Journal of Visualized Experiments

Parallel Interrogation of β-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-Tango assay.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60823R1
Full Title:	Parallel Interrogation of β-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-Tango assay.
Section/Category:	JoVE Biology
Keywords:	G-protein coupled receptors; Ligand screening; drug discovery; Deorphanization; High-throughput; Tango assay; β-arrestin2; G-protein-independent assay; GPCR-ome
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ottawa, Ontario, Canada

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September 26, 2019

Editorial Staff, Journal of Visualized Experiments

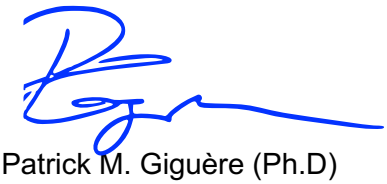
Dear JOVE editorial staff-members,

We would like to submit our publication titled "Parallel Interrogation of β-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-TANGO assay" for your consideration as a published article in JOVE. Our manuscript describes the technical application of the PRESTO-TANGO assay for parallel screening of a class A GPCR panel comprising 320 receptors. To the best of our knowledge, the PRESTO-TANGO platform is the only open-source resource to perform such GPCRome interrogation simultaneously. Moreover, the procedure described is economically and technically accessible to most laboratories.

This platform is receiving high interest in the GPCR field for the reason mentioned above, but also for a general growing awareness to polypharmacology. Indeed, this platform can be used to assay off-target activity of any pharmacological agents at the GPCRome or for the design and discovery of drugs specifically modulating multiple targets.

My colleagues and I look forward to your scientific and editorial comments on our present manuscript.

Sincerely yours and with much thanks,



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TITLE:

Parallel Interrogation of β-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-Tango Assay

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KEYWORDS:

G-protein coupled receptors, ligand screening, drug discovery, deorphanization, high-throughput, Tango assay, β-arrestin2, G-protein-independent assay, GPCR-ome

SUMMARY:

Given that GPCRs are attractive druggable targets, GPCR ligand screening is thus indispensable for the identification of lead compounds and for deorphanization studies. Towards these efforts, we describe PRESTO-Tango, an open-source resource platform used for simultaneous profiling of transient β-arrestin2 recruitment at approximately 300 GPCRs using a TEV-based reporter assay.

ABSTRACT:

As the largest and most versatile gene superfamily and mediators of a gamut of cellular signaling pathways, G-protein-coupled receptors (GPCRs) represent one of the most promising targets for the pharmaceutical industry. Ergo, the design, implementation, and optimization of GPCR ligand screening assays is crucial, as they represent remote-control tools for drug discovery and for manipulating GPCR pharmacology and outcomes. In the past, G-protein dependent assays typified this area of research, detecting ligand-induced events and quantifying the generation of secondary messengers. However, since the advent of functional selectivity, as well as an increased awareness of several other G protein-independent pathways and the limitations associated with G-protein dependent assays, there is a greater push towards the creation of alternative GPCR ligand screening assays. Towards this endeavor, we describe the application of one such resource, the PRESTO-Tango platform, a luciferase reporter-based system that enables the parallel and simultaneous interrogation of the human GPCR-ome, a feat which was previously considered technically and economically unfeasible. Based on a G-protein independent β-arrestin2 recruitment assay, the universality of β-arrestin2-mediated trafficking and signaling at GPCRs makes PRESTO-TANGO an apt tool for studying approximately 350 non-olfactory human

GPCRs, including approximately 100 orphan receptors. PRESTO-Tango's sensitivity and robustness make it suitable for primary high-throughput screens using compound libraries, employed to uncover new GPCR targets for known drugs or to discover new ligands for orphan receptors.

INTRODUCTION:

G-protein-coupled receptors (GPCRs) constitute the largest and most diverse family of transmembrane proteins, operating as communication interfaces between a cell and its environment[1]. The versatility of GPCRs is highlighted by their ability to detect a diverse array of ligands—from neurotransmitters to nucleotides, peptides to photons, and many more—as well as their ability to regulate numerous downstream signaling cascades involved in cellular growth, migration, differentiation, apoptosis, cell firing, etc.[2, 3]. Considering their ubiquity and involvement in a multitude of physiological processes, this receptor family is of utmost therapeutic importance, showcased by the fact that more than a third of currently available prescribed medications target GPCRs[4]. However, these existing therapeutics only target a small subset of the superfamily (an estimated 10%), and the pharmacology of many GPCRs remains unelucidated. Moreover, more than 100 GPCRs exist as orphan receptors, as they have not been matched with an endogenous ligand[5]. Thus, GPCR ligand screening is critical in deorphanization and drug development, as it paves the path towards lead discovery and optimization, and possibly to the clinical trial phase.

Methods for GPCR ligand screening have traditionally fallen in one of two categories, G-protein dependent or G-protein independent functional assays[6]. GPCR signaling is regulated by heterotrimeric G-proteins (Gαβγ), which are activated by the exchange of GTP for GDP bound on the Gα subunit[7]. Signals from the activated receptor are transduced by G-proteins via secondary messengers, such as cAMP, Calcium, DAG, and IP3, to mediate downstream signaling at downstream effectors[8]. The nature of the functional consequences of G-protein signaling has been exploited to create cell-based assays that reflect receptor activation. These methods, which measure proximal (direct) or distal (indirect) events in G-protein signaling, are most frequently used for GPCR ligand screening and have been principally employed in deorphanization studies[6]. Examples of assays that directly measure GPCR-mediated G-protein activation include the [35S]GTPγS binding assay, which measures binding of a radiolabeled and non-hydrolyzable GTP analog to the Gα subunit, and Förster/bioluminescence resonance energy transfer (FRET/BRET, respectively) probes to monitor GPCR-Gα and Gα/Gγ interactions, which have been gaining more traction over the years[9, 10]. Assays that monitor distal events are the most commonly used tools for GPCR profiling; for example, cAMP and IP1/3 assays measure intracellular accumulation of G-protein dependent secondary messengers, whereas [Ca^{2+}] flux and reporter assays involving specific response elements implicated in G-protein activation (CRE, NFAT-RE, SRE, SRF-RE) examine events further downstream the signaling cascade[11]. While most of the aforementioned assays can be performed at a high-throughput level, are fairly sensitive, and boast certain assay-specific advantages (e.g., discrimination between full/partial agonists, neutral antagonists and inverse agonists in the case of GTPγS binding, or assay functionality on live cells such as [Ca^{2+}] and IP1/3)[6], there are unfortunately no existing G-protein dependent methods befitting the interrogation of the entire druggable GPCR-ome. This is largely due to the native coupling of

multiple G-protein subfamilies to GPCRs, resulting in signaling at several cascades and the unknown G-protein coupling at orphan GPCRs. To mitigate this issue, assays have been developed to force promiscuous G-protein coupling through a single common signaling read-out, such as cAMP, and Ca^{2+}, albeit most of them are low-throughput[12].

An important aspect of the GPCR lifecycle is the termination of G-protein-dependent signaling, which occurs in large part through the recruitment of β-arrestins which induces dissociation of the G-protein, and ultimately desensitizing the receptor, which is targeted for clathrin-coated internalization[13]. The most ubiquitously expressed isoforms of β-arrestin are the non-visual β-arrestin1 and β-arrestin2, also denoted as arrestin-2 and arrestin-3, respectively[14]. Enter G-protein independent cell-based assays, which add a new dimension to GPCR ligand screening; receptor trafficking, label-free whole cell, and β-arrestin recruitment assays are all notable examples. GPCR trafficking assays employ fluorophore-labeled ligands or co-internalized antibodies targeting the receptor[15], whereas label-free whole cell assays use biosensors which translate cellular changes induced by ligand binding into quantifiable outputs, such as electrical or optical signals[16]. Notably, quintessential GPCR- β-arrestin interactions fashion the β-arrestin recruitment assay as an attractive tool in the repertoire of functional assays[17]. The Tango system, first developed by Barnea et al. only a decade ago, involves the introduction of three exogenous genetic elements: a protein fusion consisting of β-arrestin2 with a tobacco etch virus protease (TEVp), a tetracycline transactivator (tTA) that is tethered to a GPCR via a tobacco etch virus protease cleavage site (TEVcs) and is preceded by a sequence from the C-terminus of the V2 vasopressin receptor (V2 tail) to promote arrestin recruitment, and a reporter luciferase gene whose transcription is triggered by the tTA transcription factor translocation to the nucleus, which is freed from the membrane anchoring following β-arrestin2 recruitment (**Figure 1**)[18]. Quantitative readings of GPCR activation and β-arrestin2 recruitment can be subsequently determined by reading for luminescence. A notable distinction is that while receptor trafficking and label-free whole cell methods are relatively low-throughput, the Tango has several advantages, including selective read-out that is specific to the target receptor and sensitivity due to signal integration, which make it a suitable candidate for ligand screening on a larger scale[18].

In view of these strategic features, Kroeze et al. developed PRESTO-Tango (Parallel Receptor-ome Expression and Screening via Transcriptional Output-Tango), a high-throughput open-source platform that uses the Tango approach to profile the druggable GPCR-ome in a parallel and simultaneous manner[19]. Exploiting the “promiscuous” recruitment of β-arrestin2 to nearly all GPCRs, PRESTO-Tango is the first-of-its-kind in terms of cell-based functional assays, enabling rapid “first-round” screening of small molecule compounds at almost all non-olfactory GPCRs, including orphans, independent of the G-protein subfamily coupling.

PROTOCOL:

1. Primary screening: cell culture and plate seeding

1.1. To prepare poly-L-lysine (PLL)-coated plates, dispense 20 µL/well of a 25 mg/mL stock solution of PLL in white or black 384-well optical bottom plates using an electronic multichannel

pipette or a reagent dispenser. Incubate the plates at room temperature for 0.5–2 h.

NOTE: If using the black 384-well plates, expect the background signal to be lower compared to the white plates. Black plates are recommended to reduce bleed-through of luminescence between adjacent wells.

1.2. To preserve the coated plates and wash off the excess PLL, remove the PLL by flicking it over the sink, tap dry over a paper towel, and add 40 µL/well of diluted 1x solution of antibiotic-antimycotic using an electronic multichannel pipette or a reagent dispenser. Store PLL-coated plates at 4 °C until ready for plate seeding.

1.3. Maintain HTLA cells (kindly provided by Dr. Richard Axel)—a human embryonic kidney cell line (HEK293T) stably expressing β-arrestin2-TEV and tTA-driven luciferase—in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% of Fetal Bovine Serum, 5% of Bovine Calf Serum, 2.5 µg/mL of puromycin, 50 µg/mL of hygromycin, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO_2.

1.4. Culture HTLA cells in 150 mm dishes and pass cells twice a week at a dilution factor of 1:10, with optimal cell passage number of 5–25. Ensure that a sufficient number of 150 mm dishes are confluent the day of 384-well plate seeding, depending on the scale of the primary screen.

NOTE: Usage of HTLA cells greater than passage 25 may result in reduced viability, yielding suboptimal results.

1.5. To seed HTLA cells for the primary screen, gently rinse the confluent 150 mm dish(es) with 1x phosphate-buffered saline (PBS), pH 7.4. Detach cells with approximately 6 mL of 0.05% Trypsin/0.53 mM EDTA, and transfer to a centrifuge tube containing at least equal amount of complete Dulbecco's modified Eagle medium (DMEM) to neutralize the trypsin.

1.6. Spin down HTLA cells at 500 x *g* for 3 min and resuspend the cell pellet at a density of 0.22 x 10^6 cells/mL in complete DMEM, omitting the addition of 2.5 µg/mL of puromycin and 50 µg/mL of hygromycin as they can decrease transfection efficacy.

1.7. Incubate the necessary 384-well PLL-coated plates at 37 °C to warm them before seeding cells. Remove the storage solution of 1x antibiotic-antimycotic from the 384-well PLL-coated plate(s) by flicking the plate over the sink and taping it over a paper towel to dry.

1.8. Seed cells into the 384-well PLL-coated plates at a final density of 10,000 cells/well by dispensing 45 µL of the 0.22 x 10^6 cells/mL HTLA suspension using an electronic multichannel pipet. Incubate plates at 37 °C overnight. If a same-day transfection is preferred, seed cells at a density of 16,000 cells/well and perform the transfection 4 h later.

NOTE: For high transfection efficiency, 50–70% cell confluency is optimal.

2. Primary screening: DNA plate preparation and transfections

2.1. To prepare the 384-well DNA source plate for transfection as shown in **Figure 2**, distribute the plasmid cDNAs encoding the GPCR-Tango constructs of interest in a 96-well plate, with a different GPCR/well. The plasmid DNA should be suspended in 0.1x Tris-EDTA (TE) buffer at a concentration of 50 ng/µL.

NOTE: The 96-well DNA plates can be sealed and stored at -20 °C, and re-used for multiple screening experiments. All cDNA encoding GPCR-Tango constructs are available commercially (see the **Table of Materials**) and are cloned in the pcDNA3.1 neomycin plasmid. The PRESTO-Tango GPCR Kit consists of four 96-well plates, which include 80 GPCRs each, a couple of wells with an empty vector as negative controls, and positive control wells that hold the dopamine receptor D2 (DRD2), and wells that carry a plasmid encoding a fluorescent protein (YFP) to track transfection efficiency.

2.2. Using a multichannel pipette, manually transfer DNA solution from the 96-well to the a 384-well DNA source plate, adding 10 µL per 384-well. To ensure that each condition of the experiment is assayed in quadruplicate, half of the 96-well DNA plate (rows A-D or E-H) will cover a full 384-well plate by distributing each GPCR in two quadrants (first quadrant = – compound, second quadrant = + compound), such that the same GPCR will be transfected in 8 wells of the 384-well plate (see **Figure 2** as a guide).

2.3. Assemble the following transfection reagents needed for calcium phosphate precipitation method, as described by Jordan et al.[20]: 0.1x TE buffer (1 mM Tris-HCl and 0.1 mM EDTA); 2.5 M $CaCl_2$ solution; 2x Hepes buffer, pH 7.05 (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4). Sterilize all the solutions by filtration and store at 4 °C. The day of transfection, allow the reagents to reach room temperature before use.

2.3. Dilute the 2.5 M $CaCl_2$ stock solution in 0.1x TE (1:8 dilution) to a final concentration of 0.313 M $CaCl_2$ and vortex. Transfer 40 µL of 0.313 M $CaCl_2$ to the 384-well DNA source plate and mix by pipetting up and down with a hand-held multichannel pipette or an automated benchtop 384-channel pipettor.

2.4. Add 50 µL of 2x Hepes buffer to the 384-well DNA source plate, mix again by pipetting up and down and let stand for 1 min; each 384-well will have an adequate amount of DNA/transfection mixture for the transfection of nine 384-well plates, depending on the number of compounds that need to be tested. Transfer 10 µL of the DNA/transfection mixture from the 384-well DNA source plate to the seeded HTLA cells and incubate the plates overnight at 37 °C.

3. Primary screening: Cell stimulation

3.1. Twenty-four hours later, decant the transfected cell media by gently flicking the 384-well plate over the sink and taping it over a paper towel, or with an aspirator head. Slowly add 40 µL of starving media (DMEM supplemented with 1% dialyzed fetal bovine serum (dFBS) and 1x

antibiotic/antimycotic), being careful to avoid touching the cells directly.

3.2. Pipet 20 µL of the compound of interest at a 3x concentration (final concentration of the drug in the cell plate will be 1x) into the alternating rows with (+) stimulation, and 20 µL of vehicle buffer for the alternating rows without (–) compound. Return the cell plate at 37 °C in 5% CO_2 and incubate for at least 16 h.

4. Primary screening: Luminescence reading

4.1. Prepare the Glo reagent, modified from Baker and Boyce[21]: 108 mM Tris–HCl; 42 mM Tris-Base, 75 mM NaCl, 3 mM $MgCl_2$, 5 mM Dithiothrei-tol (DTT), 0.2 mM Coenzyme A, 0.14 mg/ml D-Luciferin, 1.1 mM ATP, 0.25% v/v Triton X-100, 2 mM Sodium hydrosulfite.

NOTE: Stock solutions of the reagents can be made in advance, except for D-Luciferin, which is always freshly added to the Glo reagent in its powdered form. If black plates were used, the amount of D-Luciferin can be increased up to 0.25 mg/mL.

4.2. At 16–24 h following stimulation, decant the transfected cell media by gently flicking the 384-well plate over the sink and taping it over a paper towel. Add 20 µL/well of Glo reagent and incubate the plate at room temperature for 5–20 min. Read the plates using a microplate luminescence counter, with an integration time of 1 s/well.

5. Primary screening: **Data analysis**

5.1. Export the saved files from the luminescence counter as a spreadsheet; results will be recorded in relative luminescence units (RLU). Based on the layout of the 384-well plate, calculate the activation (fold change) of each receptor using the following formula:

$$\text{Activation (fold of basal)} = \frac{\text{Sample RLU} - \text{Mean background RLU}}{\text{Mean basal RLU} - \text{Mean background RLU}}.$$

NOTE: Here Sample RLU refers to value of each of the four replicate wells of the stimulated (+ compound) quadrant, Mean background RLU is the mean of the negative controls on the plate, and Mean basal RLU refers to the mean of the untreated quadrant of that same receptor (- compound). In addition, calculate the standard deviation of the 4 data points to verify the quality of the results. It is recommended to perform a log2 transformation on the mean of the fold changes to rectify any heteroskedasticity; the log2 base is a practical choice to help identify positive hits. Empirically set the positive hit thresholds; it has to be noted that some receptors can have as low as 2-fold increase and up to 40-fold increase for others with full agonist.

5.2. Based on the results, select the GPCRs that are potential positive hits for secondary screening.

6. Secondary screening: Cell seeding and transfections

6.1. Subculture HTLA cells in 100 mm dishes at a total cell density of 5 x 10^6 cells in 11 mL of complete media (4.55 x 10^5/mL) and incubate at 37 °C for 24 h. If a same-day transfection is preferred, seed cells at a density of 7.5 x 10^6 cells and perform the transfection 4 h later.

6.2. Pre-warm the reagents needed for calcium phosphate precipitation at room temperature. Combine 450 µL of 0.1x TE buffer with 50 µL of 2.5 M $CaCl_2$ and quickly vortex; these amounts are specific for one 100 mm dish, based on the volume of growth medium it holds.

6.3. In a tube, add 500 µL of the TE/$CaCl_2$ solution to 10 µg of GPCR cDNA and vortex. Add 500 µL of 2x Hepes buffer solution in the tube, shake vigorously (do not vortex), and incubate for 1 min.

NOTE: 1 µg of any plasmid encoding a fluorescent protein (e.g. YFP, mCherry, etc.) can be co-transfect with 9 µg of GPCR cDNA for a total of 10 µg. The fluorescent protein is used to track transfection efficiency, and this minimal amount will not interfere with the assay.

6.4. Immediately following the short incubation, dispense the 1 mL solution dropwise onto the cells. Gently rock the plate back and forth to evenly distribute the precipitate, taking care not to swirl the plate, and incubate at 37 °C for 24 h.

6.5. The following day, observe the transfection efficiency by looking at the expression of the fluorescent protein under a fluorescent cell imager; transfections greater than 50% coverage are ideal.

6.6. Incubate the necessary 384-well PLL-coated plate(s) in the incubator at 37 °C to warm it before seeding cells. Remove the storage solution of 1x antibiotic-antimycotic from the 384-well PLL-coated plate(s) by flicking the plate over the sink and taping it over a paper towel to dry.

6.7. Gently rinse the transfected cells with Versene solution (1X PBS, pH 7.4; 0.53 mM EDTA) , and detach by adding 3 mL of 0.05% trypsin/0.53 mM EDTA to the dish. Transfer the contents to a centrifuge tube containing at least an equal amount of complete DMEM to neutralize the trypsin.

6.8. Spin down the cells at 500 x *g* for 3 min and resuspend the cells at a density of 0.4 x 10^6 cells/mL in starving media. Seed cells into the 384-well PLL-coated plate(s) at a final density of 25,000 cells/well by dispensing 45 µL of the cell suspension using an electronic multichannel pipet. Return the plates to the 37 °C for a minimum of 4 h, allowing the cells to properly attach to the wells before proceeding to the stimulation.

7. Secondary screening: Drug plate preparation for 16-point (half log) dose-curve

7.1. In a 96-well plate, add 270 µL of 1X HBSS drug buffer (1x Hank's Balanced Salt Solution [HBSS], 20 mM HEPES pH 7.4, 1x antibiotic-antimycotic), excluding the last row (row H) of the plate, as shown in **Figure 4**.

NOTE: For peptides, colloidal molecules, and poorly water-soluble compounds, the addition of 0.1–1% BSA is suggested. To prevent drug oxidation, up to 0.01% ascorbic acid can also be added.

7.2. From the drug stock, prepare a drug solution (referred to as the "High" concentration) by calculating a final 3x concentration (final concentration of the drug in the cell plate will be 1x). As an example, for a dose–response curve with 10 µM as its highest concentration, prepare the "High" concentration at 30 µM. Pipet 300 µL of "High" concentration into wells in row H.

7.3. In another tube, prepare the "Low" concentration, which represents the "High" concentration divided by 3.16 (half-log). Based on the previous example, the "Low" concentration would be 9.49 µM. Pipet 300 µL of "Low" concentration into wells in row H, adjacent to the "High" wells.

NOTE: The total number of 96-wells needed in row H will depend on the number of cells and stimulation conditions. Four wells (two "High" and two "Low") will have ample drug solution to stimulate an entire 384 well plate.

7.4. Perform a serial dilution by pipetting 30 µL of drug solution from the "High" and "Low" wells of row H to the previous row (row G) and mix by manually pipetting up and down, or as recommended, using an electronic multichannel pipette with the "Pipette and Mix" function. Repeat this step up until the first and most diluted row (row A), while discarding tips between dilutions.

NOTE: If desired, the serial dilutions can be stopped before row A, representing an internal control with no drug, in other words, a "true zero".

7.5. Using **Figure 4** as a reference, stimulate transfected cells by pipetting 20 µL of the "Low" column dilutions from the 96-well plate to rows A–O of the previously seeded 384-well plate, as well as 20 µL of the "High" column dilutions to wells B–P. Incubate the plate at 37 °C for a minimum of 16 h.

8. Secondary screening: Luminescence reading and data analysis

8.1. At 16–24 h following stimulation, decant the transfected cell media by gently flicking the 384-well plate over the sink and taping it over a paper towel. Add 20 µL/well of Glo reagent and incubate the plate at room temperature for 5–20 min. Read the plates using a microplate luminescence counter, with an integration time of 1 s/well.

8.2. Export the saved files from the luminescence counter as a spreadsheet; results will be recorded in relative luminescence units (RLU). Transfer the data of the 384-well plate to a statistics software to analyze the results using its built-in XY analysis for non-linear regression curve fit. Select the built-in 3-parameter dose-response stimulation function "Log(agonist) vs. response (three parameters)",

$$\text{Rsponse (RLU)} = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{\text{LogEC50}-\text{X}}}$$

NOTE: Here Top and Bottom are plateaus in the units of the Y axis, respectively the maximal response and basal level, EC50 is the concentration of the agonist that that generates 50% response between Top and Bottom, and X refers to the log concentration of the agonist. This model assumes that the dose-response curve has a standard Hill slope of 1.

REPRESENTATIVE RESULTS:

Using the PRESTO-Tango protocol presented herein, a chromaffin granule (CG) extract was screened against 168 non-olfactory GPCR targets, with the majority being orphan receptors. Profiling of said extract was performed by examining β-arrestin2 mobilization at the chosen receptors, based on the principle designed by Barnea et al.[18] (**Figure 1**). Plasmid cDNA of the GPCRs of interest was taken from the PRESTO-Tango GPCR Kit and assembled in two 96 well-plates in the desired layout. In total, four 384-well plates seeded with HTLA cells were transfected, as each half of the 96-well DNA plates was made into a full 384-well plate, resulting in each receptor being transfected in two quadrants (eight 384-wells total). One of the two quadrants was stimulated with the CG extract; put differently, alternating rows C–D, G–H, K–L, and O–P represented the (+) stimulation (**Figure 2**). Out of the 168 GPCRs that were interrogated in the primary screening, only two receptors were contenders as potential active targets, specifically dopamine receptor D3 (DRD3) and opsin 5 (OPN5). DRD3 produced a significant log2fold change of 4.70, whereas OPN5 produced a slightly lower response of 2.39, both meeting the threshold cut-off of log2 fold change >2. In comparison, the positive control for the primary screen was DRD2 stimulated with quinpirole, a selective agonist of this receptor, and produced a log2 fold change of 4.58 (**Figure 3**). To reproduce these signal windows and eliminate the possibility of false-positive hits, a secondary screen was conducted with the aforementioned receptors. Besides testing the CG extract, given that DRD3 is a non-orphan receptor, another condition was prepared as a positive control, specifically stimulation with quinpirole, one of its selective agonists. On the other hand, OPN5 is an orphan receptor and as such, no reference agonist can be tested alongside the CG extract as a positive control; only buffer was tested as a negative control. Further pharmacological characterization of these two GPCRs was performed by preparing 16-point dose curves ranging from 10^{-5} M to $10^{-12.5}$ M. Specifically, the CG extract and quinpirole stock solutions at 10 mM were diluted to 30 µM and 9.49 µM, the corresponding "High" and "Low" concentrations in bottom row (row H) of the 96-well drug plate; these formulations will become 10^{-5} M and $10^{-5.5}$ M once 20 µL is dispensed on to the transfected cells in 40 µL of starved medium, for a total of 60 µL within each 384-well. As previously described, serial dilutions were performed such that the most dilute drug formations for 10^{-12} M and $10^{-12.5}$ M are in the top row (row A) (**Figure 4**). Dose-response curves from the secondary screening were created using GraphPad Prism to evaluate ligand potency and efficacy. In comparison to quinpirole, the CG extract produced similar signal windows and EC50 values, confirming its validity as an active hit at DRD3. However, a flat dose-curve similar to the negative control was produced for OPN5, ruling it out as a possible target for the CG extract (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1. Modular design of TANGO constructs (A) and general scheme for the β-arrestin (Tango) recruitment assay (B). (**A**) The GPCR Tango constructs consist of various module elements in the following order: an HA signal/FLAG tag, the GPCR CDS, a Vasopressin receptor 2 C-terminal tail, TEV protease cleavage site, and a tTA transcription factor. (**B**) The principle of the Tango assay involves transiently transfecting the GPCR Tango plasmids in HTLA cells, HEK293T cells stably expressing a β-arrestin2-TEV protease fusion protein and a luciferase reporter gene whose expression is activated by tTA. Activation of the GPCR will eventually result in the mobilization of the β-arrestin2-TEV to the receptor, bringing the protease in close proximity to its cleavage site. As a result, the tTA is cleaved from the GPCR tail, freeing the transcription factor to translocate into the nucleus and activate luciferase expression.

Figure 2. Layouts of 96-well cDNA plate and 384-well cell plate for transfection and stimulation in PRESTO-TANGO primary screening. Depicting the preparation of a 384-well cDNA source plate for transfection, GPCR Tango constructs are first transferred from one half of a 96-well plate into a full 384-well plate, with each receptor being transfected in octuplicate. In this setting, stimulation of cells with (+) and without (-) the drug(s) of interest will occur in quadruplicate for each individual receptor.

Figure 3. Graphical representations of hit identification from PRESTO-Tango primary screening. As a proof-of-concept, the biological activity of a chromaffin granule (CG) extract on the GPCRome was analyzed. HTLA cells were transfected in 384 well plates with 168 GPCR Tango constructs, and either stimulated with the CG extract (+ compound) or with vehicle buffer (- compound). pcDNA3.1 was used as a negative control, and DRD2 receptor stimulated with quinpirole was used as a positive control. The signal windows (**A**) and the log2 fold change (**B**) in receptor activation was calculated between the wells in the absence or presence of CG extract. All error bars represent SD (n = four measurements).

Figure 4. Layout of 96-well drug plate preparation for stimulation in secondary screening. Depicting the preparation of a 96-well drug plate for cell stimulation, serial dilutions for a 16-point dose curve range start at 10^{-5} M (final concentration) in row H, with half-log intervals between each point until $10^{-12.5}$ M in row A. “High” and “Low” drug columns are used to stimulate alternating rows of the seeded 384-well plate.

Figure 5. Dose-curve responses for compound profiling and demonstration of β-arrestin2 recruitment to GPCRs in secondary screening. HTLA cells were transiently transfected with receptors DRD3 (**A**) and OPN5 (**B**). Both transfected conditions were stimulated with a CG extract in half-log increments, as well as the DRD3 specific agonist quinpirole as a positive control, and vehicle buffer for OPN5 as a negative control. All error bars represent SD (n = three measurements).

DISCUSSION:

The conformationally dynamic GPCRs are powerhouses of signal transduction. The physiochemical properties of the binding pockets of these heptahelical receptors, as well as their physiological relevance underscore the need for GPCR ligand screening tools. As presented

above, the PRESTO-Tango assay is rapid, sensitive and user-friendly, lending itself to drug development. Not only does this assay measure agonist-induced activation, but it can also be used to quantify the activity of antagonists and allosteric modulators[19]. In light of functional selectivity, a concept which suggests that different drug structures can elicit different receptor signaling cascades at a single receptor, comparing the activation of the G-protein pathway using G-protein dependent assays with β-arrestin recruitment using PRESTO-Tango could provide cues for the designing lead compounds with reduced negative side effects. Notably, its independence from detecting G-protein coupling helps identify coupling partners for orphan GPCRs that would not be have been previously detected by G-protein dependent assays.

To ensure consistency and robustness of PRESTO-Tango screens, care must be taken in all steps of the protocol, as perturbations introduced will be magnified due to the nature of this platform. Of course, there are general measures common to all HTS screens which should be taken into consideration, such as using reagents of the same lot/formulation to ensure identical stability and biological activity throughout, as well as keeping conditions on the HTS system consistent such as cell seeding density and drug incubation time. The miniaturized format of PRESTO-Tango demands attention to a couple of critical points: variation in cell seeding density and their homogeneous distribution (clumping versus single cell suspension) between wells, low transfection efficiency, and poor compound stimulation and delivery will prevent day-to-day and plate-to-plate reproducibility. To that effect, triturate the HTLA cell suspension to homogenize the solution before seeding and ensure a 50–70% cell confluency before transfection. The vehicle for the delivery of the compounds should be verified, with dimethyl sulfoxide being the most common carrier. Typically, the highest concentration of our dose curves is 10 µM, but this may change depending on the nature and potency of the compound; it is important to test various concentrations to deduce cellular tolerance and toxicity.

Given that some GPCRs have high constitutive activity, one issue that may arise during screening is a reduced dynamic range and a background signal that is higher than expected. This can be somewhat mitigated by ensuring that serum starvation is being performed with DMEM medium supplemented with 1% dFBS. It should be taken into consideration that if the luminescence output is high enough, there can still be bleed-through into adjacent wells, which may result in erroneously calculated fold changes. Undetectable or low signals (assuming a response is expected) can be explained in a number of ways, namely poor expression of GPCR(s) in HTLA cells, the biological activity of the compound is lost rendering it inefficacious, or the receptor(s) in question do not intrinsically recruit β-arrestin2. Respectively, assessing the quantity and quality of transfected plasmid receptor DNA, testing other preparations/lots of the inefficacious compound in question, and performing orthologous protein-protein interaction techniques such as BRET/FRET or co-immunoprecipitation are some suggested solutions to this problem. In addition, receptor expression could also be improved by subcloning Tango receptor(s) of interest into lentiviral vectors and transducing HTLA cells, generating a HTLA-GPCR stable cell line. A shift in the expected potency of an agonist during secondary screening could imply that the drug stimulation time and/or concentration of compound needed to stimulate a response is insufficient, or that the drug plate serial dilutions were incorrectly prepared. Use of an electronic multichannel pipette or an automated pipettor system without changing tips in between when

creating drug serial dilutions could be an issue when working with sticky compounds.

Notable differences between the original Tango assay developed by Barnea et al.[18] and the PRESTO-Tango platform include the design of the receptor in a modular format, consisting of codon-optimized sequences, which improves receptor expression in mammalian cells, epitope tags to validate said expression, and restriction sites which flank GPCRs, V2 tail and TEVcs-tTA, enabling for excision of parts and subcloning. Most importantly, PRESTO-Tango surpasses the Tango assay in terms of screening power and experimental design. Quadruplicate sample testing of approximately 350 GPCRs is accomplished in only 8 384-well plates, while accounting for negative background controls and positive controls to monitor transfection efficiency. While PRESTO-Tango is suitable for screening the GPCR-ome with only one compound of interest, interrogation with multiple ligands can also be performed, albeit at increased cost and use of resources, such as with pooled or arrayed small molecule compound libraries or biological samples which consist of mixtures of various chemical entities. Granted, this issue can be mitigated by reducing the number of compounds to interrogate by performing chemical similarity and diversity analyses of the compound libraries in question. While the PRESTO-Tango platform is more applicable for primary screening purposes, secondary profiling can be performed at a smaller scale, in medium or low-throughput formats, to confirm the functional consequences of ligand stimulation. However, as with all other GPCR assays, it must be acknowledged that there are no suitable positive controls for orphan receptors during secondary screening with the Tango assay. Nonetheless, potential positive hits can be identified if the output data can be fitted to a sigmoidal dose-response curve, with a computed signal window and EC50 value. It is also important to note that the mechanism of ligand activity, be it for orphan or non-orphan receptors, cannot be elucidated without running parallel assays.

With all components of PRESTO-Tango already optimized, including HTLA cell line and GPCR Tango constructs, little room for modification is required apart from choice of compound formulation(s) to be used for drug stimulation. If desired, an HTLA cell line stably expressing a receptor can be easily generated by cloning said GPCR-Tango receptor within the recommended pIRESbleo3 vector (Clonetech), and selecting clones using zeocin. With regard to the swap from pcDNA3.1 to pIRESbleo3, simply digest the GPCR Tango construct with NotI and XbaI and insert into the destination vector at restriction sites NotI and NheI. Notwithstanding, there are avenues for adapting and optimizing this technology. One of the pillars of this technology are HTLA cells, a HEK293T cell line stably expressing a β-arrestin2-TEV fusion gene and a tTA-dependent luciferase reporter, graciously supplied from the lab of Richard Axel. While a crucial component of PRESTO-Tango, there are currently no other alternatives in terms of cell line origin, or the genes they express. Moreover, future engineered cell lines can be generated to express other TEV fusion genes to track other proteins besides β-arrestin2, specifically those that have been previously shown to interact or found in residence to GPCRs, such as 14-3-3[22], SAP97[23], and β-arrestin1, which is the more prevalent isoform of non-visual arrestins in vertebrates[24]. This can be achieved by using the parental HTL cells that solely contained the luciferase reporter controlled by the tetO7 promoter. One limitation to PRESTO-Tango is non-specific activation of the reporter promoter. Based on a tetracycline-dependent regulatory system (tet system), the tetracycline-responsive element (TRE) controls expression of the downstream luciferase

reporter. However, previous studies have demonstrated "leaky" expression of luciferase due to endogenous transcription factors[25, 26]. As a result, some compounds could activate the reporter independently of the β-arrestin2 recruitment or GPCR activation, increasing the number of false positives. Another issue that emerges, also common to other HTS methods, are "frequent hitters", promiscuous compounds that stimulate substantial responses in several targets[27]. Nonetheless, the PRESTO-Tango's parallel screening set-up facilitates identification of these artifacts, which can be further tested to confirm their effect on luciferase activity. Altogether, PRESTO-Tango has provided solid foundations for the study of arrestin recruitment to GPCRs, and in the larger scheme of drug discovery, as a utile GPCR ligand screening and deorphanization tool.

ACKNOWLEDGMENTS:

This work was supported by the Canadian Institutes of Health Research (CIHR grant #MOP142219).

DISCLOSURES:

The authours declare no competing interests.

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Figure 1

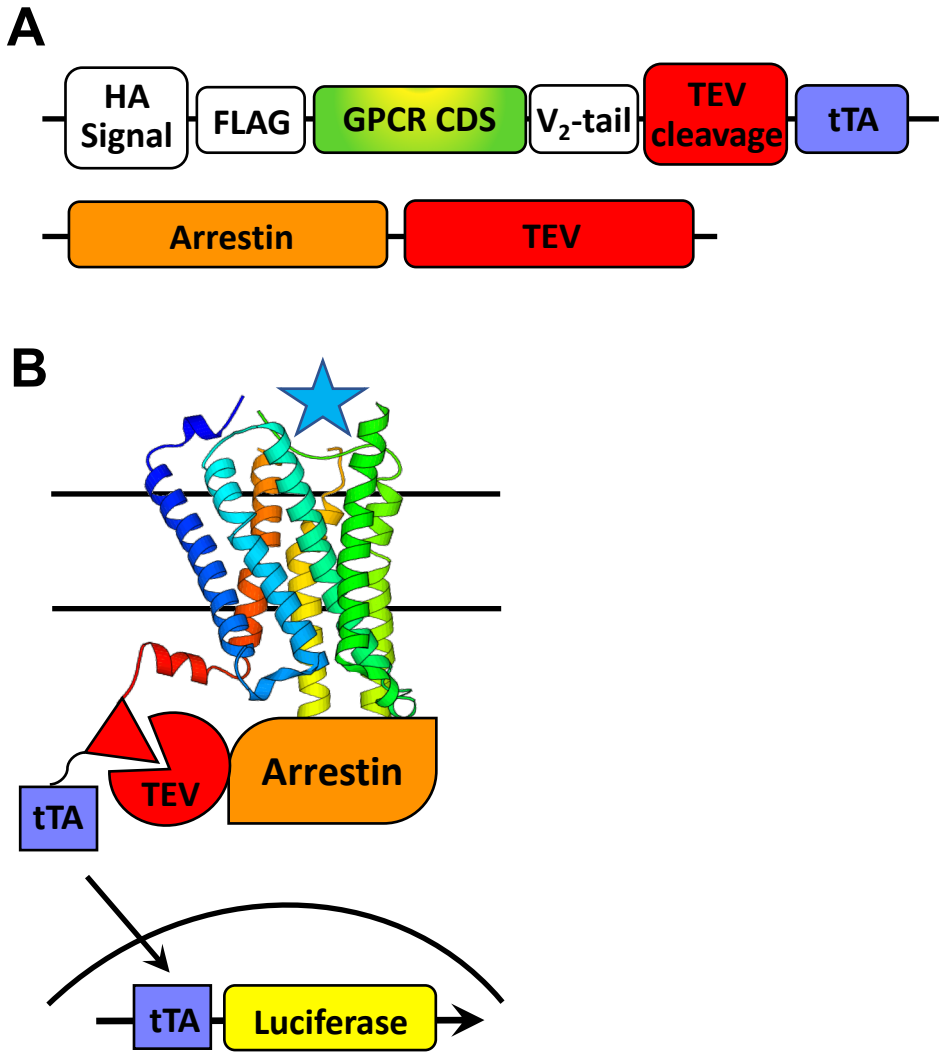

Figure 2

Click here to access/download;Figure;Fig2.pdf

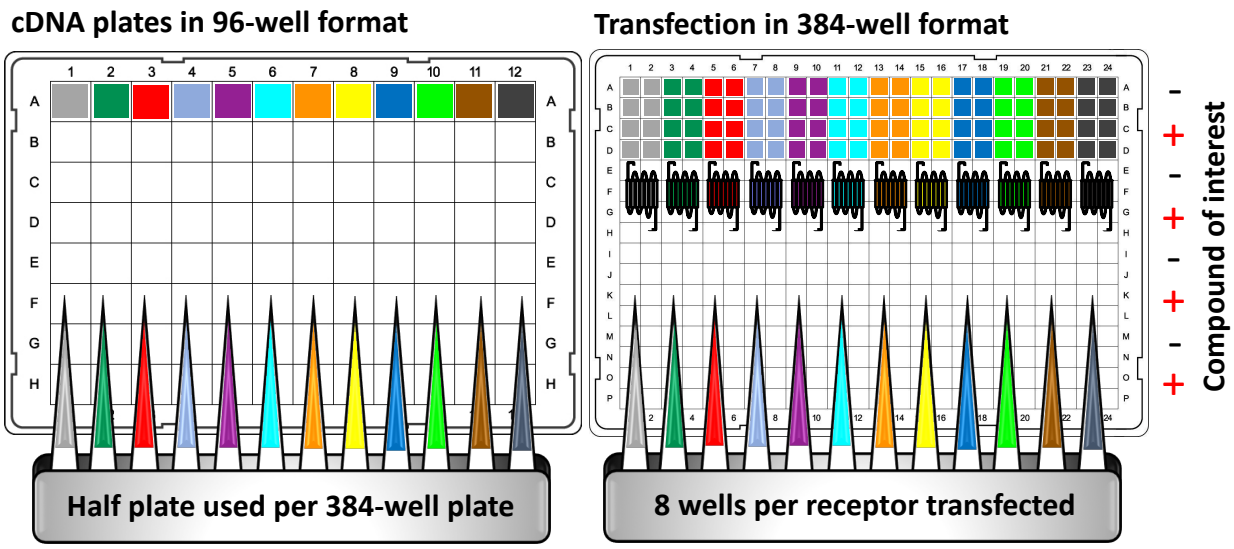

Figure 3

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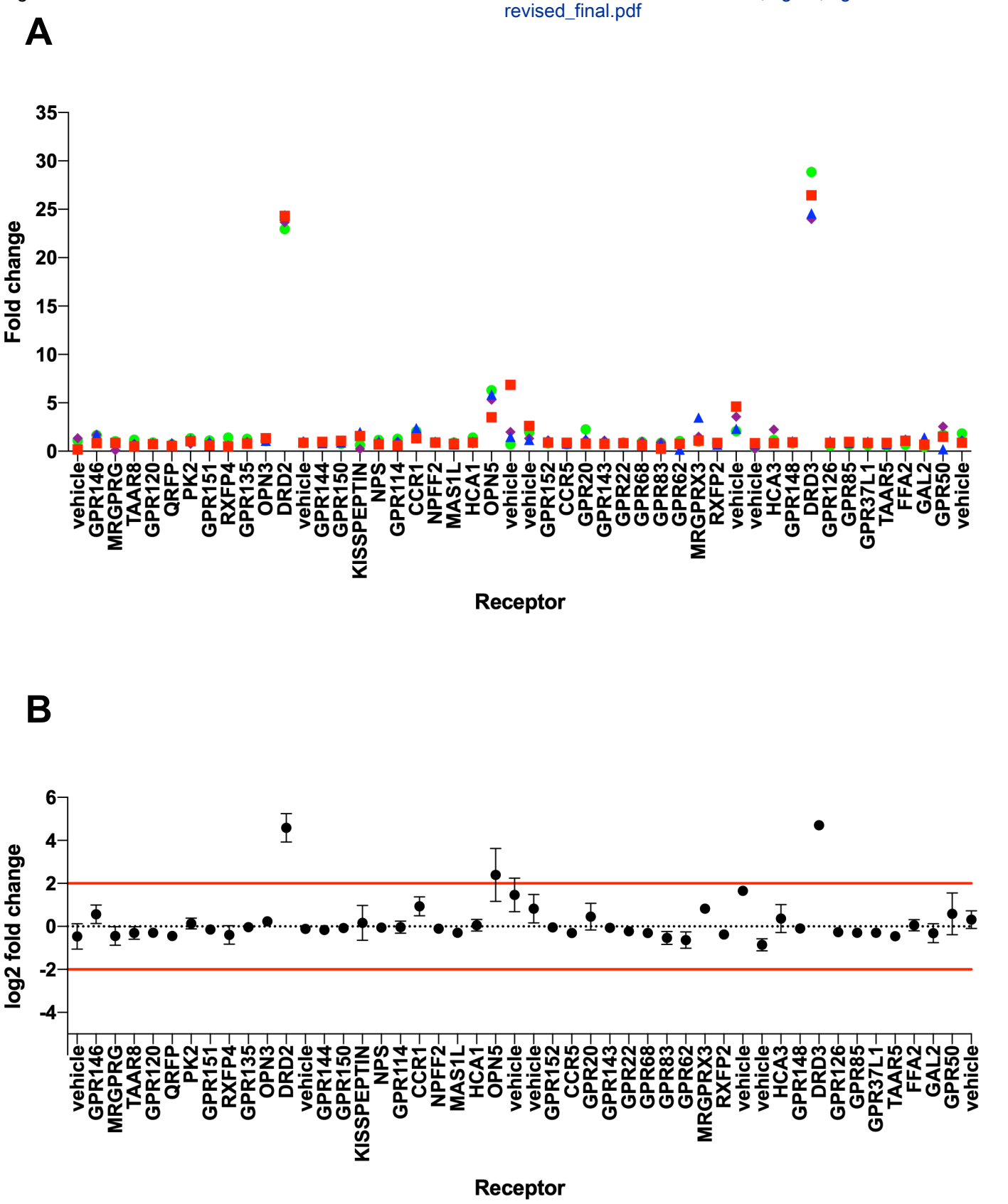


Figure 4

Click here to access/download;Figure;Fig4.pdf

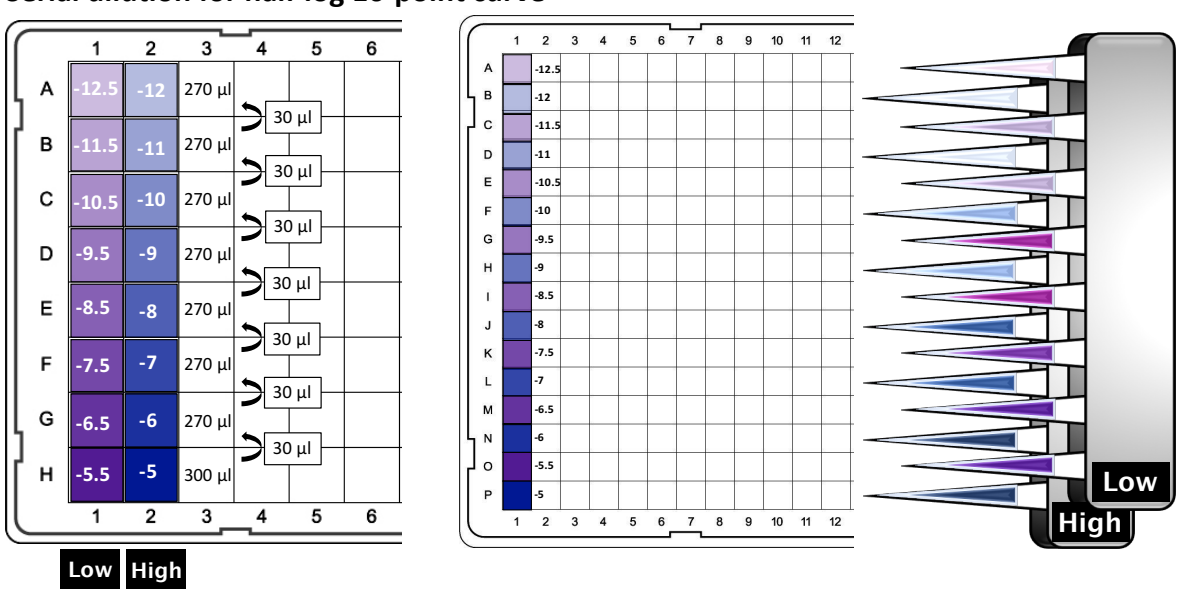
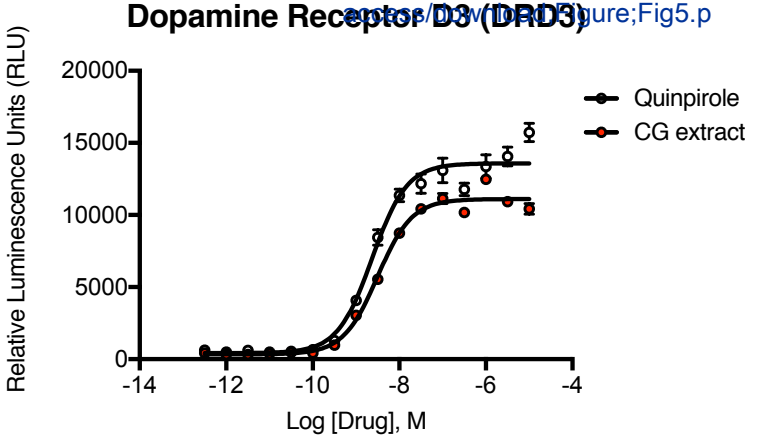


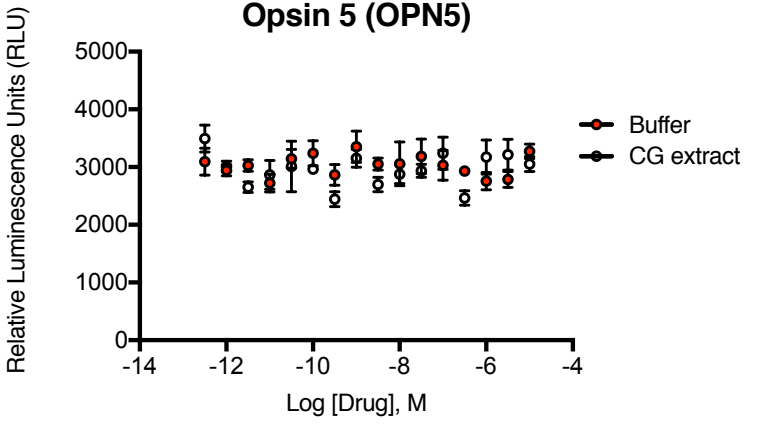
Figure 5

A



	Bottom	Top	LogEC50	EC50
Quinpirole	364.2	13666	-8.62	2.398e-009
CG extract	277.1	11175	-8.503	3.138e-009

B



Name of Material/ Equipment
384 Well Optical Bottom Plates, Polystyrene Polymer Base, Cell Culture Treated, BLACK, with lid, Sterile
384 Well Optical Bottom Plates, Polystyrene Polymer Base, Cell Culture Treated, White, with lid, Sterile
384 Well Round Bottom, Polypropylene, Non-Treated, Blue, non-sterile, without lid
Antibiotic-Antimycotic
D-Luciferin, sodium salt
DMEM with L-Glutamine, 4.5g/L Glucose and Sodium Pyruvate
Eppendorf Xplorer, 12-channel, variable, 15 – 300 µL
Eppendorf Xplorer, 12-channel, variable, 5 – 100 µL
Matrix Platemate 2x3
MicroBeta 1450 Wallac
Penicilin-Streptomycin
Poly-L-Lysine hydrobromide
Roth Lab PRESTO-Tango GPCR Kit

Company	Catalog Number	Comments/Description
NUNC	12-566	
NUNC	12-566-1	
ThermoFisher	12-565-390	
Wisent	450-115-EL	
GoldBio	LUCNA	
Corning	10-013-CV	
Eppendorf	4861000155	
Eppendorf	4861000139	
ThermoFisher	801-10001	
Perkin Elmer		
Wisent	450-201-EL	
Millipore-Sigma	P2636-500MG	
Addgene	Kit #1000000068	

uOttawa
Faculté de médecine
Faculty of Medicine

Ottawa, November 2, 2019

Dear Dr. Dsouza, JoVE editorial staff, and reviewers,

Thank you for your positive feedback regarding our manuscript entitled "Parallel Interrogation of β-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-Tango assay" (JoVE60823).

Below, we enumerate the changes we have made in this revision to our work and the responses we have made to the reviewers' requests.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors. Small errors were found, see tracking.

• **TITLE:** Please avoid abbreviations in the title where possible while meeting the 150 charaacter limit. Please focus the title on the highlighted portion of the protocol. The use of abbreviations in our title is necessary to meet the 150 character limit. More specifically, the name of the assay, the "PRESTO-Tango" was coined by the original developers (see Kroeze et al. 2015 Nat Struct Mol Bio), where PRESTO stands for Parallel Recepterom Expression and Screening via Transcriptional Output, and Tango is the name of the arrestin translocation transcriptional assay which they modified. In addition, GPCR is the conventional abbreviation for G-Protein Coupled Receptors, which should be recognized universally.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. The authors believe that the necessary material for the video has already been highlighted.

• **Protocol Highlight:**
1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next. See the tracking in the word documents, we removed and add highlighted steps to be more cohesive.
2) Notes cannot be filmed and should be excluded from highlighting. We removed the highlighting on the notes, see tracking.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. The authors have followed the suggested format of the discussion.

• **Figures:**
1) Fig 3 A, B: Please increase the axis tick label size to improve readability. Also remove the shadows from under the data points.
The authors have made the aforementioned changes.

2) Fig 3B: It is unclear why some of the error bars are laterally asymmetrical. Please use standard plotting plots.
Upon further investigation, it seems that our original Figure in Excel was plotting the standard error instead of the standard deviation, and we could not manually change this, nor resolve the issue of the laterally asymmetrical error bars. As such, we have remade this Figure in GraphPad Prism instead of Excel to resolve these issues.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
All Figures included in our manuscript are original and unpublished.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
The authors describe an assay apparently suitable for high-throughput based on earlier established and commercially available TANGO system, called PRESTO-TANGO. While the assay has its merits, it also has numerous limitations, which must be explicitly acknowledged.

Major Concerns:
Experimental issues
1. Only one of the two non-visual arrestins expressed in vertebrates is used: b-arrestin2. This subtype is a minor component (5-10%) of b-arrestin complement in most cells, with b-arrestin1 being the prevalent form (Neuroscience. 2002;109(3):421-36; J Neurochem. 2004 Dec;91(6):1404-16). The authors must comment on this limitation.

We commented on this briefly by mentioning that β-arrestin2 is the only GPCR interactor that can be monitored (lines 479-482). This is an inherent limitation of the Tango system developed by Barnea et al., and the PRESTO-Tango, being an extension of the original assay, carries this limitation as well. In order to investigate interactions with β-arrestin1, a new stable cell line would have to be created, which may be a possibility in the future.

To further highlight this issue, we also added the following to lines 493-496 (underlined): “Moreover, future engineered cell lines can be generated to express other TEV fusion genes to track other proteins besides β-arrestin2, specifically those that have been previously shown to interact or found in residence to GPCRs, such as 14-3-3, SAP97, and β-arrestin1, which is the more prevalent isoform of non-visual arrestins in vertebrates.”

2. FLIPR, where all GPCRs are forced to signal through calcium by the use of chimeric G protein a-subunits, is actually high throughput, so the statement on line 86 is false.

We agree that FLIPR is a HTS system; the absolute statement on line 86 was thus corrected to "most of them are at low-throughput".

3. The use of V2 C-terminus would invalidate the results: it is capable of engaging b-arrestins independently of the rest of the GPCR (Cell. 2016 Aug 11;166(4):907-919; Proc Natl Acad Sci U S A. 2017 Mar 7;114(10):2562-2567).

The V2 C-terminus tail was originally added to the Tango assay to increase basal β-arrestin2 recruitment. Of course, this does raise the issue that this could artificially enhance β-arrestin2 recruitment, resulting in an inaccurate signal output. However, since the V2 tail was added to all the receptors found in the PRESTO-Tango kit, any artificial increases in the Tango signal would still be proportional amongst receptors. Moreover, all drugs, including a reference, tested at the same receptor would alleviated the V2-tail effect.

While Barnea et al. did observe enhanced assay performance for multiple receptors upon addition of the V2 tail (e.g. κ-opioid receptor and the D2 dopamine receptor), it is important to note that it had no noticeable effect on the ligand specificity to the recipient receptor (Supplementary Fig. 7 and Supplementary Table 1 of Barnea et al. PNAS 2008 publication). Furthermore, PRESTO-Tango developers tested the effects of removing the V2 tail for some receptors and found variable results; notably, the removal of the V2 tail ***decreased*** the ligand-induced responses of some, e.g., the FFAR2 free fatty acid receptor (Supplementary Fig. 1f of Kroeze et al. Nat Struc Mol Bio 2015 publication), and ***had little effect*** on the ligand-induced responses of others e.g., the LTBR4 leukotriene receptor (Supplementary Fig. 1d of Kroeze et al. Nat Struc Mol Bio 2015 publication). To date, there is no complete and systematic study of including/excluding the V2 tail.

However, if desired, the modular constructs of the PRESTO-Tango have introduced restrictions sites flanking the V2 tail, allowing for its excision from the receptors. Of course, this is not a realistic option for the entire PRESTO-Tango library, but future users can modify a select number of receptors for their secondary screening purposes, if desired.

4. A general drawback of TANGO and all TANGO-based assays is high background. The authors should acknowledge that. The measures suggested on lines 431-447 cannot overcome this problem.

Compared to most assay based on resonance energy transfer, the PRESTO-TANGO assay has a better noise to signal ratio. This makes the PRESTO-TABGO assay, a better option for **screening**. The basal activity for GPCR is an intrinsic property toward b-arrestin recruitment. We cannot exclude that exogenous expression of some GPCR in HEK293 can exacerbated this effect, but for most of them, the signal window is good enough for signal detection followed agonist addition. Some orphan GPCRs seem to be constitutively active but this is out of the scope of this publication.

This being said, we found that serum starvation reduces the background level probably due to the leakiness of the TRE promoter use to develop the HTLA cells. This promoter is known to bind endogenous transcription factors that can 1- enhance TTA response or 2- activate the promoter independently of the TTA. In addition, the issue has also already been touched upon in lines 498-502. Besides the issue of high constitutive activity by some GPCRs, the leaky TRE promoter could also be a factor in the high background observed in the Tango-based assays.

The statement on line 438-441 has been changed from "this can be ***easily*** mitigated by ensuring serum starvation…" to (changes are underlined):

"Given that some GPCRs have high constitutive activity, one issue that may arise during screening is that a reduced dynamic range and a background signal that is higher than expected. This can be ***somewhat*** mitigated by ensuring that serum starvation is being performed with DMEM medium supplemented with 1% dFBS."

5. Another drawback of the assay is that it, just like parental TANGO assay, is a one-way street. Once released by even a random bystander encounter of b-arrestin with a GPCR, the transcription factor will do its job, yielding false positive signal. In contrast, biologically relevant G protein, GRK, and arrestin interactions with GPCRs are reversible and highly dynamic. This should be explicitly stated.

The authours agree that the PRESTO-Tango assay cannot truly capture the dynamics of GPCR- β-arrestin2 interactions as they would occur in a natural biological setting. As an endpoint reporter assay, PRESTO-Tango is unable to differentiate between specific and non-specific bystander reporter signals, and unlike real-time reporters, paint a rather static picture of the interaction. Nonetheless, this limitation is also a strength in that PRESTO-Tango is suitable for HTS to screen multiple targets against the GPCRome – this provides users an initial idea of potential ligand-receptor pairs worth studying in greater detail, which then undergo secondary profiling with Tango, as well as other orthologous assays which can confirm these interactions. Once positive hits have been identified, further experiments can be performed with real-time assays to study the reversible and dynamic changes in β-arrestin2 recruitment, such as Nanobit technology.
It is also important to note that the stimulation of luciferase reporter activity is not binary; for e.g. Tango-based assays are able to distinguish between levels of receptor activation by full agonists versus partial agonists, and these relative responses mirror data obtained from orthologous secondary messenger assays (Fig. 3C of Barnea et al. PNAS 2008 publication).

6. As b-arrestins preferentially bind active receptors, the authors cannot suggest a suitable positive control for orphan receptors. While this is also true for all other GPCR assays, this weakness cannot be overcome by proposed PRESTO-TANGO assay and should be acknowledged.
The following has been added (lines 475-478) to address this weakness: "However, as with all other GPCR assays, it must be acknowledged that there are no suitable positive controls for orphan receptors during secondary screening with the Tango assay. Nonetheless, potential positive hits can be identified if the output data can be fitted to a sigmoidal dose-response curve, with a computed signal window and EC50 value."

7. Some ligands of known GPCRs demonstrate bias: preferential recruitment of either G proteins or arrestins. This should be explicitly addressed. By definition, proposed assay would identify b-arrestin-biased ligands, but would miss ligands with significant G protein bias.
The authours have already addressed this point in lines 414-418: "In light of functional selectivity, a concept which suggests that different drug structures can elicit different receptor signaling cascades at a single receptor, comparing the activation of the G-protein pathway using G-protein dependent assays with β-arrestin recruitment using PRESTO-TANGO could provide cues for the designing lead compounds with reduced negative side effects."
In short, we have already stated that the PRESTO-Tango assay alone cannot capture the full picture of biased ligands – the results from PRESTO-Tango screenings should be compared with those obtained from G-protein dependent assays in order to identify the degree of ligand bias for one pathway over another; these tools would complement each other for functional selectivity studies.

8. Many GPCRs have relatively high constitutive activity, which reduces the dynamic range available for agonist screening. While this also hampers all other GPCR assays, this weakness cannot be overcome by proposed PRESTO-TANGO assay and should be acknowledged.
The authors acknowledge this weakness encounter by any assays with high constitutive activity. By having a large detection window, the PRESTO-TANGO somewhat alleviated this effect. Reducing the background level by serum starvation can somewhat mitigated this effect.
The following changes have been made to lines 438-441 to acknowledge this limitation (changes are underlined): "Given that some GPCRs have high constitutive activity, one issue that may arise during screening is that a reduced dynamic range and a background signal that is higher than expected. This can be somewhat mitigated by ensuring that serum starvation is being performed with DMEM medium supplemented with 1% dFBS."

Minor Concerns:
Presentation
9. Two systems of arrestin names are used. B-arrestin2 is also called arrestin-3, b-arrestin1 is called arrestin-2. The authors should provide translation.
The following distinction has been added (lines 91-92): "The most ubiquitously expressed isoforms of β-arrestin are the non-visual β-arrestin1 and β-arrestin2, also denoted as arrestin-2 and arrestin-3, respectively."

Reviewer #2:

The manuscript entitled " Parallel interrogation of b-Arrestin2 recruitment for ligand screening on a GPCR-wide scale using PRESTO-TANGO assay" gives a clear and quite detailed description of the already published assay that allows GPCR screening against a drug or an extract. Because this approach was already published, I wont, obviously, not comment on the assay limitation and therefore I have only few minor comments.

Point 1.3.4.: The authors should explain how cells were counted.
The authours felt that a distinction is not necessary, as we wanted to leave the choice to the users. Viable cells can be counted in any manner of their choosing, be it manually (our lab uses a hematocytometer to count the cells) or automatic measures (e.g. image-based counter, flow cytometers, etc.), without affecting the results of the Tango assay.

Point 6.2.3.: It is said to "dispense the 2ml solution dropwise onto the cells" but when I did the calculation it gave me 1.5ml: 500ul of TE/CaCl2 + 1ml of 2X Hepes buffer = 1.5ml; I guess that the 500ul missing is the DNA solution. The authors should clarify/correct that.
Thank you for pointing out this error. The ratios for the 100 mm dish (***500 µL of TE 0.1X/$CaCl_2$*** and 500 mL of HEPES) was jumbled with those of a 150 mm dish, which uses 1 mL of TE 0.1X/$CaCl_2$ and ***1 mL of HEPES)***. The following corrections (underlined) have been made to lines 259 and 265): "In a tube, add 500 µL of the TE/$CaCl^2$ solution to 10 µg of GPCR cDNA and vortex. Add 500 µL of 2X Hepes buffer solution in the tube, shake vigorously (do not vortex), and incubate for 1 minute……Immediately following the short incubation, dispense the 1 mL solution dropwise onto the cells."
Point 7.1.: second line, "excluding the last row" and not the first row.
Thank you for this correction – the appropriate changes have been made.

Figure and table legends, Figure 3, line 384, the sentence start by "The signal windows: (B) should be

(A) and (C) should (B). Also, on the same line, the authors used quinpirole as a positive control on their assay but the data is not present in Figure #3; the authors should explain why the data are absent from the graph or modify the graph accordingly.

The authors would like to point out that the positive control DRD2, which was stimulated by quinpirole (unlike the other receptors, which were stimulated by the CG extract) is present in Figure 3, specifically it is the 12th receptor from the left of the x-axis. It should be fairly easy to spot because apart from the other GPCRs that meet the hit thresholds (DRD3 and OPN5), it greatly surpasses the hit thresholds, i.e. it has a fold change of ~23 and log 2-fold of ~4.5.
The positive control DRD2 might have been missed because the font size of the axes was too small; the authours have modified the figure and increased the font size to make it easier to read.

Reviewer #3:
Manuscript Summary:
In "Parallel Interrogation of β-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-TANGO assay", Zeghal provide a step-by-step instruction on how to screen ligands ~ 350 GPCRs using the PRESTO-TANGO Kit. Aside from providing useful insights into the pros and cons of the assay, the authors further demonstrate its application via screening of a chromaffin granule extract, and subsequent identification of extract activity at the D3 Dopamine receptor.

Overall, the provided protocols are very clear to follow even in the absence of accompanying video, and the publication will greatly aid in implementing this very useful assay in the community. Overall, the manuscript reads well and provides sufficient insight into the assay and its usefulness. I only have very minor comments

Major Concerns:
N/A

Minor Concerns:
* The authors describe how the use of "starvation media" might decrease the background signal. Could the authors clarify why that reduces background signal. Also, is it possible that starvation of the cells could trigger a response/signal for some receptors? After all, the roles and mechanisms of many of the receptors in the TANGO kit are not known.
The serum starvation is used to synchronize the cells' cycles such that once the drug is introduced during starvation, it can act in the absence of proliferative signal. Therefore, compared to non-starved cells, there is diminished cell growth, which reduces the overall background signal. However, the dynamic range is greater for starved cells because there is better signal retention, as the reduced amount of growth factors in the starvation media increases the excitability of cells.
While the goal of serum starvation is to increase the dynamic range and make for a more prominent specific signal, in terms of starvation being the cause of activating a response itself for some receptors, this is unknown as the authors have not yet tested this possibility. However, we would be surprised if that were the case, as the luciferase reporter activity from Tango assay was designed such that is independent of activation of any endogenous cell signaling events that could confound receptor function.

* When comparing activation of DRD3 and OPN5, it is evident that OPN5 shows a very strong signal even in the absence of any ligand. I assume this would reflect the higher basal activity of this receptor.

Could the authors comment on whether they expect these differences to make some receptors more challenging to test in this assay?
The differences in constitutive activity of the receptors does indeed make some receptors more challenging to test in the PRESTO-Tango. As shown in the original publication by Kroeze et al., constitutive activity varied over a range of more than about 500-fold among the various GPCRs, with no apparent sequence-encoded patterns to explain high vs. low constitutive activity.
For receptors with high basal activity, for e.g. members of the serotonin and purinergic receptor families, this may reduce dynamic range observed; therefore the following suggestion on lines 438-440 have been proposed: "Given that some GPCRs have high constitutive activity, one issue that may arise during screening is a reduced dynamic range and a background signal that is higher than expected; this can be somewhat mitigated by ensuring that serum starvation is being performed with DMEM medium supplemented with 1% dFBS."
For receptors with low basal activity, for e.g. members of the adrenergic receptor families (see Supplementary Table 3 for the compiled list in the Kroeze et al. Nat Struc Mol Bio 2015 publication), the V2 tail might help increase the ligand-induced response. Moreover, the following has been added (lines 450-452): "In addition, receptor expression could also be improved by subcloning Tango receptor(s) of interest into lentiviral vectors and transducing HTLA cells, generating a HTLA-GPCR stable cell line."
* I also wonder about the caveats of universally using β-arrestin2. OPN5 signaling likely depends more on S-arrestin. I assume there is no cell line with protease-tagged β-arrestin1 or others available?
We commented on this briefly by mentioning that β-arrestin2 is the only GPCR interactor that can be monitored (lines 479-482). This is an inherent limitation of the Tango system developed by Barnea et al., and the PRESTO-Tango, being an extension of the original assay, carries this limitation as well. The reviewer is correct in assuming that to investigate interactions with β-arrestin1, a new stable cell line would have to be created, which may be a possibility in the future.
To further highlight this issue, we also added the following to lines 493-496 (underlined): "Moreover, future engineered cell lines can be generated to express other TEV fusion genes to track other proteins besides β-arrestin2, specifically those that have been previously shown to interact or found in residence to GPCRs, such as 14-3-3, SAP97, and β-arrestin1, which is the more prevalent isoform of non-visual arrestins in vertebrates."

* 99: "introduction of three exogenous genetic elements:" Maybe the authors should also point out that a V2-tail was introduced. I assume that is to boost the signal?
The following has been added to highlight the addition of a V2-tail on lines 101-106 (changes are underlined): "…a tetracycline transactivator (tTA) that is tethered to a GPCR via a tobacco etch virus protease cleavage site (TEVcs) and is preceded by a sequence from the C-terminus of the V2 vasopressin receptor (V2 tail) to promote arrestin recruitment, and a reporter luciferase gene whose transcription is triggered by the tTA transcription factor translocation to the nucleus, which is freed from the membrane anchoring following β-arrestin2 recruitment."
Indeed, the V2 C-terminus tail was originally added to the Tango assay to promote β-arrestin2 recruitment. However, while Barnea et al. did observe enhanced assay performance for multiple receptors upon addition of the V2 tail (e.g. κ-opioid receptor and the D2 dopamine receptor), PRESTO-Tango developers tested the effects of removing the V2 tail for some receptors and found variable results; notably, the removal of the V2 tail ***decreased*** the ligand-induced responses of some, e.g., the FFAR2 free fatty acid receptor (Supplementary Fig. 1f of Kroeze et al. Nat Struc Mol Bio 2015 publication), and ***had little effect*** on the ligand-induced responses of others e.g., the LTBR4 leukotriene

receptor (Supplementary Fig. 1d of Kroeze et al. Nat Struc Mol Bio 2015 publication). To date, there is no complete and systematic study of including/excluding the V2 tail.
However, if desired, the modular constructs of the PRESTO-Tango have introduced restrictions sites flanking the V2 tail, allowing for its excision from the receptors. Of course, this is not a realistic option for the entire PRESTO-Tango library, but future users can modify a select number of receptors for their secondary screening purposes, if desired.

* Lastly, have the authors ever observed indirect activation, i.e. activation of an endogeneous HTLA receptor, kinase, or other effector that would lead to activation of the tTA-tagged receptor?
The original developers of the Tango assay ensured that the luciferase reporter activity is independent of activation of any endogenous cell signaling events that could confound receptor function. More specifically, they compared Tango with an assay that monitors receptor-mediated elevations in intracellular calcium. The Tango assay was performed with the Vasopression V2 receptor in HEK293 cells that express endogenous muscarinic acetylcholine and purinergic P2Y receptors. While intracellular calcium assays revealed a response to vasopressin, carbachol (agonist for muscarinic receptors) and ATP (agonist for purinergic receptors), reporter activity in the Tango assay was only induced by vasopressin, demonstrating that only the exogenous Tango element introduced affected the signal response, without interference from endogenous pathways.

Small Errors:
* 31: increased awareness of several other G protein-independent pathway
Change to "increased awareness of several other G protein-independent pathways"
* 172: in the pcDNA3.1 Neomycin plasmid. The PRESTO-TANGO GPCR Kit consists of 4 96-well plates,
173: which include 80 GPCRs,
Change to "which include 80 GPCRs each,"
* 435: may result in erroneous calculated fold changes.
Change to "may result in erroneously calculated fold changes."
* Table of materials: Antibio-Antimycotique
Change to English term
Thank you for all of your corrections, they are greatly appreciated – the changes have been made accordingly.

Reviewer #4:
Manuscript Summary:
The protocol has been written thoroughly. The method highlights the high-throughput ligand screening for orphan receptors using the pre-validated Presto-Tango system. The introduction part is sufficient to explain the rationale for using the Tango system. However, a few queries, if addressed, will be highly beneficial for the improvement of the manuscript.

Major Concerns:
None

Minor Concerns:
1. Please include a brief workflow of the current protocol in the introduction section, preferably as the last paragraph.

The authours think that since the introduction section is fairly lengthy already and that the PRESTO-Tango method is also quite detailed, a brief workflow might not be necessary - we believe the additional video footage, along with our exhaustive step-by-step method will be more than sufficient to help users understand how to perform the assay.

2. Please include the manufacturer's name in the list of materials as it eases the process of purchasing the same material used in the protocol.
It seems that the company and catalog numbers were separated from the list of materials/equipment names into two pages. The authours used the Excel template provided from JoVE, so we assume this issue will be fixed during the editing process.

3. For HTLA cell maintenance, please provide the optimal cell passage numbers for best results and a caution note related to maximum passage usage.
The following changes were added to lines 144-150 (underlined): "Culture HTLA cells in 150 mm dishes and pass cells twice a week at a dilution factor of 1:10, with optimal cell passage number between 5-25. Ensure that a sufficient number of 150 mm dishes are confluent the day of 384-well plate seeding, depending on the scale of the primary screen.
NOTE: Usage of HTLA cells greater than passage 25 may result in reduced viability, yielding suboptimal results."

4. In the protocol section 1.2.1, authors have mentioned that HTLA cells were maintained in 5% FBS and 5% bovine calf serum containing DMEM. Is it essential to use both of them? Because we have been using FBS for HTLA maintenance and it works fine. I have primarily raised this concern because researchers love to adhere to a standardized protocol and what if labs do not use the latter one. So, it would be better to carefully draft a minimalistic and generalized protocol which can be followed by everyone and indicate which reagents are optional for use.
The authors would like to clarify that it is not essential to use both FBS and BCS. In literature, HEK293 cells (from which HTLA cells are derived) have been successfully maintained with 10% FBS and 10% BCS. In fact, the original PRESTO-Tango developers used 10% FBS for their HTLA cells, which seems to be the most common choice for serum. Our lab uses a mixture of both 5% FBS and 5% BCS, to reduce cost and because no significant changes were observed compared to 10% FBS.

5. Regarding DNA transfection, several labs use different transfection methods which have been already established in their labs. The calcium phosphate transfection method is itself a tricky one which requires several rounds of optimization. Therefore, it would be good if authors can provide optimal ratios of DNA: transfection reagent, which can generally be applied for different transfection methods.
The authours would like to point out that the amount of DNA, TE buffer, $CaCl_2$, and HEPES buffer have already been optimized by our lab and these values have been specified for 384 well plates (section 2 of the Methods) and for 100 mm plates (section 6 of the Methods). Of course, these ratios can be adjusted to fit any cell culture vessel volume using the values provided.
For example, to determine the values for a 6-well dish: Given that a 100 mm dish typically holds 10 mL of media while a 6-well holds 2 mL, the amount of DNA and transfection reagent volumes listed for the 100 mm dish can be divided by a factor of 5 (10 mL/2mL=5), resulting in 2 ug DNA, 90 uL TE, 10 uL CaCl2, and 100 uL HEPES for one 6-well.
Although we provide explanations on how to use the calcium phosphate transfection, the authours encourage labs to use the transfection methods which they are most comfortable with (e.g. PEI, Lipofectamine, Fugene, etc.), as long as there is high transfection efficiency and cell viability.

6. Receptor expression level has not been discussed. Do all receptors have the same expression levels? If not, then heterogeneity in receptor expression can lead to anomalies in data interpretation. Alternatively, receptors in the Tango library have a FLAG-tag on them. So, it is easy to check for their expression by ELISA-based approaches using conjugated anti-FLAG antibodies.
The issue of receptor expression level has been somewhat mentioned in lines 442-444: "Undetectable or low signals (assuming a response is expected) can be explained in a number of ways, namely ***poor expression of GPCR(s) in HTLA cells***, the biological activity of the compound is lost rendering it inefficacious, or the receptor(s) in question do not intrinsically recruit β-arrestin2." We have also added the following suggestion to increase receptor expression , which seems to be the major concern (lines 450-452): "In addition, receptor expression could also be improved by subcloning Tango receptor(s) of interest into lentiviral vectors and transducing HTLA cells, generating a HTLA-GPCR stable cell line".
The authours confirm that receptor expression levels do vary, but minimally – the original developers of the PRESTO-Tango transfected all the GPCRs and examined by anti-FLAG immunofluorescence for both total and surface expression. As shown in Supplementary Table 2 in the Kroeze et al. publication, 302 out of 315 receptors were surface-expressed, while those that were not efficiently surface-expressed could be visualized in permeabilized cells.
As mentioned by the reviewers, users can monitor cell surface expression by ELISA-based approaches using conjugated anti-FLAG antibodies, if they see fit.

7. Why is FBS used in the starvation medium? Although dialyzed, it still might contain large-molecule agonist/antagonist which might interfere with the results.
Dialyzed FBS is used over serum-free starvation mediums as it was finded that a small amount of FBS helps prevent cells from becoming too stressed, whilst still ensuring a prominent reporter signal.
The authours do agree that 1% dFBS might still contain larger molecules which could activate endogenous receptors, but the luciferase reporter activity of Tango is independent of activation of any endogenous cell signaling events that could confound receptor function. More specifically, Barnea et al. was performed the Tango assay with the Vasopression V2 receptor in HEK293 cells that express endogenous muscarinic acetylcholine and purinergic P2Y receptors, compared with an assay that monitors receptor-mediated elevations in intracellular calcium. While intracellular calcium assays revealed a response to vasopressin, carbachol (agonist for muscarinic receptors) and ATP (agonist for purinergic receptors), reporter activity in the Tango assay was only induced by vasopressin, demonstrating that only the exogenous Tango element introduced affected the signal response, without interference from endogenous pathways.
As such, the authours are confident this small amount of serum constituents will not interfere with the results.

8. Why have longer incubation times with ligands been kept? As luciferase reporter assays are very sensitive to expression levels of the protein, more prolonged incubation may cause a decrease in the receptor expression level. I wonder if authors have tried to optimize the duration of ligand incubation? In our lab, we incubate the cells with the ligand for 4-8 hours depending upon the receptor in use.
The developers of the PRESTO-Tango have shown examples of the effect of the length of agonist exposure on response (Supplementary Fig 5 of Kroeze et al. Nat Struc Mol Bio 2015 publication). Unfortunately, this has not been systematically studied with all GPCRs in the PRESTO-Tango, as it would require a substantial amount of work. Overall, it seems that relatively brief exposures with ligands (as short as 15 mins) are sufficient to detect responses in the Tango assay, however the minimum amount of time needed would have to be optimized for each target. Suffice to say, the Tango reporter assay relies on the amplification of the luminescence signal by accumulated luciferase protein

within cells, so our lab's past experience has found that overnight incubation (16-24 hours) allows for maximum signal amplification.
For users who want to keep ligand incubation times as short/physiologically relevant as possible, Kroeze et al. state that one or two hours is generally sufficient, provided that the signal is amplified overnight.
9. Under the discussion section for further improvement of this technique, authors, if they wish, can also suggest the generation of GPCR lentiviral libraries for efficient transduction in mammalian cells. Use of lentiviral libraries might overcome the barriers of transfection inefficiency and receptor expression.
Unfortunately, converting all Tango receptor constructs into a lentiviral expression system library would require a substantial amount of work. However, this is definitely an option for users after they have identified a select number of receptors to study in greater depth during secondary and higher-tier screening purposes. We had added this suggestion in line 449: "In addition, receptor expression could also be improved by subcloning Tango receptor(s) of interest into lentiviral vectors and transducing HTLA cells, generating a HTLA-GPCR stable cell line."

With these additional data and revisions to the manuscript text, we hope that this body of work will be accepted for publication in *JoVE*. We look forward to your scientific and editorial feedback.

Sincerely,

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Title of Article:	Parallel Interrogation of β-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-TANGO assay
Author(s):	Manel Zeghal, Genevieve Laroche, Patrick M. Giguere

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