Published in final edited form as:

Drug Discov Today Technol. 2010 ; 7(1): e37–e42. doi:10.1016/j.ddtec.2010.06.005.

Seeking Ligand Bias: Assessing GPCR Coupling to Beta-Arrestins for Drug Discovery

Laura M. Bohn, PhD and

Departments of Molecular Therapeutics and Neuroscience, The Scripps Research Institute, 130 Scripps Way #2A2, Jupiter, FL 33458, lbohn@scripps.edu

Patricia H. McDonald, PhD

Department of Molecular Therapeutics, The Scripps Research Institute, Translational Research Institute, 130 Scripps Way, #2A2, Jupiter FL 33458, mcdonaph@scripps.edu

Abstract

G protein-coupled receptors (GPCR) are the major site of action for endogenous hormones and neurotransmitters. Early drug discovery efforts focused on determining whether ligands could engage G protein coupling and subsequently activate or inhibit cognate “second messengers.” Gone are those simple days as we now realize that receptors can also couple β arrestins. As we delve into the complexity of ligand-directed signaling and receptosome scaffolds, we are faced with what may seem like endless possibilities triggered by receptor-ligand mediated events.

Introduction

GPCRs are ideal targets for pharmaceutical development with the goal of either mimicking the normal transmitter response or tempering it. In recent years, targeting GPCRs has become more complicated as we realize that drug action at receptors is “context dependent” such that activation and inhibition is limited to the response evaluated and “agonist” and “antagonist” become terms that reflect a particular condition of the experimental or physiological output [1-3]. Further, we have realized that GPCRs couple to other proteins besides G proteins. Of these, the β arrestins have proven to be critical regulators of GPCR signaling. Therefore, rather than limiting the concept of receptor activation to its coupling to a particular G protein, GPCR signaling may be envisioned as an event caused by a ligand-mediated conformational shift in receptor structure that leads to rearrangement of a signaling scaffold to recruit or dispel certain signaling players. In this sense β arrestins play a pivotal role in determining what players are recruited or dispelled, and thus, acting as facilitators, stabilizers or dampening agents, function in directing GPCR signaling.

In recent years, signaling via β arrestins has been demonstrated in cell culture models for many GPCRs [see 4 for review] and there appears to be some bias whereby certain ligands will preferentially engage β arrestins to activate particular cascades [5-7]. An interesting example of this is carvedilol, which has been known as a “beta-blocker” but has now been shown to activate ERK pathways via β arrestin-dependent signaling mechanisms suggesting that it is an “activator” at the β 2 adrenergic receptor in respect to this pathway [6]). As the

© 2010 Elsevier Ltd. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

implications of the β arrestin-directed signaling pathways begin to emerge in cellular and in animal studies [8], screening for both β arrestin interaction and second messenger activation may prove to be an important early step in drug discovery efforts.

I. High Content Screening Technology

The monitoring of β arrestin recruitment to activated receptors has become a popular mode of measuring GPCR activity and is described as a ‘universal’ GPCR platform owing to the fact that it is G-protein independent, such that GPCRs will recruit β arrestins regardless of the nature of the G protein to which they couple [9]. One of the earliest methods for detecting GPCR- β arrestin interactions evaluated the “translocation” or redistribution of a green-fluorescent protein (GFP)-tagged β arrestin from the cytosol which leads to the accumulation of intensely green punctae on the membrane or in vesicles (Figure 1) [10-12]. Oakley et al., used this approach to define two classes of GPCRs- those that recruit β arrestin to the plasma membrane yet dissociate from the β arrestin upon internalization (Class A) and those that recruit β arrestin to the plasma membrane and retain the β arrestin upon internalization into clathrin coated vesicles (Class B) [13]. This redistribution can be visualized and quantified on the high content scale using automated fluorescence-based microscopic imaging coupled to automated image-analysis software.

A. TransfluoTM by Molecular Devices

A high content screening platform based on this approach, named TransfluoTM, originally developed by Norak, Inc., is now offered by Molecular Devices (www.moleculardevices.com). The optimal cell type used in this assay grows in a monolayer, has good adherence properties and has a large cytosol to nucleus ratio to optimize visibility. A benefit of this microtiter plate-based, fluorescence bioassay is that it measures the translocation of β arrestin-GFP such that GFP acts as the probe so it is not necessary to use a fluorescent dye or secondary substrate [9]. A somewhat limiting drawback is that it requires an expensive, dedicated image analysis system; data storage and analysis, due to large imaging files, can also represent a significant challenge. However it does provide more information content than single parameter assays such as those discussed later. Most notably, the imaging assay allows for visualization of the cells, which permits for parallel detection of putative compound liabilities such as cytotoxicity. An additional benefit of this assay is that visualizing the localization of the β arrestin (whether to the membrane or to the vesicles) will provide additional information regarding the drug effect with respect to ligand-induced trafficking.

II. High Throughput Screening Technologies

More recently, other β arrestin recruitment assay methods, that do not require expensive imaging instruments and have the advantage that they can be read with conventional multimode readers, have been developed. These include, 1) Bioluminescence Resonance Energy Transfer (BRET) [14,15] a protease-activated transcriptional reporter gene (TANGOTM, Invitrogen (www.invitrogen.com, [14,16,17]) Enzyme Fragment Complementation (EFC) (PathfinderTM, DiscoverX, www.discoverx.com [18]). Each can be used to assess agonist activation of the receptor, and, in conditions where basal β arrestin-receptor interactions are high, the assays can be configured for the identification of “inverse agonists.”

A. BRET

The bioluminescent energy transfer (BRET) assay was one of the earliest approaches utilized for assessing GPCR- β arrestin interactions and can be scaled for high throughput screening (HTS) applications [19-23]. BRET involves tagging the receptor of interest at the

C-terminus with a fluorescent protein tag (such as enhanced green fluorescent protein-2, eGFP2 or GFP10, or yellow fluorescent protein, YFP) and the β arrestin with a *Renilla* luciferase (RLuc) tag (Figure 2). Alternatively, the tags can be interchanged, such that the fluorescent protein resides on the β arrestin and the RLuc tag resides on the receptor. It is best to empirically determine which protein should be tagged for each receptor: β arrestin interaction probed.

Three generations of BRET exist namely: BRET1, BRET2 and (extended) eBRET [24,25] which can be distinguished by the RLuc substrate used (coelanthrazine h, DeepBlueC™ or a protected form of coelanthrazine h, termed EnduRen™, respectively) to produce a product and release a photon the emission of which can be measured. Upon β arrestin recruitment, the two tags come into close proximity and the light emitted from the RLuc reaction is sufficient to excite the GFP which will then emit a detectable signal at higher wavelength. The assay relies on a multimode plate reader to measure the light emitted in both spectra as the RLuc emission serves as an internal control and the GFP emission reflects the degree of excitation caused by the interaction. BRET is usually reported as a ratio of excitation of the two emissions (GFP/RLuc) which are determined by the BRET pairs and the substrate used.

There are substantial practical limitations from a HTS perspective such as high backgrounds, due to potential spectral overlap and rapid substrate catalysis resulting in very brief windows in which measurements are detectable following compound addition. To optimize the sampling time, substrate injection is usually required to enable signal detection. More recently, improvements in the BRET technology have been reported describing the use of novel luminophores, variants of RLuc, that may enhance the effectiveness of BRET for HTS applications [25]. Overall, BRET between β arrestin and the receptor is a proximal readout for receptor activation. The primary limitation however, is that BRET will occur whenever the two tags are in close proximity for energy transfer, even if a functional complex is not formed between receptor and β arrestin.

Currently, there are two prominent high throughput screening (HTS) platforms commercially available to study GPCR- β arrestin interactions: Invitrogen's Tango™ GPCR Assay System [17,26] and the PathHunter™ system by DiscoverX [27,28]. Both companies offer full assay development which involves the generation of stable cell lines expressing both modified GPCR and β arrestin proteins.

B. Invitrogen's TANGO™ Assay System

The Tango™ GPCR Assay System utilizes a proprietary live-cell platform based upon transactivation of a reporter gene. Essentially, the C-terminus of the GPCR is tagged with a transcription factor (Gal4) via a protease cleavage site. The β arrestin is tethered with a TEV protease [29,30]. When the two proteins come together, the protease on the β arrestin cleaves the linker site on the GPCR thereby releasing the transcription factor which then translocates to the nucleus (Figure 3). The cells express a β -lactamase reporter gene which is subsequently activated by the transcription factor binding to a UAS promoter to produce the active enzyme. The production of β -lactamase is measured by its ability to catalyze a modified substrate tagged with two fluorophores. When the substrate is intact, the fluorophores undergo fluorescent resonance energy transfer (FRET) to generate ~520 nm emission (green cells). When the substrate is cleaved, FRET is abolished and the product fluoresces at 447 nm (blue cells) [17]. Detection of the signal can be measured by any fluorescent plate reader capable of generating 409 nm excitation and detecting ~520 nm emissions.

According to the Invitrogen's product sheet for individual cell lines, test compounds are added and then the cells are incubated for 5 (KOR, EDG6, others) to 16 (GPR119, CCR5,

others) hours to allow for the β arrestin-receptor cleavage events. The cells are then treated with substrate for an additional 2 hours. The TangoTM assay is ratiometric, such that each receptor present in the cell during the testing period can only produce one β arrestin coupling event (the transcription factor can only be cleaved once per receptor). Therefore, the amount of β -lactamase activity measured is directly proportional to the number of β arrestin-receptor collisions. On the downside, the assay depends upon several cellular events to proceed between the agonist-binding event and the induction of the substrate-to-product disruption of FRET. Over the 5-16 hours of the assay, the protease activity, the transcription factor translocation to the reporter, the transcription factor binding to the reporter gene and initiating successful transcription followed by translation of a functional β -lactamase enzyme are all events that should not be impacted upon by test compound for the results to maintain their direct correlation to GPCR- β arrestin interaction. However, the robustness of the amplified signal output may be worth the sacrifice of the proximity of the receptor- β arrestin interactions. Further, the cleavage event should capture any ligand-induced receptor- β arrestin interaction independent of the duration or stability of that complex.

C. DiscoverX PathfinderTM Assay System

The DiscoverX PathfinderTM platform utilizes enzyme fragment complementation with β -galactosidase activity serving as an indicator of β arrestin-receptor interactions. In this assay, the GPCR is fused at the C-terminus to a mutant peptide fragment of the β -galactosidase (ProLinkTM or enzyme donor) and the β arrestin is tagged with the corresponding deletion mutant of β -galactosidase (enzyme acceptor) which is provided by DiscoverX, engineered into host cells [31,32]. Agonist-induced β arrestin recruitment to the receptor brings the fragments in close enough proximity to reconstitute the functional enzyme (Figure 4). Output is measured as a chemiluminescent signal that is generated upon cleavage of exogenously added substrate by the reconstituted holoenzyme which can be detected using a standard multilabel plate reader.

DiscoverX recommends that the enzyme substrate (provided as Galacton Star SubstrateTM) is added 90 minutes after the addition of the test compound and that the spectra is read ~60 minutes after addition of the substrate; the product is measured using standard luminescence absorbance spectra plate readers. The PathfinderTM assay measures β arrestin-receptor interactions that are proximal to the receptor and do not require multiple downstream steps to produce the amplified signal. The downside of this platform is that measurements are taken at a limited time window at which only receptors and β arrestins that are actively engaged will be represented. Therefore, the assay only captures a snapshot of receptors bound to β arrestins during the period of substrate incubation.

Conclusions and Comparison of the HTS Assays

Both of the commercial assays offer the platforms for β arrestin2 specifically, although both companies will work with the researcher to develop custom cell lines, including those that assess the interaction with β arrestin1. While there is certainly much less in the research literature regarding β arrestin1-mediated regulation versus β arrestin2 mediated regulation of GPCRs, researchers should be cautioned that this does not necessarily indicate that β arrestin1 may be less important than β arrestin2 or that β arrestin1 and β arrestin2 are synonymous in their function and can therefore be interchanged in regard to significance. If one does choose to evaluate β arrestin1 interactions, which could potentially give novel information in regards to functional selectivity, a potential caveat is that β arrestin1 appears to have lower affinity than β arrestin2 for many receptors [33]. It is not clear from these studies whether the lower apparent affinity is due to low affinity of β arrestin1-GPCR interactions or if the endogenous β arrestin1 actually has much higher affinity (it is expressed at higher levels in most cells and tissues) and competes for the tagged β arrestin1 binding.

As the commercial platforms become more widely available, it becomes attractive to consider using them for studying particular receptors to elucidate the pharmacology of drug induced β arrestin interactions. However, it should be considered that these platforms are optimized for the detection of “hits” and that some sacrifice may be made in respect to normal function of the receptor. For example, the Pathfinder assay suggests a 90 minute incubation with agonist prior to substrate addition. Since most GPCRs have engaged in endocytic pathways, and since class A receptors (such as opioid receptors, adrenergic receptors, dopamine receptors, etc.) do not maintain the association with β arrestin2 during the internalization process, then one will only measure the β arrestin2-interaction within the receptor population that maintains the interaction at from the moment that the substrate is added. It is not clear what impact that time period, which can range upwards of 2 hours encompassing drug through substrate incubation periods, will have on receptor fate as most GPCRs are known to undergo internalization and down-regulation during that time frame. The internalization of the receptor may particularly impact on Class A GPCRs that do not maintain the β arrestin-receptor interaction upon internalization [13]. In this case, the only receptors that would be assessed are those that were not internalized and remain on the cell surface where they could still interact with the β arrestin. Therefore, the evaluation of the β arrestin2 interaction for a particular receptor may reflect only a small proportion of the receptors that are still activated, and may underestimate the degree of activation that was initially induced by the agonist. Furthermore, during that 90 minute period, a certain proportion of the receptors, that were activated may be downregulated by the time the measurement is made [34]. Ultimately, these regulatory events may not interfere with the detection of certain “hits” if receptor expression is in excess, but caution should be exercised when attempting to employ these approaches for basic molecular pharmacology characterization as sensitivity to temporal events is compromised.

There are several cell-based screening assays that are either commercially available or others that can be easily developed by individual laboratories without commercial restrictions, to assess GPCR- β arrestin interactions (Table 1). This approach is highly informative as it can be used to determine whether a compound activates the receptor to engage a β arrestin which may proceed independently of whatever downstream second messenger cascade is triggered. However, there may be compounds that do not recruit β arrestins that still engage G proteins, or other yet to be identified signaling cascades that will be missed if only this approach is taken. A prime example of this is the potent opioid analgesic, morphine, which under standard cellular conditions, fails to induce a robust recruitment of β arrestin to the MOR, and yet remains the gold-standard for pain management [35,36]. Finally, while an agonist may be found to engage GPCR- β arrestin interactions, it remains to be determined whether the interaction is relevant and whether it occurs in cellular environments wherein the receptor functions to control agonist-induced physiologies and behaviors.

Acknowledgments

LMB is funded by NIH/NIDA grants DA025158; DA025259 and DA018860. PHM is funded by NIH/NINDS/NIDDK grants NS067631 and DK088125. LMB is a consultant to Trevena, Inc.

References

1. Urban JD, et al. Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* 2007;320(1):1–13. [PubMed: 16803859]
2. Kenakin T. Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. *Trends Pharmacol Sci* 2007;28(8):407–415. [PubMed: 17629960]

3. Bohn, LM. Selectivity for G Protein or Arrestin-Mediated Signaling. In: Neve, K., editor. *Functional Selectivity of G Protein-Coupled Receptor Ligands: New Opportunities for Drug Discovery*. Humana Press; 2009. p. 71-85.
4. DeWire SM, et al. Beta-arrestins and cell signaling. *Annu Rev Physiol* 2007;69:483-510. [PubMed: 17305471]
5. Schmid CL, et al. Agonist-directed signaling of the serotonin 2A receptor depends on beta-arrestin-2 interactions in vivo. *Proc Natl Acad Sci U S A* 2008;105(3):1079-1084. [PubMed: 18195357]
6. Wisler JW, et al. A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci U S A* 2007;104(42):16657-16662. [PubMed: 17925438]
7. Violin JD, Lefkowitz RJ. Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* 2007;28(8):416-422. [PubMed: 17644195]
8. Schmid CL, Bohn LM. Physiological and pharmacological implications of beta-arrestin regulation. *Pharmacol Ther* 2009;121(3):285-293. [PubMed: 19100766]
9. Oakley RH, et al. The cellular distribution of fluorescently labeled arrestins provides a robust, sensitive, and universal assay for screening G protein-coupled receptors. *Assay Drug Dev Technol* 2002;1(1 Pt 1):21-30. [PubMed: 15090153]
10. Barak LS, et al. A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem* 1997;272(44):27497-27500. [PubMed: 9346876]
11. Barak LS, et al. Real-time visualization of the cellular redistribution of G protein-coupled receptor kinase 2 and beta-arrestin 2 during homologous desensitization of the substance P receptor. *J Biol Chem* 1999;274(11):7565-7569. [PubMed: 10066824]
12. Zhang J, et al. Cellular trafficking of G protein-coupled receptor/beta-arrestin endocytic complexes. *J Biol Chem* 1999;274(16):10999-11006. [PubMed: 10196181]
13. Oakley RH, et al. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem* 2000;275(22):17201-17210. [PubMed: 10748214]
14. Charest PG, et al. Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. *EMBO Rep* 2005;6(4):334-340. [PubMed: 15776020]
15. Heding A. Use of the BRET 7TM receptor/beta-arrestin assay in drug discovery and screening. *Expert Rev Mol Diagn* 2004;4(3):403-411. [PubMed: 15137906]
16. Doucette C, et al. Kappa opioid receptor screen with the Tango beta-arrestin recruitment technology and characterization of hits with second-messenger assays. *J Biomol Screen* 2009;14(4):381-394. [PubMed: 19403921]
17. Wetter JA, et al. Utilization of the Tango beta-arrestin recruitment technology for cell-based EDG receptor assay development and interrogation. *J Biomol Screen* 2009;14(9):1134-1141. [PubMed: 19726785]
18. Zhao X, et al. A homogeneous enzyme fragment complementation-based beta-arrestin translocation assay for high-throughput screening of G-protein-coupled receptors. *J Biomol Screen* 2008;13(8):737-747. [PubMed: 18660457]
19. Angers S, et al. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci U S A* 2000;97(7):3684-3689. [PubMed: 10725388]
20. Bertrand L, et al. The BRET2/arrestin assay in stable recombinant cells: a platform to screen for compounds that interact with G protein-coupled receptors (GPCRs). *J Recept Signal Transduct Res* 2002;22(1-4):533-541. [PubMed: 12503639]
21. Hamdan FF, et al. High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer 1-based beta-arrestin2 recruitment assay. *J Biomol Screen* 2005;10(5):463-475. [PubMed: 16093556]
22. Pflieger KD, Eidne KA. New technologies: bioluminescence resonance energy transfer (BRET) for the detection of real time interactions involving G-protein coupled receptors. *Pituitary* 2003;6(3):141-151. [PubMed: 14974443]
23. Boute N, et al. The use of resonance energy transfer in high-throughput screening: BRET versus FRET. *Trends Pharmacol Sci* 2002;23(8):351-354. [PubMed: 12377570]

24. Pflieger KD, et al. Extended bioluminescence resonance energy transfer (eBRET) for monitoring prolonged protein-protein interactions in live cells. *Cell Signal* 2006;18(10):1664–1670. [PubMed: 16492395]
25. Kocan M, et al. Demonstration of improvements to the bioluminescence resonance energy transfer (BRET) technology for the monitoring of G protein-coupled receptors in live cells. *J Biomol Screen* 2008;13(9):888–898. [PubMed: 18812574]
26. Hanson BJ, et al. A homogeneous fluorescent live-cell assay for measuring 7-transmembrane receptor activity and agonist functional selectivity through beta-arrestin recruitment. *J Biomol Screen* 2009;14(7):798–810. [PubMed: 19531663]
27. McGuinness D, et al. Characterizing cannabinoid CB2 receptor ligands using DiscoverX PathHunter beta-arrestin assay. *J Biomol Screen* 2009;14(1):49–58. [PubMed: 19171920]
28. van Der Lee MM, et al. beta-Arrestin recruitment assay for the identification of agonists of the sphingosine 1-phosphate receptor EDG1. *J Biomol Screen* 2008;13(10):986–998. [PubMed: 19036707]
29. Kunapuli P, et al. Development of an intact cell reporter gene beta-lactamase assay for G protein-coupled receptors for high-throughput screening. *Anal Biochem* 2003;314(1):16–29. [PubMed: 12633598]
30. Barnea G, et al. The genetic design of signaling cascades to record receptor activation. *Proc Natl Acad Sci U S A* 2008;105(1):64–69. [PubMed: 18165312]
31. Yan YX, et al. Cell-based high-throughput screening assay system for monitoring G protein-coupled receptor activation using beta-galactosidase enzyme complementation technology. *J Biomol Screen* 2002;7(5):451–459. [PubMed: 14599361]
32. Graham DL, et al. Application of beta-galactosidase enzyme complementation technology as a high throughput screening format for antagonists of the epidermal growth factor receptor. *J Biomol Screen* 2001;6(6):401–411. [PubMed: 11788058]
33. Kohout TA, et al. beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci U S A* 2001;98(4):1601–1606. [PubMed: 11171997]
34. Koenig JA, Edwardson JM. Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol Sci* 1997;18(8):276–287. [PubMed: 9277131]
35. Zhang J, et al. Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proc Natl Acad Sci U S A* 1998;95(12):7157–7162. [PubMed: 9618555]
36. Groer CE, et al. An opioid agonist that does not induce μ -opioid receptor- β -arrestin interactions or receptor internalization. *Mol Pharmacol* 2007;71(2):549–557. [PubMed: 17090705]

Abbreviations

GPCR	G protein-coupled receptor
BRET	bioluminescence resonance energy transfer
RLuc	<i>Renilla</i> luciferase
FRET	fluorescence resonance energy transfer
EFC	enzyme fragment complementation

Figure 1.
Imaging Translocation

Figure 2.
BRET

Figure 3.
Enzyme Fragment Complementation

Figure 4.
Reporter Gene Assay

Table 1

Comparison Summary Table.

Assay Technology	Transfluor	BRET	Tango	PathHunter
Assay Format	Imaging	Dual Channel Emission	Reporter Gene, FRET	Enzyme Fragment Complementation
Labels on GPCR/ β arrestin	No Tag / GFP	Luciferase / GFP	Transcription factor/Protease	“Prolink” β -gal fragment/ Complementary β -gal fragment
Equipment & Supplier	Fluorescent microscopy imaging Molecular Devices www.moleculardevices.com	Multimodal plate reader with injectors	Fluorescence plate reader Invitrogen www.invitrogen.com	Conventional plate reader DiscoverX www.DiscoverX.com
Pros	<ul style="list-style-type: none"> • Receptor proximity • Short incubation • Detection of cytotoxic & fluorescent samples 	<ul style="list-style-type: none"> • Ratiometric • Receptor proximity • Short incubation 	<ul style="list-style-type: none"> • Ratiometric- a signal is generated only once for each receptor during the assay • Amplification of signal 	<ul style="list-style-type: none"> • Signal amplification • Stable indicator • Nonspecialized equipment
Cons	<ul style="list-style-type: none"> • Costly and specialized imaging equipment • Data file storage • May not be functional interaction 	<ul style="list-style-type: none"> • Sensitive to stoichiometry • Substrate catabolism is short lived • May not be functional interaction 	<ul style="list-style-type: none"> • Long period of incubations • Relies on downstream transcription/translation events • Higher potential for compound interference 	<ul style="list-style-type: none"> • Must work with company to modify assay • Long period of agonist incubation • Delayed assay window
References	(9,10)	(19-21)	(29,30)	(31,32)