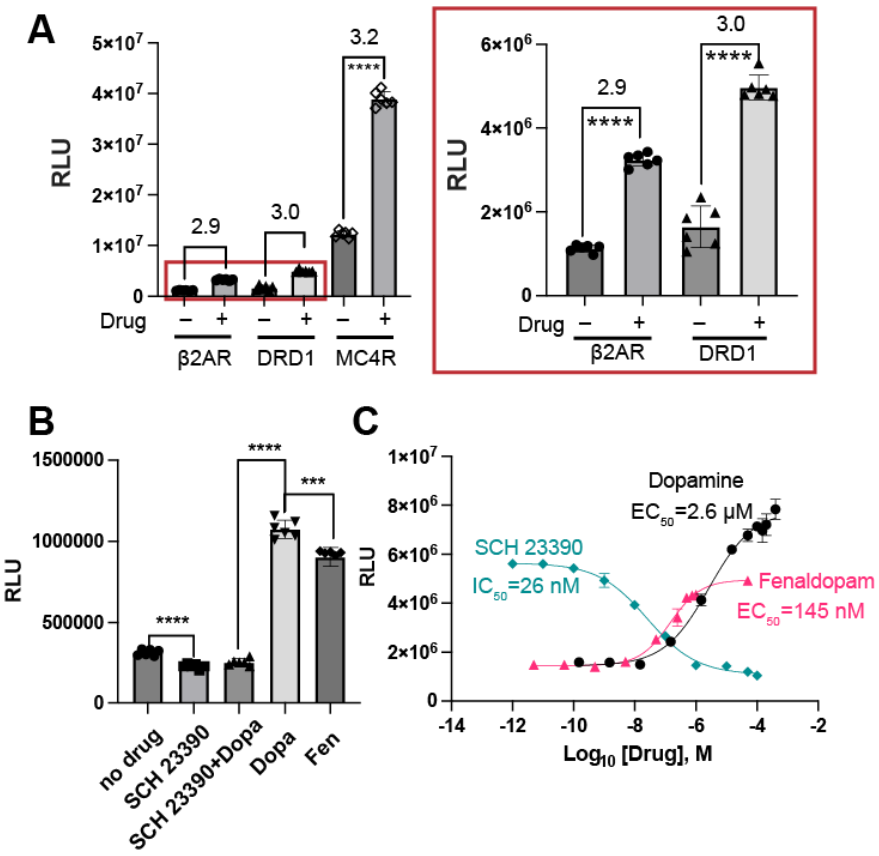
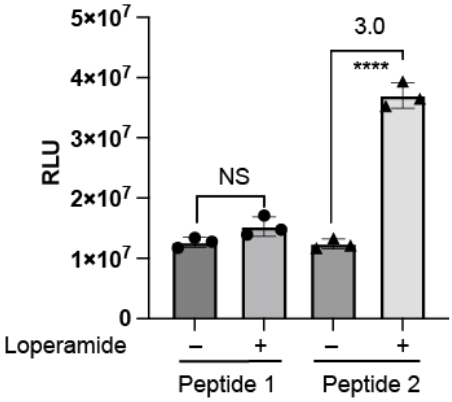


Figure 11 Characterization of the IGNiTR assay with peptidomimetics. *A.* Characterization of the IGNiTR assay with the G_s fusion peptide for β2AR, MC4R, and DRD1. G_s fusion peptide, 2 μM; drug, 10 μM. n=6. *B.* Comparison of DRD1-IGNiTR signal with a panel of drugs at saturated concentrations. SCH23390, 50 μM; SCH23390+Dopa (Dopamine), 50 and 10 μM; Dopa, 10 μM; Fen (Fenaldopam), 10 μM. Luminescence values were taken at 30 minutes post drug incubation. *C.* Dose-response curve of DRD1 IGNiTR with dopamine, fenaldopam and SCH 23390. For dopamine, EC50 range within 95% confidence is 1.8 to 4.1 μM. For fenoldopam, EC50 range within 95% confidence is 114 nM to 181 nM. For SCH 23390, IC50 range within 95% confidence is 20 nM to 33 nM. n=4. Stars indicate significance after performing an unpaired Student's t-test. ****$P \leq 0.0001$.

Expanding versatility of IGNiTR to include G_i fusion peptide

To test engineer a G_i-mimic peptide, we evaluated 2 peptides to assess the importance of the incorporation of the unnatural amino acid, Cha, at the penultimate position of the peptide mimic (**Figure 12**). A leucine (Leu) residue at the penultimate position of the α5-helix is conserved in all the G-proteins, indicating its importance in binding to GPCRs.[25] Changing this Leu to Cha was found to improve the binding affinity of G_s-protein mimic for β_2AR.[25] We tested whether this Cha replacement could enhance the binding affinity for the other G-protein peptidomimetics as well.

We tested IGNiTR with a G_i fusion peptide and a G_i-coupled GPCR, the μ-OR. A significant DDR was observed for μ-OR IGNiTR peptide 2 (**Figure 12)** while much like the behavior of the SmBiT-G_s peptide 1, the cysteine residues of SmBiT-G_i peptide 1 failed to facilitate helix formation and thus resulted in no significant DDR. The result validates the G_i peptide 2's selective binding to the agonist-activated μ-OR and establishes the use of G_i fusion peptide in IGNiTR for G_i -coupled GPCRs.



Natural G_i peptide	FDAVTDVIIKNNLKDCGLF
SmBiT-G_i peptide 1	VTGYRLFEEILGSKKKFD-C-VTD-C-IIKNNLKDSG-{Cha}-F
SmBiT-G_i peptide 2	VTGYRLFEEIL-GSKKK-FDAVTDIIIKENLKDCG-{Cha}-F

Figure 12 Comparison of drug dependent activation (DDR) between two sequence versions of the G_s fusion peptide. Peptide 1 incorporated cysteine residues in an attempt to facilitate backbone stabilization of the α helix. Stars indicate significance after performing an unpaired Student's t-test. ***$P \leq 0.001$. *$P \leq 0.05$.

2.5 Conclusions

In summary, we developed a protocol for successfully preparing GPCRs with preserved functionality, especially for in vitro conditions. The IGNiTR assay design was tested using a variety of CSBs for detection of drug dependent GPCR activation. The development of both G_s and G_i fusion peptidomimetics established a novel approach for characterization of the GPCR conformational change responsible for inducing coupling with G-proteins. The control gained through using a peptidomimetic includes using unnatural amino acids to fine-tune the affinity of the peptide for the hydrophobic binding pocket as well as fine tuning concentrations of the peptide within small reaction volumes. The benefits of component regulation will be exhibited in

the further development of IGNiTR for applications in the next chapter. Overall, the development of the fusion peptide offers distinct advantages over using cellularly expressed CSBs, in both categories of consistency and versatility. The peptidomimetic has the potential to be further developed for a variety of applications to harness their capacity to detect coupling for receptors within classes of GPCRs.

Contributions: Ruby Miller conceived the idea for the project, design, and experimental plans. Wenjing Wang advised on the details of carrying out necessary experimentation. Jennifer Sescil aided with the characterization (such as titrations) and with producing biological replicates.

Chapter 3: Implementing the IGNiTR Assay in a Variety of Applications

Some of the work presented in this chapter was previously published in BioRxiv

3.1 Introduction

Thus far in the thesis I have established the precedent of working with cellular components to track function based on distinctive signaling pathways. Often the expectation assumed when working with cellular components is that the best way to study them would be within in their endogenous environments (i.e., within a living cell), where the activations, couplings, and downstream effects all operate in sequence. However, there are certain contexts where it becomes advantageous to study cellularly expressed components outside the living cell. Examples of these specific cases include isolation of proteins for highly specialized characterization, such as binding interactions between a protein and a ligand.[44] When adapting cellular components to a biochemical context, it is important to understand that each assay offers advantages and disadvantages that are entirely dependent upon the target(s) and information expected to be gained from the testing. Thus, there is a need for a wide diversity of methodology to draw on, especially when analyzing a category of receptors as diverse and individually unique as GPCRs.

One parameter that becomes relevant for GPCRs is their status as drug targets and a need to screen large quantities of molecules against the receptors, investigating their interactivity. The most economical and convenient approach has been high throughput screening (HTS) models

which enable rapid collection of robust data sets. Depending on the HTS model employed, readouts can report on a wide variety of details about the GPCR-ligand interaction. For example, some HTS can be prepared to report on the binding of the ligand to a specific GPCR pocket or cavity compared to another HTS platform designed to report on protein couplings elicited by GPCR-ligand interaction. A variety of these assays will be explored in the following sections.

Another parameter relevant for testing GPCRs is the accessibility of the method. For example, we explore harnessing the GPCR as a detection method for synthetic opioid derivatives which are often highly potent and thus have the potential to cause lethal overdoses.[114] IGNiTR could be packaged as an accessible kit for detecting GPCR agonists, outside of a biosafety level 2 laboratory space. And finally, there are demands for increased diversity in method availability for characterization and detection of GPCR activity under a variety of conditions. Explored here are the challenges particular to tracking GPCR structural integrity and function, especially throughout the Nanodisc assembly process.[115]

3.2 In vitro applications

High throughput assays (for scaling up)

High throughput screening (HTS) is a generic term for a format of testing that scales up from individual experiments to testing thousands of reactions in an accelerated timeframe. The most common application for HTS has commonly been drug screening which is particularly applicable and relevant for GPCRs.[66] When little is known about the target (GPCR) or when looking for novel binding effectors (such as allosteric vs orthostertic), HTS of libraries of compounds becomes the most effective way to find desired compound activity.[116] While it is true that structure-based strategies such as docking and virtual screening are viable methods based on growing amounts of structural information, HTS remains extremely accessible with the number of sensor-based

methods that have been developed.[13] Within this category, there are cell-based assays (refer to sensors designed for monitoring activation discussed in the introduction chapter 1) and biochemical assays.

Biochemical assays for HTS of binding interactions

The standard biochemical assay format requires purified protein receptor (GPCR), and involves competition binding, or displacement of a known binder.[117] The reactions are carried out in 384-well plates for ideal reaction volume and measured via optical readouts, typically either absorbance, fluorescence, or luminescence.[116] Examples include total Fluorescence intensity (FLINT) where the dye in a substrate is released by enzymatic reaction and resulting fluorescent signal is integrated over time.[118] This method can be affected by autofluorescence and quenching. Another example, ratiometric fluorescence, overcomes some of these issues by measuring a change in signal.[119] The HTS technology fluorescence anisotropy/polarization (FA/FP) connects the change in rotational correlation time of bound vs unbound states through a dye bound to the ligand.[119] Excited by polarized light, the fluorophore tumbles freely before slowing rotation upon binding to the larger protein component.[120] The resulting change in the polarization of the light can be quantified and correlated to the binding interaction.[120] A final example in this grouping is Förster resonance energy transfer (FRET) which can be used to track transfer of energy from a fluorescent donor to an acceptor within a radius of 2-6 nm.[116] When applying this method to GPCRs, geometry and placement of the donor and acceptor must be carefully considered which can often preclude the use of the technique with less-well known receptors.

In contrast to the biochemical assays previously mentioned, binding-based assays measure only the binding interaction between GPCR and ligand, not any subsequent protein coupling binding.[121] These are examples of biophysical techniques, primarily under the category of

Fragment-Based Drug Design (FBDD) which are often limited to smaller library sizes.[122,123] HT-Surface plasmon resonance (HT-SPR) is an example of one such technique used in FBDD.[124] The technique features purified proteins covalently immobilized on a sensor over which small molecules can flow. Binding kinetics and stoichiometry are measured as a result in a change of the refractive index at the gold, glass, or solvent interface (sensor surface).[116] The refractive index changes proportionally to the mass of the bound components in unbound vs bound form.[124] While there is a lot of information to be gained from the experiment, the rate of testing can only occur at 100s of molecules/day which is not ideal for HTS.[116]

The last binding-based assay discussed here will be affinity selection-mass spectrometry (AS-MS). In this method, purified protein is incubated with a molecule pool followed by separating out only the protein-ligand bound complexes.[125] The isolated bound ligands are then dissociated from their protein and analyzed using liquid chromatography, electrospray ionization, and mass spectrometry.[126] Excellent sensitivity and a linear dynamic range are advantages of using this method, but ionization of different proteins or molecules can be an issue when comparing across experiments.[116]

Many cell-based biosensors have been discussed in the introduction. Highlighted here is one main cell-based assay that has been scaled up for HT detection of increased cAMP levels connected to GPCR coupling: the GloSensor.[127] The high sensitivity and dynamic range of this assay is achieved through the design which features a protein kinase A (PKA) cAMP binding domain coupled to a circularly permutated luciferase.[127] Binding of the cAMP molecule to the PKA allows for a conformational change, generating a functional luciferase. The resulting luminescent signal can report on the kinetics and magnitude of the cAMP release. The GloSensor assay has been scaled up for HTS ligand screening.[128]

Opioid detection: IGNiTR for visualizing change

We envisioned adapting the IGNiTR system for increased accessibility to broaden the diversity of drug detection methods available to be used within the context of "harm reduction".[129] Drug and pill testing are becoming crucial to the "harm reduction strategy" where healthcare workers aim to mitigate harmful effects of substance use.[129] For example, point-of-care workers need methods to help rapidly detect drugs such as opioids.[130] The best methods are highly technical and require great skill, training, and time to accurately process the data.[130] The following are examples of rapid detection tests for opioid drugs.

Colorimetric detection or color spot tests are the result of chemical reactions of the analyte with a detection reagent which produces a colored spot or band.[131] Detection methods are cheap and accessible without requiring high skill levels. The color change can be detected with simple technology such as a smartphone camera.[130] However, interpretation of the results does require a trained eye and accounting for changes in light quality or intensity or a thorough database for comparative analysis.[129] The main limitations of this method are that it only identifies the presence or absence of the drug and that it lacks specificity making false positive results likely and difficult to overcome. For example, several different assays may be required to determine the contents of a drug mixture.[129] Yet, optical detection has proven to be rapid and convenient.

Another common method for opioid detection involves the binding of an antibody selective for the analyte of interest and using an Enzyme-linked immunosorbent assay (ELISA)-based detection method (like fluorescence) to report the binding.[130] These assays can be commonly formatted as lateral flow assays (LFA) and packaged in convenient strips to which the biological sample can be applied.[131,132] In an immunochromatographic type of configuration, if the analyte is present, a band of color will appear. Gold nanoparticles (AuNP) functionalized with analyte-

specific binders have also been used as a component of these detection strips.[130] For example, AuNPs have been functionalized as part of a strip for fentanyl detection in urine.[133] The limits of detection (LOD) have been reported in the low nanomolar range, indicating high sensitivity.[131] However, specificity and false positives can still be challenges, since antibodies have demonstrated off-target binding.[129]

We envisioned utilizing IGNiTR as a complementary assay for existing colorimetric detection or lateral flow tests. There is an increasing need for point-of-care detection methods and an additional test could prove useful for determining opioid components of unknown drug mixtures.

Nanodiscs: adapting IGNiTR for changing environments

It has been demonstrated that the components surrounding the GPCR will directly affect its integrity.[134-136] Staus et al demonstrated that a micelle made with detergent components affected the functionality of β_2AR compared to the same GPCR encompassed in a phospholipid bilayer (HDL particle).[134] Nanodiscs are an example of a technology developed to accommodate the challenge of creating an ideal environment to promote protein stability.[137] They were first characterized by the Sligar group in 2002 and featured a lipid bilayer enclosed by a membrane scaffold protein wrapped around like a belt.[138] Nanodiscs are uniquely positioned to provide a vehicle for studying delicate proteins in various environments, as an alternative to typical harsh isolation strategies, which lack in condition adaptivity.[54]

For the average GPCR isolation in a biochemical context, the protein solubilization method may be chosen dependent upon the application or end goal for the isolated protein. The conditions selected are based on the structural integrity of the GPCR.[54] For example, if the

protein is not very stable outside of the lipid membrane (which is the case for most GPCRs) special tailored solubilization conditions are tested and optimized.[73] Common failure modes can occur in the form of low expression levels, lack of stability, inefficient solubilization, low yield from purification strategies, aggregation, and loss of functionality.[56] The issues can be avoided by optimization at each of the stages of production and isolation with special consideration given to preserving the structure in the most functionally conserved state.[73] For example, the GPCR can be truncated, mutated, or modified to improve stability by removing disordered sections.[54] Moving beyond the stabilization of the protein itself, it becomes necessary to solubilize the proteins and examine them in various environments.

For a particular set of biological studies of the protein, such as gaining structural information, it becomes necessary to isolate the 7TM protein from native lipids.[139] However, in some cases it is more beneficial to study the proteins within the context of their lipid interactions to replicate their native environments most closely.[44] Mammalian cells tend to be easy to lyse with a hypertonic buffer or a freeze-thaw cycle to disrupt the cell wall. Many strategies employ methods of adding detergents for solubilization. These detergents must be carefully selected as mild enough for surrounding the protein in mixed micelles incorporating lipid components.[73] A delicate balance must be achieved between effective solubilization and degradation. Therefore, the ideal concentration and structure of the detergent must be experimentally determined i.e., charged hydrophilic heads and length of hydrophobic tails in addition to amount of cholesterol derivatives incorporated for increased stability.[54] Most of the time, the GPCRs encompassed inside the micelles are supported by the amphiphilic detergent, but there are certain cases/GPCRs where that environment will not be sufficient for preserving the endogenous functionality or nature.[139] The many rounds of purification also increase the exposure of the protein to detergents,

which increases the likelihood of damaging the protein's tertiary structure by the end of the process.

Nanodiscs have been shown to stabilize the GPCR because of the way it mimics a cellular membrane.[140] These vesicles can incorporate the protein shortly after initial solubilization and isolation of the GPCR from its natural membrane environment using a mild detergent. Membrane scaffold proteins (MSP) and additional lipids are added to the detergent-protein mixture and incubated to allow the formation of the Nanodiscs.[140] Detergents are removed from the mixture using nonpolar polystyrene beads followed by pull down of the Nanodiscs using affinity binding. Further purification steps can be performed on the discs to isolate only those that are filled (have successfully incorporated GPCR) standardly confirmed in the form of a size exclusion chromatography (SEC) trace.[135]

Once the Nanodisc has been formed, the next step requires characterizing functionality of incorporated GPCRs. Recruitment of binding partners such as Gα-coupling is perhaps the most accessible proof of activity, which includes GTP hydrolysis measurement where the ability of the GPCR to couple with downstream G-protein components is measured by GTP turnover.[134,141] Some examples include GTPγS binding experiments, which use an analog whose fluorescence increases upon binding to the G-protein. Another alternative would be radiolabeled [35S]-GTPγS Binding Assays.[142] Additionally, the use of a fluorescently labeled GTP analog (BODIPY FL GTPγS) along with purified Gαqβ1γ2 trimer confirms activation when the fluorescence of the GTP analog is restored upon binding the G_s protein (after being quenched in the free-floating aqueous solution).[140] The exchange of GTP for GDP does not fully occur in this scenario but the binding does represent a consequence of effective GPCR-G-protein coupling.[142]

Other examples of functional characterizations performed on the GPCR in the Nanodisc are tracking β-arrestin recruitment as well as competition ligand-binding with fluorescence energy transfer.[143] These assays are all meant to confirm that the protein is fully capable of activation by a ligand and can undergo subsequent conformational shifts.[137] The confirmation of functionality ensures proper expression and folding of the protein and that the environment in which it is incorporated is favorable for the protein.

Another common method for confirming the protein maintains functional selectivity, especially in binding, is pharmacological characterization of the various ligands known to affect the GPCR.[136] For example, assays can be used to track how the membrane environment affects the binding properties (EC_{50} and IC_{50} values) of the GPCR-ligand interaction. These pharmacological profiles change depending on the environment and thus an easy comparison to perform is the activation of the GPCR incorporated in a Nanodisc versus the endogenous GPCR in its cellular membrane.[54] If different ratios of lipids or any native lipids are used in the formation of the Nanodiscs, the effects these lipids can have on the GPCR can also be measured through ligand binding assays.[136]

An area for further refinement could be analyzing how various lipid components and their incorporation can affect the functionality of the protein within the Nanodisc. Testing or tracking the results of incorporation would require optimization of the Nanodisc assembly process for each lipid variation. During the steps of assembly, it would be convenient to track the functionality of the protein as it is incorporated into the Nanodisc. Most of the characterization assays are multi-step processes and involve extreme purification of protein components.[137] Therefore, it would be ideal to have the option of adding a simple peptide into the solution to verify that the protein being incorporated is not adversely affected by the assembly process and

can respond to ligand activation. For example, as discussed previously, the use of various detergents and their concentrations for solubilizing the GPCR from the membrane prior to Nanodisc assembly could disturb fragile proteins and it would be useful to know if any denaturation had occurred.[137] Additionally, IGNiTR incorporates the best aspects of existing characterization methods, most of which are limited to reporting either binding only or limited coupling. By measuring a direct readout of GPCR conformational changes the assay can provide both functionality and binding information.

3.3 Methods

DNA constructs and cloning

Standard cloning procedures, including NEB restriction enzyme digest, Q5 polymerase PCR amplification, T4 ligation and Gibson assembly, were used. Oligonucleotide primers were purchased from Sigma Aldrich.

Plasmid constructs were transformed into *Eschericha coli* cells via heat shock. XL1-Blue competent cells were used for all constructs, except for MBP-LgBit, which is described in "Expression and Purification of MBP-LgBit" below. Sequences were confirmed by Sanger Sequencing (Eurofins, GeneWiz)

Cell culture and transfections

HEK 293T/17 cell lines (ATCC, cat#: CRL-11268) used in these experiments were cultured at 37 °C and 5% CO_2. Cells were grown in complete growth media (1:1 MEM (Eagle's Minimal Essential Medium): DMEM (Dulbecco's Modified Eagle Medium, Gibco): with 50 mM HEPES (Gibco), 10% Fetal Bovine Serum (Sigma), and 1% Penicillin-Streptomycin (Gibco).

Cells at 80-90% confluence were plated into a flask that had been pre-incubated with human fibronectin for 10 minutes at 37 °C. An hour after seeding, these cells were transfected using FBS-free MEM and polyethlenimine (PEI, 1 mg/ml, Polysciences) with a ratio of 1:10 between µL of PEI and µg of plasmid DNA. The cells were then incubated at 37 °C for 20 – 24 hours.

The cell pellet was harvested by aspirating the media and resuspending the cells with a cell scraper in Dulbecco's Phosphate Buffered Saline (DPBS) buffer. 18 mL of DPBS was used to resuspend the cells within a T-75 flask, with this ratio kept constant for other flask sizes. 1.5 mL of resuspended cells were placed into an Eppendorf tube, which was centrifuged at 6,010 g for 3 minutes. The supernatant was aspirated, and the pellet was resuspended in DPBS, then centrifuged again under the same conditions. The supernatant was aspirated again, and the cell pellet was flash frozen in liquid N_2 and stored at -80 °C until ready for use.

Preparation of cell pellet

The cell pellets were prepared immediately before the assay, by first placing the cell pellet onto ice. For cell pellets containing GPCR constructs, the pellet was treated with 210 µL of Membrane Resuspension Buffer (MRB). MRB is comprised of incomplete membrane resuspension buffer (resuspension buffer comprising 20 mM HEPES, pH 7.5 and 2 mM $MgCl_2$) and benzoase (EMD Millipore, 70746) in a 4.5 mL to 1.8 µL ratio. For cell pellets containing cytosolic proteins (Nanobody 80 or miniGs), the pellet was treated with Mammalian Protein Extraction Reagent buffer. After this, 2 µL of 100X protease inhibitor (Sigma Aldrich, P1860#) was added to a final concentration of 1X.and the pellet was resuspended by pipetting up and

down. The solution was then sonicated using Model 50 *Sonic* Dismembrator (Fischer Brand) at a 20% amplitude (3 x 1 sec pulse) and returned to ice.

IGNiTR assay

A master mix containing Nano-Glo Buffer and substrate (Promega, N2012), GPCR construct, and G-protein mimic was prepared. For one well, the ratio was 9.75 µL of Nano-Glo Buffer, 0.25 µL of Nano-Glo substrate, 5 µL of GPCR pellet and 5 µL of the conformation specific binder. The conformation specific binder may be either peptidomimetic (LifeTein), miniGs pellet or Nanobody 80 pellet. The GPCR construct cell pellet was prepared separately and added to the master mix immediately prior to adding the master mix to the well. The concentrations were varied for the optimization assays (Figure 4), but the volumes remained constant. Concentrations for each experiment are indicated in the figure legends. The 384 well cell culture plates were preloaded with 10 µL of drug per well, followed by loading of the 20 µL of the master mix per well. Times reported on figure captions were recorded from the addition of the master mix to the first well of the plate. The luminescence values for all the conditions were measured using an EnVision 2104 Multilabel Reader (Perkin Elmer) (except for the Nanodisc experiments which were measured using the PHERAstar plate reader).

High throughput screening

To scale up the IGNiTR assay for high throughput screening, we used the Echo 655 (Labcyte) to load 150 nL of drug to each well of the 384 well plate. Then, the Multidrop Combi Reagent Dispenser (Thermo Scientific) was used to add 10 µL of MRB to each well. The same

machine was used to add 20 µL of master mix, prepared as described in "*IGNiTR Assay"* using 2 µM fusion peptidomimetic and the 0.5x dilution of DRD1-LgBiT found to be optimal from the characterization in Figure 4. After a 30-minute incubation, the luminescence values were measured using an EnVision 2104 Multilabel Reader (Perkin Elmer).

$$Corrected\ Relative\ Luminescence = \frac{lum\ of\ sample - mean\ lum\ of\ negative\ ctrl}{mean\ lum\ of\ positive\ ctrl - mean\ lum\ of\ negative\ ctrl} x\ 100\%$$

We validated the drug hits from an initial screen of six 384 well plates by testing four replicates of each compounds using the same method. From this validation, a dose response curve was constructed for promising candidates by loading 150 nL at a range of concentrations, using a mosquito X1 (SPT Labtech) to obtain final concentrations in well from 661 nM to 25.1 µM. 10 µL of MRB was loaded to the compounds, followed by 20 µL of master mix. After a 30 minute incubation, the luminescence values were measured using an EnVision 2104 Multilabel Reader (Perkin Elmer).

Expression and purification of MBP-LgBiT

The DNA encoding MBP-LgBit was transformed into BL21 cells. A colony of these cells was inoculated in 5 mL Luria-Bertani broth with ampicillin at 37 °C overnight. The culture was then transferred to a 500 mL flask of Luria-Bertani broth with ampicillin and placed in a 37 °C shaker until OD-600 reached 0.4 to 0.8. Protein expression was induced by addition of 1000X 0.1g/mL IPTG, to a final concentration of 1X. The culture was then shaken overnight at room temperature.

The cells were centrifuged at 4,248 g for 5 minutes at 4 °C. The cell pellet was lysed by resuspension in cold Bacterial Protein Extraction Reagent (B-PER, Fisher) buffer with 1 mM dithiothreitol (DTT) and 1X protease inhibitor (BioBasic, BS386). 15 mL of B-PER was used for every 500 mL of bacteria culture. 3-4 µL of benzoase was added to the cells, followed by a 5 min incubation on ice to ensure full cell lysis. The cells were then centrifuged at 16,994 g for 10 minutes at 4 °C.

50 mL of clear lysate was added to 2 mL of Ni-NTA resin slurry and incubated at 4 °C for 10 minutes. This mixture was then purified via an Ni-NTA column. The purity of MBP-LgBiT was then established using gel electrophoresis and a Coomassie stain analysis.

Determining the concentration of MBP-LgBiT

The molar extinction coefficient of MBP-LgBiT was calculated using Expasy PratParam to be 89,270 M^{-1} cm^{-1}. This value was then used in conjunction with Bier's Law to establish the concentration of the protein by the absorbance at A_{280}. The absorbance was re-calculated for each time MBP-LgBiT was used.

Standard curve

The standard curve was created using the same 384 well plates. First, a master mix containing a ratio of 5 µL of 30 µM HiBiT, 0.125 µL furimazine and 4.875 µL NanoGlo Buffer was prepared. 10 µL of this master mix was added to each well. 5 µL of MBP-LgBit or GPCR-LgBit was added, in the dilution ratio indicated in the figure legends. Times reported on figure

captions were recorded from the addition of the MBP-LgBit or GPCR-LgBit to the first well of the plate.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 9 software. This software was also used to construct plots. The sample size is indicated in figure legends (where n is the number of independent replicates). The mean and standard error of the mean were calculated for each condition. Two-sided Student's t-tests were used to evaluate the significance between data points. Z' values were calculated using the following equation:

$$Z' = 1 - \frac{3SD\ of\ positve\ control + 3SD\ of\ negative\ control}{|mean\ of\ positive\ control - mean\ of\ negative\ control|}$$

where SD is the standard deviation.

Fentanyl detection using IGNiTR

The frozen pellet expressing μ-OR-LgBiT was thawed, resuspended, and mixed with the G_i fusion peptide and NanoLuc substrate. The reaction mix was aliquoted to separate wells of an opaque white 96-well plate. A range of concentrations of fentanyl were added to the wells and the plate was imaged using an Azure Biosystems c600 for chemiluminescence. The resulting images were analyzed using imageJ.

Nanodisc assembly and purification

The pellet (β2AR-LgBiT) was resuspended in 400 uL membrane resuspension buffer (MRB) and sonicated. The lysate was quantified using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). The protein concentration was calculated using an average molecular weight of 40kDa for membrane proteins. Nanodiscs were made as described previously.[140] Phosphatidylcholine (POPC) (Avanti Polar Lipids) was dried under nitrogen and stored in a desiccator overnight. POPC was solubilized to 50 mM with 100 mM sodium cholate. Nanodiscs were assembled by adding MSP1E3D1 (Millipore Sigma) and lysate to the solubilized lipids up to a final volume of 350 µL in standard disc buffer (20 mM Tris, 100 mM NaCl, 0.5 mM EDTA, 0.01% NaN3) supplemented with sodium cholate to a final concentration of 20 mM. The final lysate concentration in the mixture was 10 uM, the MSP:lysate was 4:1, and the lipid:MSP was 90:1. The component mixture was incubated on an end over end mixer at 4°C for 45 minutes. 150 mg of Amberlite XAD-2 beads (Millipore Sigma) were added, and the component mixture was incubated at 4°C overnight before the beads were removed. The resulting Nanodiscs were then purified with Ni-NTA spin columns (NEB). The purified Nanodiscs were then exchanged into standard disc buffer with Bio-Spin P-6 Gel Columns (Bio-Rad) to remove imidazole.

3.4 Results and discussion

High throughput screening

Finetuning of the IGNiTR assay with G_s and G_i fusion peptides

The advantage of an in vitro, lysate-based assay is that we could modulate the concentration of each of the components in the assay and fine tune the conditions. We therefore characterized the performance of the IGNiTR assay concerning following conditions: GPCR-LgBiT

concentration, ligand incubation time, and G_s fusion peptide concentration. To characterize IGNiTR with different GPCR-LgBiT concentrations, we first estimated the relative concentration of GPCR-LgBiT by creating a standard curve of LgBiT with its high-affinity peptide partner, HiBiT[144,145] (**Figure 13**). We then varied the dilution factor for the GPCR-LgBiT cell lysate while the peptidomimetic concentration was held constant at 10 μM. Among the dilutions, the 1x and 0.5x dilutions yielded the best DDR of >3 when measured at 30 minutes for DRD1 (**Figure 14**). Since the signal tends to stabilize around 25-30 minutes after ligand incubation, we therefore measured the luminescence at 30 minutes for all DRD1 characterizations. For the μOR-LgBiT, we observed that the DDR ratio peaks around 10 minutes, with all three dilutions, 0.25X, 0.5X and 1X, producing comparable DDR of ~ 4 (**Figure 14**).

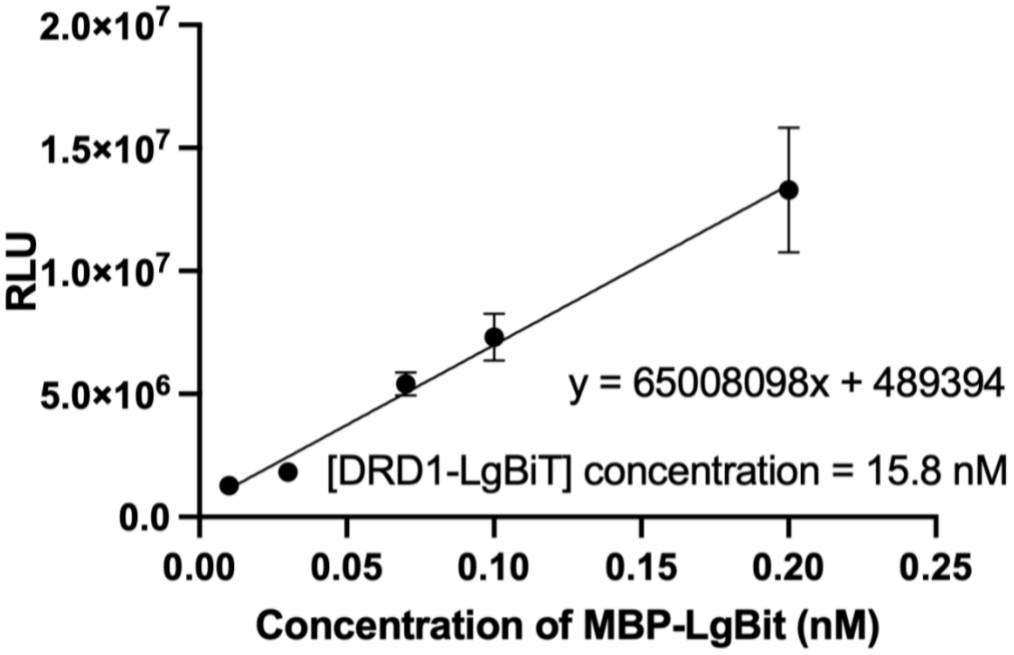


Figure 13 Standard curve using the luminescent signal of the high-affinity binder HiBiT with known concentrations of MBP-LgBiT to determine the concentration of DRD1-LgBiT.

We next characterized the IGNiTR assay with different concentrations of the fusion G_s and G_i fusion peptides. To minimize the cell pellet needed for characterization, we used the 0.5x of the

GPCR-LgBiT lysate dilutions. We varied the concentration of fusion peptides added. For both G_s and G_i fusion peptides, the highest concentration (10 μM) resulted in a lower DDR compared to the lower concentrations due to higher background luminescence (**Figure 14**). Drug-dependent ratios (DDR) of ~ 3 were observed with 5 μM and 2 μM $G_{\alpha s}$ fusion peptidomimetic and the highest DDR of ~ 4 was observed with 2 μM G_i peptidomimetic. We therefore used the 2 μM fusion peptide in the subsequent DRD1 and μ-OR IGNiTR assays to maximize DDR and to reduce the volume of fusion peptides needed for HTS.

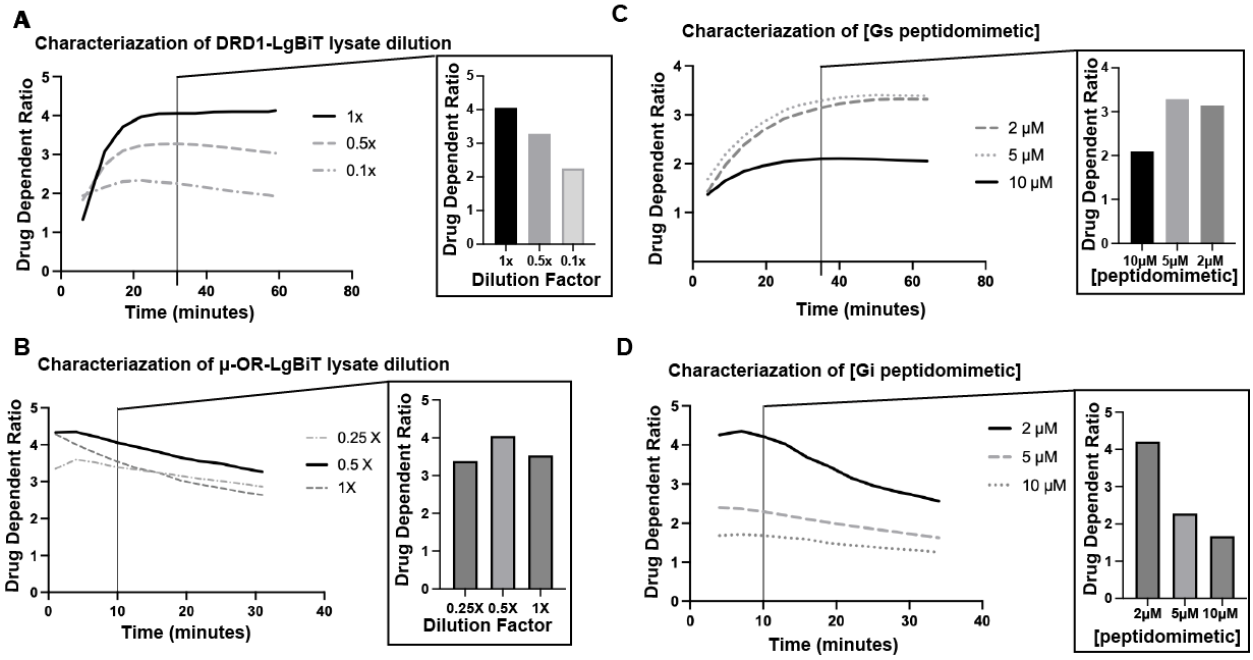


Figure 14 Characterizing the IGNiTR assay with G_s and G_i fusion peptides. *A.* Characterization of the effects of GPCR cell lysate dilution factors on IGNiTR using DRD1. *B.* Characterization of the μ-OR-based IGNiTR with cell lysate in a range of dilutions. *C.* Characterization of the effects of a range of G_s peptidomimetic concentrations on IGNiTR using DRD1-LgBiT. DDR were taken around 30 minutes after drug incubation for both. N=6 *D*. Characterization of the effects of a range of G_i peptidomimetic concentrations on IGNiTR using μ-OR-LgBiT. DDR were taken around 10 minutes after drug incubation for both. n=3.

High throughput screening (HTS) with robust Z' values

IGNiTR could be used as a beneficial HTS platform for GPCR ligands, especially because IGNiTR components can be mixed in a batch, ensuring consistency across large-scale screens. The approach could eliminate inconsistencies of protein component expression across large quantities of cell populations. As a proof-of-concept, we performed a small-scale screen using DRD1 IGNiTR. Using the conditions optimized previously (**Figure 14**) DRD1-IGNiTR assay to scale up to screen for potential agonists using 1,916 compounds from an FDA-approved & Passed Phase I Drug Library from SelleckChem library (**Figure 15A**). The Z' value was consistent across the plates with an average of 0.79 (Figure 15**B**) which is within the range of optimal Z' value for HTS ($1 > Z' > 0.5$).[146] Even though no lead compounds were identified from this library, the proof-of-principle screening demonstrated the feasibility and robustness of IGNiTR in GPCR ligand screening. Due to the high consistency of IGNiTR, the standard deviation (SD) in the primary screen is low, which allows for a higher threshold to define a "hit" (**Figure 15A**). Dose-response curves were constructed for the five compounds with the highest S/B. Four of them showed dose-dependent responses and one did not, demonstrating IGNiTR's capability to differentiate between ligands of varied efficacies (**Figure 15C**). This proof-of-principle screening demonstrated the feasibility of applying IGNiTR for GPCR ligand screening. The low SD of IGNiTR is highly advantageous because it enables us to identify compounds with low activation efficacy, which is advantageous for discovering partial agonists.

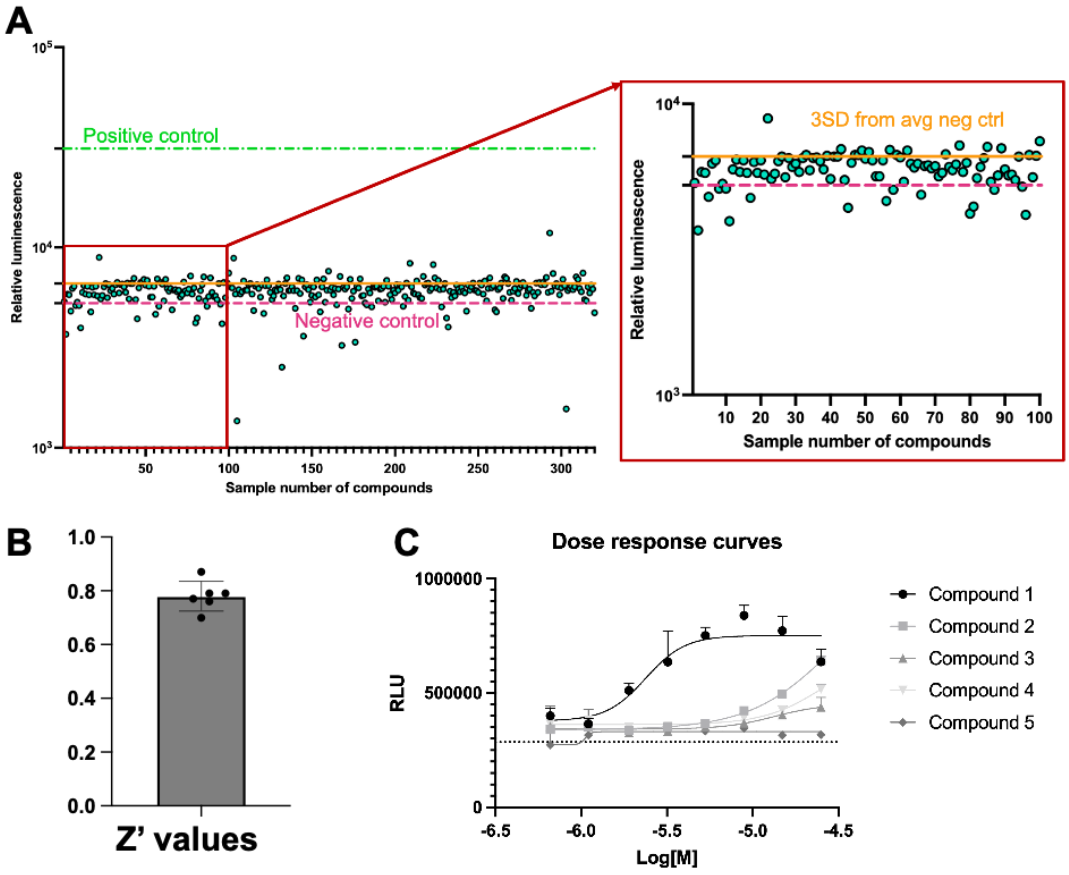


Figure 15 Proof-of-principle screening for DRD1 agonists. A. High-throughput screening of drug-repurposing library, showing the relative corrected luminescent signal as a percentage of the difference between the mean luminescent signal of the sample and the negative control to the difference between the mean luminescent signal of the positive control and the negative control. B. Z' values measured across a proof-of-concept drug screening for DRD1-LgBiT and G_s fusion peptide across 6x 384-well plates. C. Dose-response curve of the molecules with the top five relative corrected luminescence from the high throughout screening. n=4.

Rapid detection of opioids

To increase the accessibility of performing the IGNiTR assay, for detection we used a less sophisticated gel-imaging camera rather than a plate reader to measure IGNiTR luminescence. First, we began with the optimized μ-OR and G_i fusion peptide concentrations for μ-OR IGNiTR (**Figure 14)**.

IGNiTR reagents containing the μ-OR-LgBiT expressing cell pellet can be prepared and stored frozen until usage. A range of concentrations of fentanyl were added to the wells and the

plate was imaged using a gel imager for chemiluminescence. As shown in **Figure 16 A & B**, higher concentrations of fentanyl results in higher luminescence and the signal plateaued at around 500 nM. μ-OR-based IGNiTR was able to detect 50 nM fentanyl reliably with high significance, while 10 nM fentanyl also provide a significantly different luminescence compared to the no drug control.

The results indicate that IGNiTR can successfully detect various levels of opioid agonists. Notably, IGNiTR provides the general information of the presence of opioids, which can complement existing assays for detecting specific synthetic opioid molecules.[147] The detection sensitivity of the IGNiTR assay can potentially be further improved by optimizing the assay conditions, such as tuning the concentrations of both GPCR-LgBiT and peptidomimetics.

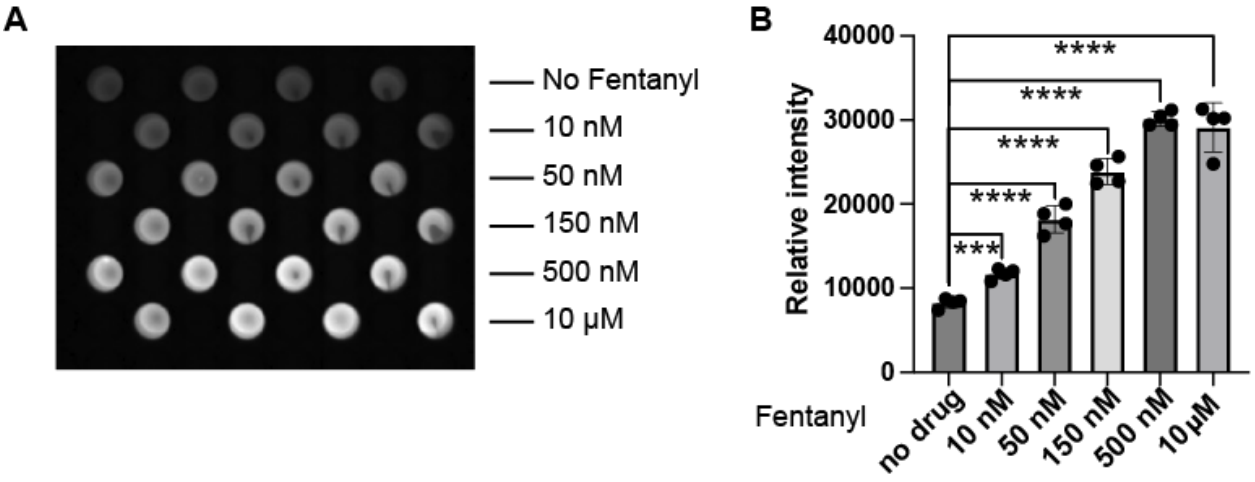


Figure 16 A. Imaging and *B.* quantification of the IGNiTR assay performed with μ-OR LgBiT and the G_i fusion peptide to detect varied concentrations of fentanyl. n=4. Stars indicate significance after performing an unpaired Student's t-test. ****P value<0.0001. ***P value<0.001.

Characterizing GPCR functionality during Nanodisc-based GPCR extraction and reconstitution

We tested IGNiTR's ability to characterize GPCR functionality during several crucial steps of POPC-based Nanodisc formation.[140] As established, Nanodiscs have been applied for GPCR

reconstitution by embedding the GPCR and lipids inside the Nanodisc and forming stable GPCR-lipid complexes.[137] As seen in **Figure 17A** β2AR-LgBiT pellet was resuspended, the solution was divided into aliquots for varied treatment conditions. One aliquot was kept at 4°C without any further treatment for the same incubation period as the other samples. Two aliquots were treated with detergent (sodium cholate) but only to one of them was added the necessary components for the formation of Nanodisc (MSP and POPC). After a 45 minute-incubation period, a portion of the samples was set aside for later IGNiTR analysis. To the remaining solutions, Amberlite XAD-2 beads were added and incubated overnight to extract cholate from the samples. The reconstituted Nanodisc was further purified using Ni-NTA column since the MSP protein that constitutes the Nanodisc has a Histag on it. The relative concentrations of the β2AR-LgBiT in in all the steps were estimated using the LgBiT standard curve as before to normalize the concentrations of the samples before NTA purification (same procedure as demonstrated in **Figure 13**). All the samples, including post NTA purification samples, were then analyzed using the IGNiTR assay (**Figure 17B**). We mixed the protein samples at different stages with G_s fusion peptide and the NanoLuc buffers and substrate with or without agonists to evaluate the agonist-dependent DDR in these samples. The agonist-dependent DDR could indicate the structural integrity of the β2AR protein. Higher DDR suggests higher content of functional β2AR that can undergo agonist-dependent conformational change and bind to the G-protein peptidomimetics to reconstitute the split NanoLuc.

As shown in **Figure 17B**, β2AR reconstituted in Nanodisc with detergent cholate removed (sample 2) and its subsequent Ni-NTA purified sample (sample 3) produced a significant agonist-dependent 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 for the

correct folding and functionality of β2AR during its incorporation into the Nanodisc. The study establishes that IGNiTR could be used to monitor GPCR functionality throughout the protein extraction and reconstitution process, which is useful for optimizing these protocols.

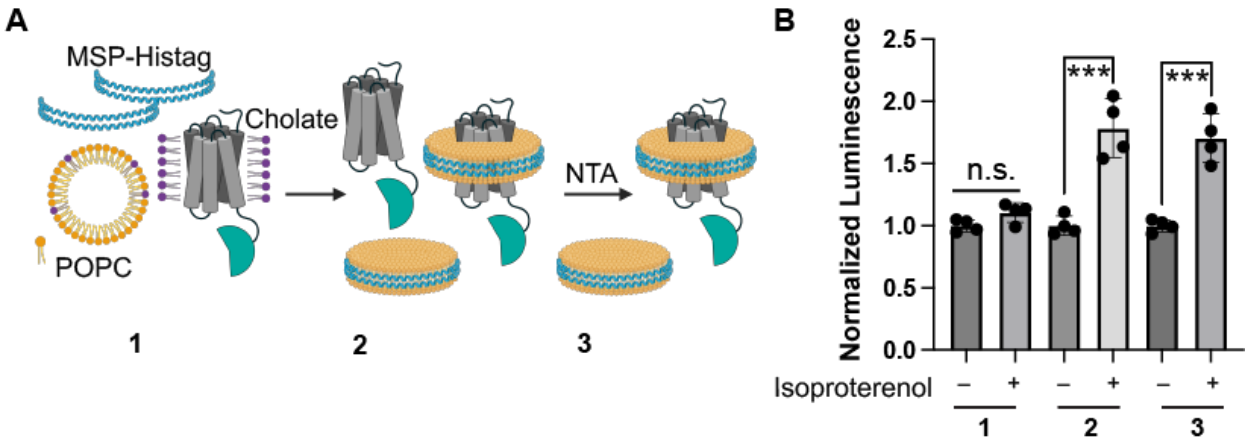


Figure 17 A. Workflow for the incorporation of β2AR-LgBiT into POPC-based Nanodiscs. "NTA" represents Ni-NTA column purification. B. Analysis of the β2AR-LgBiT samples in A using IGNiTR with G_s fusion peptide. Stars indicate significance after performing an unpaired Student's t-test. ***P value<0.001.

3.5 Conclusions

To summarize the results from this chapter, we first demonstrated a proof-of-principle HTS of DRD1 ligands using IGNiTR, showing that IGNiTR has an excellent Z' value of 0.79 and can identify low-efficacy GPCR ligands. The study showed that IGNiTR is highly sensitive, demonstrates low standard deviation of controls and produces a consistent dynamic range which all could be useful for discovering molecules with lower efficacy. The results also establish that IGNiTR can successfully detect various levels of opioids. Notably, IGNiTR reports on the general presence of opioids, which complements existing assays for detecting synthetic opioid derivatives.[148,149] Because the IGNiTR reagents can be readily stored frozen, we envision the components of IGNiTR being packaged into a kit for detecting μ-OR agonists in a variety of

settings. Once fully realized, the kit could become point of care method for detecting opioids with much less sophisticated instrumentation. Finally, we applied IGNiTR for characterizing GPCRs at multiple steps during the process of incorporation into POPC-based Nanodiscs. These results corresponded to the amount of protein that was able to undergo drug-dependent activation, a conformational change that indicates functionality confirmed through conformation specific binding. Overall, these applications validate our claim that the in vitro characterization provided by IGNiTR is both versatile and generalizable.

Contributions: Ruby Miller developed the assay methods, experimental plans for high throughput screening, and idea for collaboration with the Bailey laboratory on the Nanodisc application. She also performed all the experimentation and method development for opioid detection. Wenjing Wang conceived the idea for using a gel imaging camera to detect the bioluminescence and convert the in vitro method for opioid detection. Jennifer Sescil aided with scaling up the method for high throughput drug screening. Marina Sarcinella (Bailey laboratory) performed all the Nanodisc sample preparation prior to characterization experiments performed by Ruby.

Chapter 4: Concluding Remarks and Future Work

4.1 Summary of major findings

I have described the development of 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. IGNiTR has features which can be both complementary to and advantageous over live cell-based assays. First, IGNiTR components, including the GPCR and the conformation-specific binder components can be prepared in advance and stored frozen until usage. Second, IGNiTR can be performed without the restrictions of working with live 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 optimization of DDR. Fourth and finally, mixing of the components could standardize the reaction conditions in thousands of wells to achieve consistency across HTS plates.

IGNiTR has advantages over existing in vitro assays: it can measure binding interactions while also reporting a quantitative measure of induced GPCR activation or ligand efficacy. IGNiTR's bioluminescent readout is quantifiable in a single step, and therefore can be easily scaled up and accessibly carried out, while the existing in vitro GPCR assays, including the radioligand assay, requires a complicated set up.[75,76] We demonstrated diverse applications in: 1) HTS of GPCR ligands; 2) characterization and detection of GPCR ligands in the lab and in the field; as well as 3) verifying GPCR structural integrity for in vitro GPCR characterizations. In

future work, IGNiTR can potentially be adapted to detect other GPCR agonists, enabling biosensor development for a wide range of molecules.

4.2 Opportunities for improvement

For improving HTS method robustness

Included in the major revisions requested by our reviewers were more comparative studies that would make it easier to evaluate some of the claims about capacity of this system for HTS. The most significant claim that needs supporting is validating that the high throughput screening process performs competitively, with the same quality readouts and analysis that a live-cell assay would produce. There was no side-by-side comparison for consistent results compared to a live-cell assay (such as GloSensor). This will be addressed in future experimentation, including confirmation that IGNiTR can identify known hits for a GPCR target in a library of compounds. It is important to note that while IGNiTR was successfully scaled up for high throughput screening, the application has yet to demonstrate productive sorting of a library of compounds against DRD1.

The proposed experimentation to address these questions would involve acquiring access to a drug or small molecule library with known agonists for a particular receptor. The IGNiTR system has been optimized for three GPCRs thus far, with the most well-studied targets for therapeutics being DRD1 and μ-OR. Ideal libraries would include multiple known “hits” or molecules that trigger responses (for DRD1 for example) providing a basis for evaluating yielded response in the form of IGNiTR produced signals. The most significant obstacle to overcome is establishing the reported values based on previously performed screenings and setting up a parallel platform that has been established to report favorably under the desired testing

conditions. Theoretically, the live cell-based assay would report GPCR activity for the known activators similarly to the activity reported by the IGNiTR system for the same molecules, confirming that both lytic and living systems can trace the GPCR's functionality.

While expression levels of protein components do become unpredictable and difficult to maintain quality control over large batches of cells, it is also challenging to quantitatively compare the effects this has compared to the results output, if any. We recognize that there are limitations to the IGNiTR approach and that the advantage of testing the behavior of GPCRs in their native complex environments provides more information about activity in a screening compared to any benefits gained through disruption of endogenous systems. However, while live cell assays are well established for HTS, there are certainly niche biochemical applications that could benefit from activity characterization methods under in vitro conditions.

For opioid detection method robustness

Harnessing the opioid receptor as a method of detection has the advantage of being universal for all drugs that would activate the receptor endogenously and therefore could detect even novel opioids. A detection method based on luminescent readout of activation could be useful from a drug development standpoint. If IGNiTR were to be packaged into a kit, chemists could test and visualize if a compound they have synthesized has any activation efficacy on an opioid receptor as an initial screening. Applying a gel imager pared with ImageJ makes luminescence readout analysis even more accessible, especially for settings without more extensive quantitative instrumentation. The use of more accessible equipment complements the need for additional assays that can appropriately deployed in place of extensive mass spectrometry techniques for point-of-care support. Adding to the number of detection-based assays such as colorimetric or later flow assays increases the chances of identifying compounds in mixtures of drugs (as

increasing amounts of opioids are laced with molecules more potent at lower quantities and thus more likely to cause potential overdoses).[147] However, the LOD for this assay still needs to be experimentally determined for a robust comparison to existing methods.

For characterization of proteins incorporated into Nanodiscs

Nanodisc preparation with GPCR incorporation has been a noteworthy application of these characterization strategies, since they deal directly with membrane extracts. Part of the major revision requests was for further testing of GPCR activation and coupling post assembly of the Nanodisc. Crucial comparative information can be gained by performing one of the standard methods in parallel with the use of IGNiTR to 1) confirm the activity observed using the new IGNiTR method and 2) observe how competitive the IGNiTR method is compared to well-established standards. Further methods optimization will be required for the Nanodisc formation which could benefit from the use of higher concentrations of receptors to achieve a better incorporation uptake of GPCR. Having a higher concentration of GPCR would also allow for further quantification of activity and optimization of the ratio of IGNiTR components necessary to effectively measure functionality.

4.3 Future work

Short term goals: applying IGNiTR for collaboration experiments

An advantage of this system is that it could prove to be very useful for untangling any activity-inducing properties within a mixture of natural products. Preliminary experiments are underway for a funded collaboration project with the Center for Chemical Genomics and the Natural Products Core in the Life Sciences Institute. The goal of this project is to screen a library of extracts against both Nociceptors (NR) and μ-OR to find any activators among the mixtures

and untangle the levels of induced activation for one receptor compared to the other (i.e., partial or full agonist activity). A compound with some degree of activity towards both would be ideal, eliciting a strong response from NR while only inducing partial activity from μ-OR. These two receptors present an ideal testing ground for this type of comparison since agonists for μ-OR are widely established as having analgesic effects with unwanted side effects, including addiction.[150] Therefore, greater selectivity towards NR would be ideal and IGNiTR could be useful as part of the discovery and characterization of natural product candidates.

Long term goals

Performing comparative assays

Examples of strategies for parallel confirmation assays requires both live cell-based methods as well as standard in vitro procedures. The 2022 review by Vandeputte et al. highlights many standard practices used for evaluating coupling and GPCR activity in vitro.[147] Mentioned in **Chapter 3** but meriting further discussion is the use of GTPγ[^{35}S] binding assay which enlists a radioactive GTP analog to track hydrolysis. The products of hydrolysed [^{32}Pi] and Gα-GTPγ[^{35}S] complex are measured using a scintillator and this monitoring of nucleotide exchange is a standard in the literature for quantification in membrane extracts.[147] Using radioactive reagents is not ideal for anything large scale, and even for smaller scale experiments, it requires specialized equipment, training, safety regulations, as well as safe disposal of waste biproduct. These factors result is accessibility barriers.

The need for less toxic mechanisms of tracking activity in vitro has ushered in development of biochemical methods that rely on other protein coupling components paired with

luminescent signals for monitoring those interactions. The PathHunter® cell line method was originally established in 2007 as a way to measure complementation of fragments of beta-galactosidase enzyme brought together through the recruitment of β-arrestin upon GPCR activation.[151] It's important to acknowledge that while this system requires the use of a stable cell line which could pose challenges for labs that do not work with cell cultures, it has been employed in efforts to deorphanize GPCRs.[152] PathHunter® (commercialized by DiscoverX) preceded the split NanoLuc system (NanoBiT® assays commercialized by Promega) which has also been used for in vitro characterization through functional complementation produced bioluminescence. It would be informative to compare the PathHunter® method to IGNiTR to see if it can perform competitively side-by-side with an industry standard.

Experiments needed for expanding the assay's application capacity

Regarding increased generalizability of the IGNiTR components, there are more options to be explored. Envisioning expansion of the IGNiTR system to additional peptidomimetics has led to the design of a G_q peptidomimetic (soon to be tested). In theory, these peptidomimetics could be optimized for utility within a mixture. For example, the peptidomimetics could be used for deorphanization of a GPCR or for determining the relative preferred coupling protein. Further testing would be required to determine the peptides' binding specificity and preference for one active binding conformation over another. Additionally, optimization of the peptide structure/sequence could potentially help to reduce any non-specific binding and decrease the amount of background signal. The linker region between attaching the α-5 helix mimic to the SmBiT could be shortened and lengthened to determine if that has any effect on the signal output.

The opioid detection sensor application requires additional testing to fully realize the strategy. While we envision that it could theoretically be adapted for a kit to use in a "point-of-care" capacity, the components need to be thoroughly optimized, standardized, and stress-tested against a variety of molecule mixtures. Particularly lacking (and under consideration now) is acquiring additional analytical quantification to establish metrics like LOD for determining if the method is sensitive enough to be able to detect opioid agonists in realistic levels (i.e., those found in real world samples). Rigorous testing would include a panel of known low-affinity activators subjected to pharmacological characterization using the IGNiTR system with direct comparison to both lab-based standard methods as well as the more field-specific testing methods to determine whether the outputs are comparable. The development of an accessible kit also requires preparation of the materials to make the testing as accessible as possible, even for someone who has no experience performing this type of experiment. We would also need to develop interpretation guidelines for the results and determine whether any conclusions can be drawn beyond presence or absence of a μ-OR activator.

4.4 Final thoughts

Overall, the IGNiTR method offers a unique perspective on tracking receptor activation within an in vitro environment, and I hope the assay can be added to the toolbox of existing strategies for studying GPCRs to enhance our understanding of these critical and enigmatic proteins.

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