The luminescent HiBiT peptide enables selective quantitation of G protein–coupled receptor ligand engagement and internalization in living cells

G protein–coupled receptors (GPCRs) are prominent targets to new therapeutics for a range of diseases. Comprehensive assessments of their cellular interactions with bioactive compounds, particularly in a kinetic format, are imperative to the development of drugs with improved efficacy. Hence, we developed complementary cellular assays that enable equilibrium and real-time analyses of GPCR ligand engagement and consequent activation, measured as receptor internalization. These assays utilize GPCRs genetically fused to an N-terminal HiBiT peptide (1.3 kDa), which produces bright luminescence upon high-affinity complementation with LgBiT, an 18-kDa subunit derived from NanoLuc. The cell impermeability of LgBiT limits signal detection to the cell surface and enables measurements of ligand-induced internalization through changes in cell-surface receptor density. In addition, bioluminescent resonance energy transfer is used to quantify dynamic interactions between ligands and their cognate HiBiT-tagged GPCRs through competitive binding with fluorescent tracers. The sensitivity and dynamic range of these assays benefit from the specificity of bioluminescent resonance energy transfer and the high signal intensity of HiBiT/LgBiT without background luminescence from receptors present in intracellular compartments. These features allow analyses of challenging interactions having low selectivity or affinity and enable studies using endogenously tagged receptors. Using the β-adrenergic receptor family as a model, we demonstrate the versatility of these assays by utilizing the same HiBiT construct in analyses of multiple aspects of GPCR pharmacology. We anticipate that this combination of target engagement and proximal functional readout will prove useful to the study of other GPCR families and the development of new therapeutics.

G protein–coupled receptors (GPCRs) remain an important target class for drug development. Despite only making up a small portion of the human genome, they are the target of almost a third of drug discovery efforts (1). Vital to these drug discovery campaigns is the development of simple, robust, and sensitive assays to gauge compound potency in a physiologically relevant environment. Evaluation of drug candidates intended to modulate GPCR function usually involves biochemical quantification of compound binding under equilibrium conditions (2). However, there is increasing interest in evaluation of binding dynamics under nonequilibrium conditions, which are postulated to be more relevant for predicting in vivo compound efficacy (3, 4). Additionally, because ligand-binding assays cannot differentiate agonists from antagonists, binding evaluations typically require complementary cell-based functional assays that monitor the biological consequences of receptor engagement.

In general, biochemical assays use radioligands in a competitive binding format to measure interactions between ligands and their unmodified cognate GPCRs (5). The specificity of these assays relies on the use of overexpressed GPCRs in combination with highly selective radioligands, which limits the versatility of each radioligand for engagement with multiple GPCRs (5). In addition, these nonhomogenous assays require separation of unbound from bound radioligand, which can be cumbersome and time-consuming, particularly for kinetic analyses (6). Consequently, potential loss of bound radioligand during separation, as well as limited kinetic resolution and throughput, can result in missing critical information, especially for interactions with fast association or dissociation.

A wide variety of complementary cell-based functional assays can be used to evaluate ligand-induced GPCR activation (7). Assays that measure GPCR internalization provide an attractive readout because they offer unambiguous information on receptor behavior separate from other downstream signaling events. These internalization assays often rely on antibody-based quantification of changes in cell-surface receptor density. However, such assessments by imaging, flow cytometry, or ELISA require complex multistep protocols (8), which limits the resolution and throughput of kinetic analyses.

Taken together, the field can benefit from a simple and robust cellular format that enables real-time measurements of GPCR ligand engagement and receptor internalization. Proximity binding assays relying on time-resolved (TR)-FRET or bioluminescent resonance energy transfer (BRET) offer promising alternatives to radioligand-binding assays (6, 9). These
assays typically use fluorescently labeled ligands (fluorescent tracers) in a competitive binding format to measure interactions between ligands and their cognate GPCRs that are genetically fused to an energy donor (i.e., a lanthanide-labeled protein tag or a luciferase). The high specificity of these approaches, which is governed by the proximity required for efficient energy transfer, allows for homogenous cell-based assays and real-time kinetic measurements.

Even though proximity binding assays provide number of advantages over radioligand binding, these approaches can be improved. TR-FRET requires prelabeling of the GPCR fusion with a lanthanide energy donor, adding an extra step to the workflow. In addition, TR-FRET measurements require external excitation of the energy donor, which upon repeated excitations can lead to photobleaching, a potential limitation for kinetic measurements (10, 11). In contrast, the BRET approach requires no external excitation or additional labeling of the GPCR fusion. Stoddart et al. (12) reported that GPCR target engagement assays using the bright NanoLuc luciferase as the energy donor enabled BRET evaluations previously impossible with other luciferases. Additional studies further demonstrated the feasibility of this BRET-based target engagement analysis for a few GPCRs (13–15). However, the use of full-length NanoLuc as a reporter results in signal generation regardless of NanoLuc’s cellular localization. This could potentially lead to an elevated background from NanoLuc-tagged receptors that are present in intracellular compartments and are unable to engage the ligand (16). Such interference from intracellular background signal could decrease overall assay performance and limit the capacity to detect interactions with low affinity and/or selectivity. Likewise, intracellular luminescence could also limit the sensitivity and dynamic range of NanoLuc–GPCR internalization assays in which a decrease in total luminescence corresponds to receptor endocytosis (17).

To this end, we sought to use GPCRs genetically fused to the newly described HiBiT tag, a small 11–amino acid peptide that can produce bright luminescence upon high-affinity complementation with LgBiT, an 18-kDa subunit derived from NanoLuc (18, 19). Generally, small tags like HiBiT are perceived to minimally interfere with protein function and trafficking to the cell surface. More importantly, because no luminescence is emitted without LgBiT complementation and LgBiT is membrane-impermeable (20), signal generation in end-point assays is limited to HiBiT-tagged receptors expressed on the cell surface. Elimination of background signal from receptors accumulated in intracellular compartments should increase the sensitivity and dynamic range of ligand-binding and receptor internalization analyses. Finally, the small size of HiBiT enables simple and efficient generation of endogenously tagged receptors in relevant cell models using CRISPR/Cas9-mediated genome editing (19). The sensitivity afforded by the HiBiT/LgBiT reporter allows analyses of those endogenous receptors (19), which are frequently expressed at low levels (21).

In this study, we introduce a versatile suite of assays utilizing HiBiT-tagged GPCRs for analysis of ligand-binding pharmacology and receptor internalization, in equilibrium and real time. Briefly, our BRET assay utilizes fluorescent tracers in a competitive binding format to quantify dynamic cell-surface interactions between ligands and their cognate HiBiT-tagged GPCRs (Fig. 1B). The second bioluminescent assay employs the same HiBiT-tagged GPCRs to monitor ligand-induced internalization through changes in cell-surface receptor density (Fig. 1A). We used the β-adrenergic receptor (β-AR) family to demonstrate the power of these assays utilizing the same HiBiT construct to gauge ligand-binding affinity and kinetics, allosteric regulation, and receptor internalization. The β2-AR has been extensively characterized for ligand-induced internalization (17, 22), as well as target engagement using radioligands (23, 24) and proximity-based approaches (12, 25), making it an excellent model for assay validation. Conversely, β1-AR and β3-AR have been studied almost exclusively in radioligand-binding assays (23, 26) and only at equilibrium. Using β3-AR as an initial model, we generated ligand binding and internalization analyses in transient and endogenous expression setups, which were in general agreement with reported values. These results not only validated but also demonstrated the sensitivity and robustness of our approach, particularly for analyses of endogenously tagged receptors. The elimination of intracellular background luminescence afforded by HiBiT/LgBiT allowed for further binding analyses across the entire β-AR family using a single fluorescent tracer exhibiting a wide range of affinities to those receptors. Furthermore, our study captured comprehensive kinetic evaluations for all β-AR receptors. These evaluations revealed for a panel of agonists and antagonists a direct correlation between binding affinity and association rate but not for binding affinity and residence time. We foresee these simple, homogeneous, cell-based assays being
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applied broadly to the study of other GPCR families and to evaluations of new drug candidates.

**Results**

**Measuring target engagement in transient and endogenous assay formats**

The sensitivity and robustness of a BRET target engagement assay stems from its capacity to reproducibly and specifically measure ligand binding across a range of expression levels and in different assay formats. To this end, we used β2-AR as a model to compare ligand binding at transient and endogenous GPCR expression setups. For transient expression we generated a DNA construct expressing β2-AR that is genetically fused to an N-terminal HiBiT tag. IL6 secretion signal sequence was incorporated upstream of HiBiT for efficient translocation to the cell surface (12). Additionally, to ensure HiBiT integrity upon signal peptide removal (27), a two amino acid valine–serine spacer (VS) was inserted between the signal sequence and HiBiT. For endogenous expression, we used CRISPR knock-in to tag β2-AR in PC3 cells with either VS–HiBiT or IL6–VS–HiBiT. We chose PC3, a human prostate cancer cell line expressing β2-AR, which was suggested to play a role in prostate cancer (28).

A BRET target engagement assay also requires a ligand modified with a fluorophore (fluorescent tracer), which serves as an energy acceptor. Accordingly, we appended the NanoBRET 590 dye to the potent β-adrenergic antagonist propranolol (propranolol-NB590) (Fig. 2A). We chose this red-emitting NanoBRET 590 dye (Ex = 576 nm; Em = 589 nm) because it is a well-suited energy acceptor to the HiBiT/LgBiT complementation reporter (29).

First, we used imaging experiments to verify specific engagement of propranolol-NB590 with HiBiT–β2-AR in endogenous and transient expression setups (Fig. 2B). Complementation with LgBiT, followed by treatment with propranolol-NB590 (20 μM), produced observable BRET. Subsequent treatment with excess unmodified propranolol (20 μM) resulted in a significant decrease in BRET. This attenuation in BRET confirmed specific and reversible interaction between propranolol-NB590 and HiBiT–β2-AR. The ability to carry out such analyses with transiently and endogenously expressed HiBiT–β2-AR demonstrated the sensitivity of this approach. Furthermore, the cell impermeability of LgBiT (20) suggests that signal originated only from HiBiT-tagged receptors localized to the cell surface.

We continued testing the capacity of the BRET assay to reproducibly quantify ligand engagement over a wide range of expression levels. Accordingly, HEK293 and PC3 cells were transfected with HiBiT–β2-AR construct serially diluted into promoterless DNA and then tested alongside the two endogenously tagged PC3 clones for their donor luminescence signals. The transiently transfected HEK293 and PC3 cells exhibited a range of luminescence, which was close to the signal originating
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from endogenously tagged receptors (1.5–2.5 × 10⁵ relative light units) at 1,000-fold or 10,000-fold plasmid DNA dilutions, respectively (Fig. S1). The same transient and endogenous set-ups were further evaluated for saturation binding using increasing concentrations of propranolol-NBS90 (10 pm to 1 μM). Similar binding constants (K_D) for propranolol-NBS90 were reproducibly derived from analyses of endogenously and transiently expressed HiBiT−β₂-AR having donor luminescence that is greater than the endogenous signal (Figs. S1 and S2). Analyses of transiently expressed HiBiT−β₂-AR, having donor luminescence that is similar to the endogenous one, derived comparable K_D values but with increased variability between biological replicates (S.E. > 10%). It is likely that homogeneous expression of endogenously tagged β₂-AR allows for reproducible BRET measurements at a lower donor luminescence signal. We opted to use in further experiments transfections with the highest plasmid DNA dilution that could deliver reliable BRET analysis (100- and 1,000-fold plasmid DNA dilutions for HEK293 and PC3, respectively). Notably, these transient expression conditions and the endogenously tagged PC3 clones yielded highly similar saturation and competitive binding analyses for propranolol-NBS90 (Fig. 2, C and E) and unmodified propranolol (Fig. 2, D and E), respectively. Together, these results demonstrate the capacity of this HiBiT/LgBiT approach to measure GPCR ligand engagement at low expression levels and to avoid overexpression that could lead to biological artifacts.

The endogenously tagged PC3 clones were further used to test for potential impact of HiBiT tagging on ligand binding and subsequent downstream signaling. To this end, these PC3 clones and unedited PC3 cells were transiently transfected with a CRE-Luc2P reporter construct. The transfected cells were subsequently tested for inhibition of isoproterenol-stimulated CRE signaling by increasing concentrations of either propranolol or propranolol-NBS90 (Fig. S3). All three cell populations displayed similar IC₅₀ values for propranolol and propranolol-NBS90−mediated inhibition of CRE signaling. These results imply that neither VS−HiBiT nor the IL6 signal sequence had significant impact on β₂-AR activity.

To further test the robustness of the HiBiT/LgBiT BRET target engagement assay, we expanded the analysis to a panel of unmodified agonists/antagonists and determined their binding constants (K_D) for β₂-AR. Endogenously tagged PC3 clones, as well as transiently transfected HEK293 and PC3 cells, were tested for competitive displacement of propranolol-NBS90 at a fixed EC₅₀ concentration by increasing concentrations (1 pm to 30 μM) of unmodified ligands (Fig. 3, A−D). Binding constants calculated according to the Cheng–Prusoff equation (30) revealed pKᵦ values (negative log of the Kᵦ) that were highly reproducible and in agreement with previously reported values (Fig. 3E and Table S1) (23, 31−33). We obtained the same rank order affinities from endogenous and transient expression set-ups in different cell backgrounds, further demonstrating the reliability of the assay. Because the two endogenously tagged PC3 clones exhibited similar activity in signaling and ligand-binding experiments, only the HiBiT−VS−β₂-AR clone was carried for further analysis.

Comparing HiBiT/LgBiT versus NanoLuc as the BRET energy donor

To investigate the benefits afforded by HiBiT/LgBiT as the BRET energy donor, we compared it with NanoLuc and extended the analysis to additional members of the β-adrenergic family. HEK293 cells transfected with serial dilutions of

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**Figure 3.** Binding affinities for β₂-AR. A−D, competitive displacement of propranolol-NBS90 by increasing concentrations of unmodified ligands for β₂-AR expressed transiently in HEK293 (A) and PC3 (B) or in endogenously tagged PC3 clones PC3 VS−HiBiT−β₂-AR (C) and PC3 IL6-VS−HiBiT−β₂-AR (D) cells. Error bars indicate S.E. E, unmodified ligand affinities (pKᵦ) in all cell types tested. Ligands are ranked based on affinity in HEK293 cells, with darker coloring indicating higher affinity. The data represent the means ± S.E. of four independent experiments. **A** HEK293 (transient) **B** PC3 (transient) **C** PC3 VS-HiBiT−β₂-AR **D** PC3 IL6-VS-HiBiT−β₂-AR

| Ligand       | HEK293 (transient) | PC3 (transient) | PC3 VS-HiBiT−β₂-AR | PC3 IL6-VS-HiBiT−β₂-AR |
|--------------|--------------------|-----------------|--------------------|-----------------------|
| timolol     | 10.1 ± 0.1         | 10.34 ± 0.09    | 10.7 ± 0.1         | 10.64 ± 0.08          |
| salmeterol  | 9.8 ± 0.1          | 10.2 ± 0.1      | 10.4 ± 0.1         | 10.31 ± 0.07          |
| pindolol    | 9.55 ± 0.05        | 10.05 ± 0.08    | 10.3 ± 0.1         | 10.4 ± 0.1            |
| propranolol | 9.49 ± 0.08        | 10.1 ± 0.1      | 10.4 ± 0.1         | 10.4 ± 0.1            |
| alpranolol  | 9.39 ± 0.09        | 10.04 ± 0.09    | 10.43 ± 0.09       | 10.50 ± 0.09          |
| carvedilol  | 9.3 ± 0.1           | 10.1 ± 0.1     | 10.5 ± 0.2         | 10.49 ± 0.09          |
| formoterol  | 8.32 ± 0.06        | 8.37 ± 0.05     | 8.5 ± 0.1          | 8.50 ± 0.09           |
| isoproterenol| 6.39 ± 0.08        | 6.6 ± 0.1       | 6.7 ± 0.1          | 6.7 ± 0.1             |
| xamoterol   | 6.37 ± 0.03        | 6.60 ± 0.09     | 6.9 ± 0.2          | 6.9 ± 0.2             |
| salbutamol  | 6.3 ± 0.1           | 6.54 ± 0.08     | 6.62 ± 0.08        | 6.6 ± 0.1             |

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**J. Biol. Chem. (2020) 295(15) 5124–5135 5127**
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β-AR constructs were tested for donor luminescence and saturation binding of propranolol-NB590 (10 pm-1 μM) (Fig. S4 and S5). We observed minimal variation in $K_D$ values for all three HiBiT-tagged β-ARs across a wide range of expression levels and related donor signals (Fig. S5). Such consistency was not observed in a similar analysis using NanoLuc-tagged receptors. We speculated that background luminescence originating from unoccupied intracellular NanoLuc–β-ARs decreased the sensitivity and robustness of the assay. Indeed, we found that increased expression levels of NanoLuc–β-ARs were associated with disproportional increase of intracellular luminescence (Fig. S6). Interference of intracellular luminescence with assay performance was clearly revealed for the low selectivity interaction between β₁-AR and propranolol-NB590. Accordingly, the $K_D$ for NanoLuc–β₃-AR could be determined with just one of the four transient expression condition tested, and this $K_D$ value was significantly higher than the one obtained using HiBiT–β₃-AR. Together, these results suggest that elimination of intracellular background afforded by the HiBiT/LgBiT reporter can translate into assays with overall greater sensitivity and robustness.

Binding analysis for the entire β-AR family in equilibrium and real time

The ability to use a single fluorescent tracer to survey an entire family of GPCRs reduces the burden of developing a specific tracer for each member of the family. Saturation binding analysis revealed that although propranolol-NB590 exhibited a wide range of affinities for HiBiT-tagged β-ARs (β₂-AR > β₁-AR > β₃-AR; Fig. 4, A and E), it was still adequate for additional binding analyses with the entire family.

Kinetic analyses can reveal additional binding characteristics that may be more relevant for predicting in vivo efficacy (6). We first determined the kinetic parameters of propranolol-NB590 for the entire β-AR family. To do this, we used varying concentrations of propranolol-NB590 and monitored binding over time to HiBiT–β-ARs in the presence and absence of excess unmodified propranolol (Fig. 4, B–D). Binding affinities derived from these kinetic analyses closely matched those from saturation binding experiments (Fig. 4E). At the same time, kinetic analyses revealed that association rates ($k_{on}$) for NanoLuc–β₁-AR > β₂-AR > β₃-AR) and to a lesser extent dissociation rates were correlated to the rank order potencies of propranolol-NB590 for members of the β-AR family (Fig. 4E).

With the binding of propranolol-NB590 characterized for all β-ARs, we proceeded to gather binding affinities, as well as association and dissociation rates, for the panel of agonists and antagonists. Fig. 5 shows a representative example of equilibrium and kinetic analyses for unmodified propranolol. Equilibrium $K_I$ values (Fig. 3A and Fig. S7) and rank order affinities for the three receptors were in general agreement with reported values (Fig. 3E and Tables S1 and S2) (23, 31, 33–37). The same unmodified ligands were then tested for their binding kinetics to the three β-ARs. Kinetic-derived binding affinities for unmodified ligands were generally comparable with affinities derived from equilibrium analyses (additional kinetic binding data shown in Figs. S8–S10 and Table S3). Correlation between equilibrium and kinetic derived binding affinities ($pK_I$ and $pK_{D,K}$, respectively) yielded regression lines with $R^2$ values of 0.97, 0.96, and 0.89 for β₁-AR, β₂-AR, and β₃-AR, respectively (Fig. S11). Furthermore, high-affinity $K_I$ values were generally associated with a high $k_{on}$ but not with a prolonged residence time (Fig. 6). Indeed, correlation plots between kinetic derived $pK_I$ and $k_{on}$ values yielded regression lines with $R^2$ values greater than 0.8, whereas $pK_{D,K}$ plotted against $k_{off}$ values showed very little correlation (Fig. S11).

Allosteric modulation

We next sought to determine whether our ligand engagement assay could measure the influence of an allosteric modulator on the affinity of a given receptor for an orthosteric agonist. Ahn et al. (38) recently highlighted a positive allosteric modulator (PAM) that is specific to β₂-AR (referred to as com-
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We tested the influence of this allosteric modulator (at 300 nm to 30 μM concentration range) on the competitive displacement of propranolol-NB590 by unmodified β2-AR agonists. The analysis revealed for all the tested agonists a significant leftward shift in the competitive displacement curves, indicating an increase in affinity for agonists with increasing concentrations of PAM (Fig. 7 and Fig. S12). As expected, we observed no PAM-induced modulation for partial agonists or antagonists (Fig. S13). Interestingly, the fold shifts in apparent IC50 values did not correlate with agonist rank order affinities (Fig. 7C). The greatest shift was observed for formoterol, which binds β2-AR with a pKᵢ of 8.3, whereas salmeterol, which binds with a pKᵢ of 9.8, exhibited the smallest shift. Similar analysis for β1-AR revealed very little PAM-induced modulation for isoproterenol (Fig. S14), which was consistent with the study of Ahn et al. (38).

Ligand-induced receptor internalization

Lastly, we tested the capacity of the HiBiT/LgBiT approach to monitor ligand-induced internalization, focusing on HiBiT–β₂-AR as a model. We first gauged whether transient expression level would influence the extent of ligand-induced internalization. HEK293 cells transiently transfected with a range of DNA dilutions were treated for 90 min with increasing concentrations of the agonist formoterol (70 pM to 1.2 μM). Subsequent
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complementation with LgBiT was used to monitor formoterol-induced changes in cell-surface receptor density, measured as decreased luminescence. We found that extent of maximal internalization was inversely correlated with expression levels (Fig. 8A). However, the concentration of 50% maximal internalization or Int50 remained consistent regardless of expression level. Still, because high cell-surface receptor density may affect rate and extent of internalization, we opted to use transfection condition (1,000-fold DNA dilution) that maximized the dynamic range of the internalization assay. Treatments with serial dilutions of the four agonists in our study revealed similar dose-dependent internalization profiles for transiently and endogenously expressed HiBiT–β2-AR, which were in general agreement with previously published studies (Fig. 8, B and C, and Table S4). To further verify that decreased luminescence corresponds to agonist-induced β2-AR internalization, we performed a similar analysis using antagonists and partial agonists from our study (Fig. S15). This analysis not only showed no luminescence loss but also revealed increased luminescence at high ligand concentrations, which is likely due to inhibition of basal, constitutive internalization. Taken together, these results correlate decreased luminescence with agonist-induced endocytosis. In addition, they suggest that the HiBiT/LgBiT approach could also be used to estimate the degree of basal GPCR internalization.

We also monitored internalization kinetics in transiently transfected HEK293 cells and in the endogenously tagged PC3 clone. Unlike end-point assays, kinetic analyses require pre-complementation of HiBiT/LgBiT, which is necessary for continual luminescence reads. These assays monitor real time changes in total luminescence and exploit the decrease in signal upon receptor translocation into endosomes (17). In these experiments, the cells were first treated with LgBiT and a protected HiBiT/LgBiT substrate, which is deprotected over time via a slow-rate ester hydrolysis catalyzed by cellular and serum esterases. The continual release of a cell-permeable substrate allowed for prolonged measurements without significant changes in luminescence because of substrate consumption or degradation. Upon agonist addition, rapid internalization was observed for both formoterol (Fig. 9, A and B, and Fig. S16) and isoproterenol (Fig. 9, C and D, and Fig. S16) at concentrations that exceeded the Int50 for each compound. Again, both setups exhibited similar extents of maximal internalization. However, we noticed differences in the internalization rates, with the endogenously tagged PC3 clone internalizing HiBiT–β2-AR nearly twice as fast as transiently transfected HEK293 cells (Table S5). It is possible that the homogeneous expression of HiBiT-tagged β2-AR in the endogenously tagged PC3 clone allowed detection of faster internalization.
To gain better understanding of real-time internalization measurements, we first verified that agonist-induced loss of luminescence corresponds to receptor endocytosis. We showed that pretreatment with a competing antagonist (propranolol) or dynamin inhibitors (MiTMAB and OcTMAB), two independent approaches known to inhibit receptor internalization, eliminated or significantly reduced the loss of luminescence, respectively (Fig. S17, A and B). We speculated that the decreased luminescence upon endosomal trafficking is caused by lower pH and/or rapid substrate consumption in the microenvironment of endosomes. Biochemical analysis further substantiated the influence of pH on luminescence signal (Fig. S18). It also suggested that the pH profile of HiBiT/LgBiT luminescence is well-suited to reveal receptor translocation from neutral pH to lower pH of endosome, as well as receptor recycling back to the cell surface (Fig. S17C).

**Discussion**

In this study we introduced a versatile cellular platform that takes advantage of the HiBiT/LgBiT complementation reporter to provide robust analyses of GPCR ligand engagement and receptor activation measured as internalization. Using the β-AR family as a model, we demonstrated the capacity of these assays to survey with the same HiBiT-tagged receptor multiple aspects of GPCR–drug interactions, including binding affinity and kinetics, allosteric regulation, and receptor internalization. Together, such complementary assessments have the potential to advance GPCR research and to facilitate development of new drugs with improved efficacy and safety.

These assays utilize GPCRs tagged with a small HiBiT peptide that had no significant influence on β2-AR activity in a phenotypic assay and is not expected to interfere with the function and translocation of other GPCRs. The sensitivity and large dynamic range of these assays stem from the bright luminescence of HiBiT/LgBiT and the cell impermeability of LgBiT, which limits signal generation to the cell surface and eliminates background luminescence originating from unoccupied receptors accumulated in intracellular compartments. Such sensitivity and dynamic range are necessary not only for analysis of binding interactions with low affinity and/or low selectivity but also for quantification of limited receptor internalization. These features also enable analyses at relatively low expression levels, which minimize biological artifacts related to overexpression. The high sensitivity of these assays was further demonstrated by the robust measurements of ligand binding and internalization using HiBiT–β2-AR expressed from an endogenous locus at native low levels. Such sensitivity can potentially permit evaluations of drug candidates in disease relevant cell models under native GPCR expression levels.

BRET specificity coupled with sensitivity and inherent cell-surface localization of HiBiT/LgBiT allows quantification of binding interactions with selective GPCRs on the surface of living cells. The same rank order potencies of ligands for β2-AR obtained from endogenous and transient setups in different cell backgrounds demonstrate the reliability of the assay. Still, we note subtle ligand affinity differences between cell types that may be due to variations in protein expression, membrane components, or cell physiology. Other studies have shown that expression host differences such as membrane lipid content and G protein availability can influence GPCR behavior (39, 40). Further work is required to understand cell-specific facets that could influence such interactions.

The ligand-binding analysis was readily expanded to additional members of the β-AR family using a single fluorescent ligand exhibiting a range of affinities for those receptors. β1-AR and β2-AR have been significantly less studied than β3-AR, and the HiBiT/LgBiT approach allowed systematic analyses of binding properties for both receptors. Although propranolol-NBS90 exhibited lower affinity and selectivity for β3-AR, consistent binding constants were determined across a range of expression levels. Such consistency and dynamic range could not be achieved with a comparable NanoLuc fusion, presumably because of interference by background luminescence originating from intracellular NanoLuc–β3-AR. These results clearly demonstrated the inherent benefit of the HiBiT/LgBiT approach for analysis of low affinity and/or low selectivity interactions. We anticipate that HiBiT/LgBiT will likely facilitate the development of GPCR-binding assays utilizing promiscuous fluorescent tracers that target multiple GPCRs from the same family or different families. Such tracer versatility could facilitate a multitude of GPCR-binding assays while reducing the burden of developing a large number of highly selective tracers.

Kinetic analyses are emerging as important means to understand ligand-binding pharmacology and potency (6). The HiBiT/LgBiT BRET target engagement assay facilitates real-time kinetic analysis in a live cell format. Our kinetic study revealed for all tested ligands a general correlation between binding affinity and $k_{on}$. This observation is in agreement with work by Sykes and Charlton (24), who established a similar relationship for clinically used β2-AR agonists. Our work further extends the trend to the tested antagonists and to other receptors in the β-AR family. It was suggested that higher $k_{on}$ rates may lead to faster drug rebinding, which in turn would influence in vivo efficacy (41). The ability to readily monitor such kinetic parameters might prove valuable for drug screenings.

There is a significant amount of interest in allosteric modulators of GPCRs. These modulators bind allosteric pockets, which are often protein-specific. Therefore, allosteric drugs may be able to distinguish more readily between GPCR family members than orthosteric drugs (42). We demonstrated in this study that the HiBiT/LgBiT BRET engagement assay is suitable to probe allosteric modulations. Although the PAM used in this study is predicted to bind an intracellular pocket distant from the HiBiT tag (43), the BRET approach still captured PAM-induced modulations of β2-AR affinity for agonists, which were comparable with those observed in radioligand-binding assays (38). We anticipate that the simplicity of the HiBiT/LgBiT assay will help with further development of allosteric modulators.

Agonist-induced receptor internalization is a major autoregulatory mechanism for GPCR function (44). The HiBiT/LgBiT internalization assays facilitate simple luminescence measurements of changes in cell-surface density of HiBiT-tagged GPCRs. The bright luminescence and inherent cell-surface localization of HiBiT/LgBiT allow quantification of end-point and real-time internalization with high degree of sensitivity and
large dynamic range. Using our end-point assay, we generated internalization analysis for β2-AR that was comparable with a published NanoLuc-based analysis with respect to rank order internalization profile (17). However, we note that the HiBiT–β2-AR construct provided significantly greater dynamic range than was observed with NanoLuc. By adding LgBiT at the end of the assay, signal generation is limited to the cell surface, producing an accurate profile of HiBiT-tagged GPCR remaining on the cell surface.

Real-time measurements of GPCR internalization exploit the decrease in luminescence upon endosomal trafficking, presumably because of the lower pH environment of endosomes. It worth considering that rapid exhaustion of available substrate by elevated reporter levels in the microenvironment of endosomes may further decrease the signal. In this kinetic assay, the extent of internalization is less apparent, probably because of precomplementation with LgBiT, which is necessary for continual luminescence measurements. Still, HiBiT/LgBiT likely provides a greater dynamic range than NanoLuc because its intracellular signal is not only restricted to endosomes but is also more sensitive to low pH environments (Fig. S18). In addition, this simple HiBiT/LgBiT method generated real-time internalization profiles that correlated closely with those produced via antibody detection but with no protein overexpression, cell fixing, or limited time points (8, 22).

Interestingly, we note that the PAM-dependent increase in β2-AR affinity for agonists correlated with the extent of internalization induced by those agonists. Agonists exhibiting a larger fold shift in IC50 values displayed greater receptor internalization. Salmeterol in particular has a unique receptor activation profile relative to other agonists, likely because of its lipophilicity, which reduces the recruitment of β-arrestin for internalization (22, 45). This unique ligand-binding mode may in turn influence the ability of the PAM to shift salmeterol’s binding behavior. Several studies have similarly shown that allosteric modulators can have an impact on GPCR internalization, whereas the PAM-induced increase in affinity for agonist correlated with an increased rate or extent of endocytosis (46–50).

In conclusion, our data show the broad utility of the HiBiT/LgBiT approach in furthering the study of GPCR pharmacology. We note that related assays utilizing similar recombinant complemented HiBiT/LgBiT have recently been introduced, emphasizing the power of HiBiT-tagged GPCRs in reducing background from intracellular luminescence (20, 51). The assays highlighted herein are simple, versatile, and robust tools that capitalize on a luminescent HiBiT peptide. Furthermore, these assays could easily be adapted to high-throughput evaluations of drug candidates. We foresee this approach combining quantitative assessment of GPCR engagement with functional internalization readout being extended to other GPCR families in due time.

Experimental procedures

Reagents and cell culture

Unmodified compounds and additional chemicals were purchased from Tocris, Sigma–Aldrich or Abcam. HEK293, HeLa, and PC3 cells were obtained from ATCC and cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Gibco) or Ham’s F-12K (Kaighn’s) medium (Gibco) containing 10% fetal bovine serum (Seradigm) and 100 units/ml of penicillin–streptomycin (Gibco).

Endogenous tagging using CRISPR/Cas9

Ribonucleoprotein complexes comprising synthetic guide RNA, purified Cas9, and synthetic single-stranded oligodeoxynucleotide donor DNA were prepared as previously described (19). The synthetic single-stranded oligodeoxynucleotide donor DNA sequence included the IL6 signal sequence, as well as VS–HiBiT, or VS–HiBiT alone. Clonal populations were generated by sorting single edited live cells into a 96-well plate using FACS Melody cell sorter (Becton Dickinson). Following 3 weeks of outgrowth, clonal populations were screened for luminescence. Genomic DNA samples for the top 12 clones were collected using the Wizard Genomic DNA purification kit (Promega) and analyzed by digital droplet PCR (ddPCR). Properly edited clones identified by ddPCR were also confirmed by Sanger sequencing, performed as previously described (52).

Additional details on DNA analysis can be found in the supporting information.

Construction of expression constructs

cDNA for human ADRB1 (P08588.2), ADRB2 (AAA88017.1), and ADRB3 (NP_000016.1) were obtained from Kazusa DNA Research Institute (Chiba, Japan) and subcloned into a modified pF5 CMV-neo Flexi vector (Promega) using the Flexi cloning system (Promega). The pF5 vector was modified to enable the generation GPCR constructs tagged at their N termini with either NanoLuc or HiBiT tag. NanoLuc vectors contained NanoLuc with an N-terminal IL6 secretion tag (MNSFSTSAFGPVAFSLGLLLPVPAFAP), followed by a Gly-Ser-Ser-Gly (GSSG) linker. HiBiT vectors contained the IL6 tag, followed by a VS linker, a HiBiT tag, and a 2× GSSG linker.

Cell transfection and manipulation

Transfections for cell-based assays were carried out in HEK293 (ATCC) or PC3 cells (ATCC) using Viafect (Promega) at either a 1:3 (HEK293) or 1:4 (PC3) DNA/transfection reagent ratio. Generally, DNA encoding HiBiT fusion was diluted 1:100 (HEK293) or 1:1,000 (PC3) in promoterless carrier DNA. If other DNA dilutions were used they are specified within the Results section and the figures (Fig. 8 and Figs. S1 and S4). The cells suspended in Opti-MEM (without phenol red) supplemented with 2% FBS and 100 units/ml penicillin–streptomycin (Gibco) at a final concentration of 220,000 cells/ml, were combined with DNA–transfection reagent complexes, seeded in white 96-well plates at 18,000 cells/well, and grown for 18 h at 37 °C/5% CO2.

BRET assays

Serial dilutions of the fluorescent tracer were generated as 100× solutions in DMSO and subsequently diluted into 0.3× tracer dilution buffer (Promega) to generate 20× serial dilutions in a final solution of 10% DMSO, 60% Opti-MEM, and 30% tracer dilution buffer. The cells were treated with 5 µl of propranolol-NB590 dilutions in the presence or absence of 30
μM competing propranolol (prepared as a 20× solution, 10% DMSO in Opti-MEM). The plates were mixed briefly and incubated for 90 min at room temperature. To measure BRET, the cells were treated with 11 μl of a 10× detection solution comprising 1:20 dilution of LgBiT (Promega) and 1:10 dilution of furimazine live cell substrate (Promega) in Opti-MEM. The plates were mixed for 10 min to allow HiBiT/LgBiT complementation. Filtered luminescence was then measured using GloMax Discover microplate reader (Promega) equipped with a 450-nm (8-nm band pass) filter (donor) and a 600-nm long pass filter (acceptor). BRET was calculated by dividing the acceptor >600 nm light output by the donor 450-nm emission. The values were background corrected by subtracting the BRET values from samples treated with excess unmodified ligand then normalized.

For competitive displacement experiments, 20× dilutions of unmodified ligand were prepared in Opti-MEM supplemented with 10% DMSO. The cells were then treated with 5 μl of serially diluted ligand alongside 5 μl of 20× fluorescent tracer at a fixed EC60–EC80 concentration (prepared as 20× in a solution of 10% DMSO, 60% Opti-MEM, and 30% tracer dilution buffer (Promega)); the plates were mixed briefly and incubated for 90 min at room temperature. The cells were then treated with 11 μl of a 10× detection solution, and BRET measurements were performed as previously described. Affinity values (Ki) were calculated from the observed IC50 values according to the Cheng–Prusoff equation (30).

**Kinetic measurements of ligand binding to HiBiT-GPCR fusion**

For binding kinetics of propranolol-NB590, the cells were treated with 95 μl of a 2× detection solution comprising 1:100 dilution of LgBiT (Promega) and 1:50 dilution of furimazine live cell substrate (Promega) in Opti-MEM without phenol red. To determine specific binding, the control wells were also treated with a fixed EC60–EC80 concentration of a fluorescent tracer (prepared as 20× solution in Opti-MEM, 10% DMSO). The plates were mixed for 15 min prior to the addition of 10 μl of serially diluted propranolol-NB590 (prepared as 20× solution in 10% DMSO, 60% Opti-MEM, and 30% tracer dilution buffer (Promega)). Following brief mixing, kinetic reads were immediately collected on a GloMax Discover microplate reader (Promega).

For the binding kinetics of an unmodified ligand, as described above, the cells were first treated with a 2× detection solution, and control wells were additionally treated with excess propranolol. Following 15 min of incubation, the cells were treated with 10 μl of serially diluted unmodified ligand (prepared as 20× in Opti-MEM with 10% DMSO) and 10 μl of a fixed EC50–EC80 concentration of a fluorescent tracer (prepared as 20× in a solution of 10% DMSO, 60% Opti-MEM, and 30% Tracer dilution buffer (Promega)). Following brief mixing, kinetic measurements were immediately collected on a GloMax Discover microplate reader (Promega).

**Bioluminescence imaging**

HeLa cells (ATCC) were transfected with HiBiT:β2-AR construct at a 1:10 dilution with a promoterless carrier DNA as described above and grown for 18–24 h at 37 °C/5% CO2. Transfected cells as well as endogenously tagged PC3 clones were plated in 35-mm tissue culture–treated imaging dishes (Ibidi) at a density of 4 × 105 cells/dish in 2 ml of appropriate media and incubated 18–24 h at 37 °C/5% CO2. Following incubation, the medium was replaced with 2 ml of warm CO2-independent medium, and the cells were treated with 10× detection solution comprising 1:10 dilution of LgBiT (Promega) and 1:20 dilution of furimazine live cell substrate (Promega) to allow complementation and substrate equilibration. The cells were then treated with 10× EC50 concentration of propranolol-NB590 for 15–20 min at 37 °C and imaged. Subsequently, the cells were treated with 20 μM propranolol for an additional 30 min at 37 °C and imaged again. Images were taken on a LV200 bioimaging imaging system (Olympus) equipped with an ImagEM X2 EM-CCD camera (Hamamatsu) and a temperature-controlled stage. Images were taken using a 20×, 0.75 objective, and each image is an average projection of five individual frames (donor channel: 450/80-nm band pass, EM1200; acceptor channel: 600-nm long pass, EM1200; HeLa acquisition time: 1 s for donor and 8 s for acceptor; PC3 clones acquisition time: 5 s for donor and 40 s for acceptor).

**Internalization assays**

Agonist solutions were prepared as a 10× solution in Opti-MEM supplemented with 10% DMSO. Following the addition of agonist, the plate was briefly mixed and incubated at 37 °C and 5% CO2 for 90 min. Subsequently, the plate was cooled to room temperature and then treated with 11 μl of 10× detection solution comprising 1:20 dilution of LgBiT (Promega) and 1:10 dilution of furimazine live cell substrate (Promega) prepared in Opti-MEM without phenol red. Following 10 min of mixing, luminescence was measured on a GloMax.

For kinetic internalization reads, the cells were treated with 50 μl of 4× detection solution comprising 1:25 dilution of Nano-Glo Vivazine live cell substrate (Promega) and 1:50 dilution of LgBiT (Promega) in Opti-MEM without phenol red supplemented with 2% DMSO. The plate was incubated at 37 °C and 5% CO2 for 1 h to allow substrate deprotection by cellular and serum esterases, after which the plate was moved to a 37 °C plate reader (Thermo Scientific Varioskan Flash) for initial luminescence reads. Upon treatment with 50 μl of agonist solution (prepared as a 4× solution in 2% DMSO and Opti-MEM without phenol red), the plate was thoroughly mixed and transferred back to the 37 °C plate reader, and luminescence was measured over time.

**Data analysis**

Data were analyzed using GraphPad Prism software. Saturation binding analyses of the fluorescent tracer were normalized to the highest corrected BRET signal in each assay and graphed using a one site-specific binding fit. Competitive displacement analyses were normalized to the no compound negative control and graphed using log(inhibitor) versus response-variable slope fit. The resulting IC50 values were used to calculate Kf values according to the Cheng–Prusoff equation (30),

\[
K_f = \frac{IC_{50}}{[L]} + \frac{1}{K_D}
\]

(Eq. 1)
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where [L] is the concentration of fluorescent ligand in the assay, and $K_D$ is its affinity in a saturation binding experiment.

Kinetic analyses for the fluorescent tracer were graphed using the association kinetics: two or more concentrations of hot fit. Kinetic constants ($k_{on}$ and $k_{off}$) and binding constant ($K_D$) for the fluorescent tracer were determined from the resulting curves (53). Kinetic analyses for unmodified compounds were graphed using the kinetics of competitive binding fit (Motulsky–Mahan model for kinetics of competitive binding) (54). Binding affinity and other kinetic constants were determined from the resulting curves.

For end-point internalization, all treatments were normalized to a no-compound control and graphed with a log(inhibitor) versus response-variable slope fit. Kinetic internalization reads were normalized to the initial luminescence reads for each well and then baseline-corrected to the no-compound control reads at each time point. Fig. S16 describes the step-by-step normalization of luminescence measurements. Internalization half-lives were calculated using a one-phase decay fit.

Author contributions—M. E. B., K. V. W., and R. F. O. conceptualization; M. E. B. and R. F. O. data curation; M. E. B. and R. F. O. formal analysis; M. E. B., S. L., K. Z., T. M., R. H., and R. F. O. investigation; M. E. B., S. L., K. Z., T. M., R. H., B. L. B., C. T. E., and R. F. O. methodology; M. E. B. and R. F. O. writing—original draft: M. E. B., S. L., T. M., R. H., C. T. E., T. A. K., and R. F. O. writing—review and editing.

Acknowledgments—We kindly thank Emily R. Lackner and Mary P. Hall for providing molecular biology assistance.

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