A NanoBiT assay to monitor membrane proteins trafficking for drug discovery and drug development

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Internalization of membrane proteins plays a key role in many physiological functions; however, highly sensitive and versatile technologies are lacking to study such processes in real-time living systems. Here we describe an assay based on bioluminescence able to quantify membrane receptor trafficking for a wide variety of internalization mechanisms such as GPCR internalization/recycling, antibody-mediated internalization, and SARS-CoV2 viral infection. This study represents an alternative drug discovery tool to accelerate the drug development for a wide range of physiological processes, such as cancer, neurological, cardiopulmonary, metabolic, and infectious diseases including COVID-19.
Membrane receptors participate in the detection of various environmental stimuli. Their internalization follows the functional process of receptor binding to agonists. Receptor trafficking is also part of cell signaling. Alterations in receptor trafficking have been reported in neurological diseases such as human startle disease hyperkplexia. Also, alterations in the rate of constitutive degradation and on the trafficking of GPCRs receptors in epilepsy have been reported. Membrane receptor trafficking is also seen during viral infection, one good example is the molecular mechanism of how the Human Immunodeficiency Virus (HIV) enters the white blood cells through chemokine receptor 4 (CXCR4) and 5 (CCR5). In cardiovascular diseases, we have the case of the internalization of angiotensin II type I receptor (AT1R) which plays an important role in maintaining cardiovascular homeostasis. Decreased internalization of AT1R is closely related to hypertension, induced by abnormal activation of this receptor.

Antibody-drug conjugates (ADCs) are an excellent therapeutic approach in cancer therapy because they can directly bind to cancer cells and deliver the drug to kill them. In this case, the target membrane receptors are required to be highly and selectively expressed on cancer cells but also susceptible to be internalized by antibody binding. To date, some studies of membrane proteins internalization have been reported. However, some of the methodologies that monitor internalization have been costly and limited to a specific internalization mechanism. In some cases, to monitor physiological processes such as receptor internalization, it is necessary to label the components that participate in such a process. The first labeling agents used to monitor receptor internalization were radioactive tags. Later on, cell-based assays employing fluorescence were developed. However, such technologies require the use of expensive equipment; such as gamma radiation counters and confocal microscopes, and costly conjugated antibodies with a fluorophore. Recent studies reported in the literature have started to use bioluminescence to study receptor internalization.

In this work, we hypothesized that, due to the high sensitivity of bioluminescence, we could devise a new assay using blue light emission to monitor a wide variety of internalization processes by targeting the early endosomes and using NanoLuc Binary Technology (NanoBiT). Bioluminescence resonance energy transfer (BRET) between a Renilla luciferase-inserted GPCR and a GFP10-fused FYVE domain of human Endo receptor 4 (Dynasore), consistent with the role that β-arrestins have in agonist-dependent and mediated internalization of GPCRs. Finally, a dose-response stimulation curve was obtained from (Fig. 1d) to quantify the internalization potency of the epinephrine at the β2AR (Fig. 1f).

Assessing recycling and forward trafficking of a prototypical GPCR. By using the same approach, we were able to study not only GPCR internalization but also receptor recycling. After treating the cells expressing β2AR-NanoBiT with the final concentration of 10 µM epinephrine, the internalization process occurred. This internalization process was observed by a rapid increase in luminescence, indicating the receptor was being removed from the cell surface, subsequently localized in early endosomes, and then finally recycled into the cell membrane.
epinephrine, and continued measuring the luminescence signal. We observed a gradual decay of signal in the absence of ligand, indicating the ligand–receptor complex was being dissociated and the receptor was localized back to the plasma membrane (Fig. 2, number-3). It is interesting to note that the β2AR recycling back to the cell surface was slightly slower as compared to its internalization kinetics.

Luminescent signal is originated from early endosomes. To demonstrate that the luminescent signal is generated from early endosomes, we performed a proximity ligation assay (PLA). The PLA assay is a powerful tool to detect close proximity (about 30 nm) between two entities with high specificity and sensitivity. In this case, the protein targets we used were (1) NLuc, attached to the FYVE domain, (2) EEA1, at the early endosome, and (3) the β2AR (Fig. 3). We then used two primary antibodies, raised in different species (rabbit and mouse), to detect two unique protein targets (β2AR and EEA1 or NLuc and EEA1). A pair of oligonucleotide-labeled secondary antibodies (PLA probes) were bound to the primary antibodies (Fig. 3a, d). Next, hybridizing connector oligos joined the PLA probes. If the PLA probes were in close proximity to each other and, the ligation process formed a closed circle, serving as
the DNA template required for the rolling-circle amplification (RCA). This allowed up to a 1000-fold amplified signal that was still tethered to the PLA probe, allowing localization of the signal. Lastly, labeled oligos hybridized to the complementary sequences within the amplicon which were then visualized and quantified as discrete red spots (PLA signals) by microscopy image analysis (Fig. 3c, f).

**Live cell imaging: GPCR internalization.** In order to visualize the GPCR internalization in real-time living cells, we used a bioluminescent microscope to characterize the receptor localization within the cells. This trafficking visualization is unique in that being able to measure receptor internalization in living cells and in real-time by using luminescence. Currently, most other technologies that quantify receptor internalization do not offer information on spatiotemporal live-cell imaging since they are not performed in real-time and living systems. Here, we were able to visualize the trafficking of a prototypical GPCR (β2AR) in real-time and in living cells (Fig. 4). This trafficking was observed as small luminescent spots moving through the cytosol (highlighted in arrows). After ligand addition, we recorded the β2AR internalization by capturing total luminescence every 2 min. We observed ~25% decrease in bioluminescent signal intensity along the experiment (Supplementary Fig. 6), presumably as a consequence of furimazine (NLuc substrate) depletion.

**Non-GPCR membrane receptor internalization.** To continue exploring potential applications of our technology, we next focused on a prototypical non-GPCR receptor, the human epidermal growth factor receptor 2 (EGFR2), also referred to as the HER2 receptor (Fig. 5a). In this study, we were able to observe that the internalization rate was slightly slower compared to some GPCRs, suggesting that agonists that activate membrane receptors follow similar internalization kinetics (Fig. 5b).

**Antibody-mediated internalization.** Being able to measure antibody-mediated internalization is currently one of the most exciting receptor trafficking mechanisms to study in medicine. To test the versatility of our methodology, we set up a strategy to monitor antibody-mediated internalization. We used a membrane protein recently discovered termed FAM19A5 Isoform II (also called TAFA5). It has been described that FAM19A5 plays a key role in neurological disorders. We decided to use FAM19A5 Isoform II as a prototypical system to study antibody-mediated internalization using two antibodies currently under development. We tagged FAM19A5 Isoform II with SmBiT at the N-termini. SmBiT-FAM19A5 isoform II was co-expressed with the FYVE domain.
tagged with the LgBiT at the N-termini (Fig. 6a). In this experiment, we observed a slower internalization kinetics than observed for the GPCRs (Fig. 1). This slower internalization kinetics suggested a slow conformational change in the receptor induced by the binding with the antibody (Fig. 7b). We achieved to quantify and compare the internalization potencies for the two antibodies, highlighting that there is no statistical difference between them (Fig. 6c).

SARS-CoV2 viral entry. Finally, since internalization is also present in the process when a virus enters into the cell, we devised a simple system to monitor that molecular mechanism by adapting the SARS-CoV2 Spike protein to a lentiviral system as a model of viral infection (Fig. 7a). In this experimental approach, we observed viral entry into the cells is mediated by early endosomes and that its kinetics is also much slower as compared to ligand-activated receptors (Fig. 1). The viral entry trafficking, using the SARS-CoV2 Spike protein (Fig. 7b), was similar to the antibody-mediated receptor trafficking (Fig. 6b, c). In ongoing experiments, we are exploring whether new variants (delta and Omicron) of SARS-CoV2, increase the internalization rate and kinetics of the Angiotensin-Converting Enzyme 2 (ACE2) internalization.

Discussion

In this study, we described several strategies to accurately quantify membrane receptor internalization across different systems by setting up a structural complementation assay based on NLuc. Other studies have been reported in the literature that also used the structural complementation of NLuc but adapted to different physiological contexts such as GPCR dimerization and oligomerization40,41. The approach of structural complementation has great potential in drug discovery and structural biology. It has been successfully applied for several applications in biological research. One of the most interesting applications has been reported by Duan and colleges42. In this report, they used HiBiT technology (another structural complementation approach of NLuc). They used the strong affinity that exists between the two fragments of NLuc to stabilize a GPCR protein complex; the vasoactive intestinal polypeptide receptor and the heterotrimeric G-protein that were used for posterior structural studies involving Cryo-EM. Finally, another interesting work was made by Inoue and co-workers43 where they designed a dissociation assay, to follow in real-time, the activation of Ga protein by monitoring its dissociation from Gβγ subunits and observed by a decrease of bioluminescence.

At the beginning of our study, we aimed to develop a drug discovery tool that can be applied to several types of receptors susceptible to undergo internalization. We hypothesized that it would be possible to monitor the activity of a particular receptor by observing in real-time, its trafficking in living systems. In this regard, we continued exploring different membrane receptor’s
systems to extend the potential of our assay in studying membrane protein internalization induced by external stimuli.

For this purpose, we used the FYVE domain of endoﬁn, since this domain selectively binds to early endosomes. We covalently linked the FYVE domain to the large fragment of NLuc (Supplementary Fig. 1). As the ﬁrst application of our methodology, we decided to explore how internalization occurs across the class A GPCRs; the largest class of GPCRs, accounting for nearly 85% of the GPCR genes that encode for rhodopsin-like receptors (i.e., β2AR), olfactory and orphan receptors44. We were able to observe that the internalization kinetics reached a maximum within about 10 min after ligand stimulation and the fold induction (previously normalized against vehicle) was around 2, depending on the GPCR (Fig. 1c).

To validate our methodology, we pretreated the cells expressing our system with internalization inhibitors (Fig. 1e). We were able to verify that the luminescent signal was abolished in the presence of those inhibitors. Such observation suggests that the increase in the luminescent signal after ligand stimulation is originated from the GPCR internalization process.

One of the advantages of real-time assays is that we can study different conditions in the same well of the assay plate. To illustrate this, we monitored the GPCR internalization and recycling in the same sample (Fig. 2a, b). On the other hand, we consider this technique useful in the de-orphanization of GPCRs, especially in cases where the receptor signaling is unknown, as well as in the development and characterization of agonists and antagonists for GPCRs. However, since the current development of our assay relies on transient transfections, the expression levels of the receptors are not at endogenous levels. Therefore, future directions will focus on developing assays that will express receptors at endogenous levels by using CRISPR gene editing techniques; thus, being able to better mimic the physiological conditions.

The potential application of this internalization assay is that it can be applied to other classes of membrane receptors beyond the study and characterization of the GPCRs, where GPCRs account for approximately 35% of the Federal Drug Administration (FDA) approved drugs45. In addition to GPCRs, we set out to apply our assay to receptor tyrosine kinases (RTKs), another class of membrane receptors. Specifically, we applied our methodology to the human epidermal growth factor receptor 2 (HER2), a member of the receptor tyrosine-protein kinase ErbB family of receptors that promote cell proliferation and opposes apoptosis46. The application of our technology to the HER2 receptor is highly significant since HER2 is known to have therapeutic importance.
in breast and ovarian cancers. Thus, we studied HER2 receptor internalization, where this membrane receptor trafficking pattern represents pivotal internalization steps towards the development of treatments of HER2 positive cancer patients. Our results demonstrate that we can monitor membrane protein trafficking of RTKs, specifically HER2, using an approach similar to the methodology described for GPCRs.

As further validation regarding to the versatility of our membrane protein internalization assay, we applied this technology to monitor antibody-mediated internalization. As such, we studied the Family with sequence similarity 19 (chemokine (C-C motif)-like) member A5 (FAM19A5) receptor protein, a member of the TAF4 family a chemokine-like protein that regulates cell proliferation and migration. Specifically, FAM19A5 is a novel gene with multiple physiological functions (i.e., neurokine, adipokine) recently being discovered. For this membrane protein, we were able to study antibody-mediated internalization of FAM19A5 by two newly develop monoclonal antibodies (Fig. 6a, b). In contrast to GPCRs or RTK receptors, antibody-mediated internalization of FAM19A5 displayed much slower kinetics, reaching a maximum of internalization at two hours after antibody treatment. The fold induction was slightly lower than previously seen in GPCRs or RTK receptors, presumably because the binding between the antibody and FAM19A5 induced minor conformational changes on the receptor as compared to the ligand–receptor interactions observed for β2AR and HER2.

Finally, we wanted to extend our membrane protein internalization assay to monitor viral infections. Specifically, we set out to monitor SARS-CoV2, the seventh coronavirus known to infect humans and able to cause severe respiratory disease, can cause multiorgan infection and cell tropism in the human body. Moreover, the SARS-CoV2-mediated receptor trafficking can be studied and characterized for drug discovery. Thus, when SARS-CoV2 binds into the cell, the virus/plasma membrane receptor can be monitored as an internalization process mediated by virus particles. For this purpose, we produced lentivirus expressing the SARS-CoV2 spike protein. We then added the viral suspension containing the SARS-CoV2 spike protein to cells expressing the corresponding human receptor. In the case of GPCRs, the assay was performed using the corresponding endogenous ligands (i.e., epinephrine).

Methods

Isolation of the positive clones. We picked 3–10 individual bacterial colonies and transferred them into 1 ml of LB medium containing ampicillin (100 µg/ml) and incubated them for 6 h. Then, we used 200 µl of bacterial suspension and transferred it to 5 ml of LB medium containing the same concentration of ampicillin and incubated overnight at 37.5 °C with shaking at 200 rpm. We performed miniprep DNA purifications using 5 ml of LB grown overnight following the manufacturer’s instructions (Life Technologies). To identify successful ligations, we set up PCR reactions with the DNA obtained from mini-preps as a template with the same primers as during the first PCR used for cloning. Positive clones produced the PCR products with the corresponding insert size. We verified the construct sequence by sequencing using the primers shown in Supplementary Table 1.

Proximity ligation assay (PLA). HEK293 cells were seeded in an 8 well Lab-Tek II Chamber Slide (Life Technologies) with a density of 2 x 10^5 cells per well. The next day, cells were transfected with 200 ng of β2AR-SmBiT and 200 ng of LgBiT-PTV constructs using Viafect (Promega Corporation). The next day, samples were treated with 10 µM epinephrine final concentration for 5 min, and immediately after, cells were incubated with 4% paraformaldehyde for 15 min at room temperature.

Nanobit Technology. The NanoBit starter kit contains the plasmids and the necessary reagents for the development of the structural complementation assays used in this study was obtained from Promega Company (Madison, Wisconsin, USA).

Materials. Cell culture medium and cell culture additives were from Promega and Life Technologies.

Chemicals and peptides. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The restriction enzymes were obtained from New England Bio Labs (Ipswich, MA, USA). All ligand peptides were synthesized by AnyGen (Gwangju, Korea). The synthesized peptide purity was greater than 98% as determined by high-performance liquid chromatography analysis. All peptides were dissolved in dimethyl sulfoxide and then diluted in media to the desired working concentrations.
temperature. After the cells were rinsed with PBS and permeabilized with PBS containing 0.1% Tween 20 (PBST), then, cells were incubated with blocking buffer (Dulbecco blocking buffer for PLA) at 37.5 °C for 1 h and followed by incubation with the primary antibodies, anti-(2AR (ThermoFisher catalog number MA5-38441), anti-NLuc (Promega catalog number N7000), or anti-Early Endosome Antigen 1 (Abcam, catalog number ab109110) at 1:1000 by diluting in the Dulbecco antibody dilution buffer at 4 °C overnight. After, three washes (5 min each) with PBST, the cells were incubated with PLA probes (PLUS and MINUS PLA probes) in a pre-heated humidity chamber for 1 h at 37.5 °C. Three washes (5 min each) were then performed. Ligation of the two PLA probes was performed by incubation of the slides in a pre-heated humidity chamber for 30 min at 37 °C in the presence of ligase and 1× ligation buffer. Confocal images were taken using a Nikon A1R-s.

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The next day the cells were transfected using 1 µg of plus-2AR-SmBiT plasmid plus 1 µg of LgBiT-FYVE and ViaFect Transfection Reagent (Promega, catalog number E4981). Twenty-four hours later, on the day of imaging, medium was removed, and the cells were incubated with 1 mL of Opti-MEM containing furimazine for 10 min at 37 °C before epinephrine (10 µM final concentration) was added and allowed to equilibrate for 5 min at 37 °C. Luminescence images were taken by capturing total luminescence for (120 s exposure time).

**Internalization assay using NanoBit Technology.**

HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin (Invi- trogen; Carlsbad, CA, USA). At 1 day before transfection, the cells were seeded in 96-well plates at a density of 2.5 x 10^4 cells per well. A mixture containing 100 nM-receptor construct containing the LgBiT or SmBiT and 50 ng of the Endo domain domain containing one of the two domains of Nano luciferase and 0.3 µl Viafect (Promega) was prepared and added to each well. We tested four Endo-receptor spatial orientations. The one with the highest signal was chosen for further experiments to obtain maximum sensitivity. At 24 h post-transfection, the medium was aspirated and replaced with 100 µL OPTIMEM (Life Technologies, Grand Island, NY, USA). After a 10 min incubation, 25 µl substrate (furimazine) was added, and once every minute for 1–3 h (Synergy 2 Multi-Mode Microplate Reader Bio-Tek, Winooski, VT, USA). Fold induction was calculated by normalizing the luminescent signal against vehicle.

**Lentivirus-mediated expression of the spike protein of SARS-CoV2.** All manipulations were taking place in a biosafety cabinet at all times. HEK293 cells were transfected with the plasmids containing SARS-CoV-2, Wuhan-Hu-1 (GenBank: NC_045512), spike-pseudotyped lentiviral vector (NR-52948, from Be Resources) designed to generate pseudotyped lentiviral particles expressing the spike (S) glycoprotein gene, as well as luciferase (Luc2) and green fluorescent protein (GFP). Seventy-two hours after transfection, the medium was collected in a 500 µl tube and stored at –80 °C for further applications. This protocol only requires Biosafety Level 1 (BSL1) conditions and the viruses used in this protocol were replication-defective.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data set corresponding to dose–response curves and Z-factors for each assay is available for download at Dryad (https://doi.org/10.5061/dryad.7fw3x7x). Some plasmids have been deposited in Addgene (Deposit number 8697). Additional data that support the findings of this study are available in supplementary data and from the corresponding author upon request.

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