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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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