

A High-Throughput Luminescence-Based Live Cell Assay to Measure β -Arrestin Recruitment in Real Time



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Key Words:

- Luminescence
- β -arrestin
- GPCR
- HTS
- 1536-well
- CX3CR1
- CX3CL1

Abstract

Characterizing protein:protein interactions (PPI) in relation to G protein-coupled receptor signaling (GPCR) is of great interest to those investigating potential druggable targets, however, endpoint assays lack valuable information about the cellular interactions over time. By incorporating a novel high-throughput assay workflow with hands-free kinetic luminescence-based measurements over time, PPI dynamics in living cells can be more fully defined in an efficient manner.

Introduction

G protein-coupled receptors (GPCRs) and their involvement in cellular signaling remain a focal point of concerted efforts to identify druggable targets. Investigation of protein:protein interactions (PPIs) involved in GPCR signaling, such as the GPCR: β -arrestin2 interaction, provides a means to better understand pathways involved with inflammatory diseases, fibrosis, and cancer¹.

Kinetic live cell measurements of these interactions capture important real-time information that may be otherwise missed using end point measurements. NanoLuc[®] Binary Technology (NanoBiT[®]) from Promega Corporation is a two-component structural complementation reporter that enables real-time measurement of PPI dynamics in living cells in the Nano-Glo[®] Live Cell Assay System and uses a simple bioluminescence readout. In the assay, large BiT (LgBiT; 18 kDa) and small BiT (SmBiT; 11 amino acid peptide) subunits are fused to proteins of interest as indicated by Protein A and Protein B in Figure 1. As the tagged proteins interact, the NanoBiT subunits come together to form an active enzyme that

generates a bright luminescent signal detectable by a luminescence-based microplate reader. The non-lytic assay allows real-time measurements of protein interaction dynamics for one to two hours.

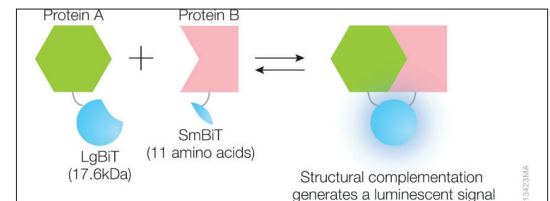


Figure 1. NanoBiT assay overview.

Here, we use specialized NanoBiT CX3CR1/ARRB2 cells, derived from the stable expression of CX3CR1/ARRB2 in HEK293 cells, and activated by CX3CL1 ligand (fractalkine) addition, to demonstrate the GPCR: β -arrestin2 recruitment assay (Figure 2). The assay is performed in 1536-well microplate format, using an automated liquid handler and the luminescence mode of a multi-mode microplate reader. A dose response titration of CX3CL1, as well as Z'-factor determination, are used to assess automated assay performance.

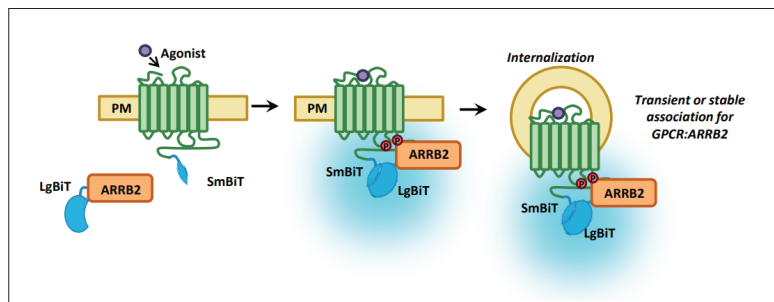


Figure 2. NanoBiT CX3CR1/ARRB2 cells. NanoBiT CX3CR1/ARRB2 cells, derived from the stable expression of CX3CR1/ARRB2 in HEK cells, allow the real-time investigation of GPCR: β -arrestin2 recruitment.

Materials and Methods

Materials

Cells and Reagents

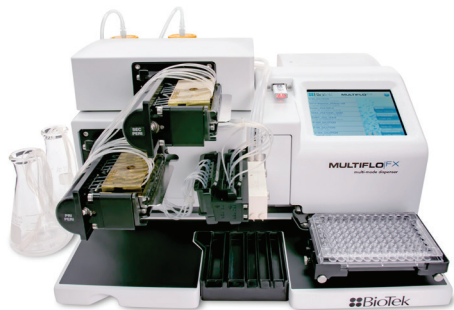
NanoBit CX3CR1/ARRB2 cells (Catalog No. CS208104A) and the Nano-Glo Live Cell Assay System (Catalog No. N2012), consisting of Nano-Glo Live Cell Substrate and Nano-Glo LCS Dilution Buffer, were generously donated by Promega Corporation (Madison, WI). Blasticidin S HCl (Catalog No. A11139-03), TrypLE™ Express Enzyme (1x), phenol red, and Opti-MEM™ reduced serum medium (Catalog No. 11058-021) were purchased from Thermo Fisher Scientific (Waltham, MA). Recombinant human fractalkine (CX3CL1, Catalog No. 300-31) was obtained from PeproTech, Inc., (Rocky Hill, NJ).

Synergy™ Neo2 Hybrid Multi-Mode Reader



The Synergy Neo2 Multi-Mode Reader is designed for speed and ultra-high performance, incorporating BioTek's patented Hybrid Technology™, with independent optical paths that ensure uncompromised performance. Continuously variable bandwidth quadruple monochromators, sensitive high transmission filter-based optics and up to four photomultiplier tubes (PMTs) provide ultra-fast measurements with excellent results. Advanced environment controls, including available CO₂/O₂ control, incubation to 65 °C and variable shaking, support live cell assays while cell-based detection is optimized with direct bottom illumination.

MultiFlo™ FX Multi-Mode Dispenser



MultiFlo FX is an automated multi-mode reagent dispenser for 6- to 1536-well microplates. MultiFlo FX incorporates several unique technologies in its modular design, such as Parallel Dispense, RAD™ (Random Access Dispense) and the patent-pending AMX™ (Automated Media Exchange) modules to facilitate a variety of liquid handling applications from 2D and 3D cell culture to concentration normalization assays, ELISA, bead-based assays and more. A fully configured MultiFlo FX replaces up to five liquid handlers, saving space, time and instrumentation budgets. MultiFlo FX integrates to the BioSpa™ 8 Automated Incubator and a BioTek imager or multi-mode reader, for complete workflow automation for many cell imaging and biochemical applications.

Methods

Cell Preparation

NanoBiT CX3CR1/ARRB2 cell line, HEK293 were cultured using standard tissue culture methods per the manufacturers recommendations. Blasticidin, prepared at a concentration of 5 µg/mL, was used to maintain selective pressure. Cells were harvested at 80-90% confluence using TrypLE dissociation reagent with gentle handling. The cells were then collected by centrifugation and resuspended at the desired cell density in Opti-MEM media for serum starvation during incubation at 37 °C, 5% CO₂ for 4-6 hours.

NanoBiT Assay Workflow

Nano-Glo Live Cell Reagent was prepared as a 5x stock by diluting the Nano-Glo Live Cell Substrate with the Nano-Glo LCS Dilution Buffer per the manufacturers recommendations and added to the cell suspension with mixing. The MultiFlo FX was used to dispense cells into a 1536-well, solid white microplate in a volume of 5 µL at a concentration of 600,000 cells/mL resulting in 3000 cells/well.

Baseline luminescence measurements were taken for approximately 10 minutes, at ambient temperature using a Synergy Neo2 with the parameters listed in Table 1, before proceeding with agonist addition.

Synergy Neo2 Parameters	
Mode	Lum
Light Path	Single PMT
Optic Position	Top
Gain (PMT1, PMT2)	135
Integration Time	0.2 sec
Read Height	8 mm
Delay	0 msec

Table 1. Synergy Neo2 Reader Parameters. Synergy Neo2 equipped with a luminescence filter cube fitted with a 1536-well aperture (3.5 mm) was used, with the above settings, to rapidly capture kinetic measurements.

The known CX3CR1 agonist, fractalkine, was prepared as an 8-point 1:3 serial dilution series ranging from 30-0 nM in Opti-MEM media at 2x concentration and added as eight replicates in an equal 5 μ L volume using MultiFlo™ FX. Luminescence measurements were taken using Synergy™ Neo2 and the minimal read time interval of approximately every 90 seconds for 60 minutes.

NanoBiT Calculations

Data normalization to account for cell plating differences was performed by dividing each data point, relative luminescence units (RLUs) post-fractalkine addition, by the final RLU measurement taken just prior to fractalkine addition. The integral under the curve was then calculated using kinetic measurement data post-fractalkine addition.

Z'-factor was calculated using thirty-six replicate data points for negative and positive control wells using vehicle alone and the highest concentration of fractalkine from the dose response titration, 0 and 30 nM, respectively.

Results

Fractalkine Titration

The EC_{50} concentration was determined using a four-parameter dose-response curve fit in Prism as shown in Figure 3 (GraphPad Software, Inc., La Jolla, CA). The EC_{50} value of 0.75 nM correlates well with previously published data² (Table 2).

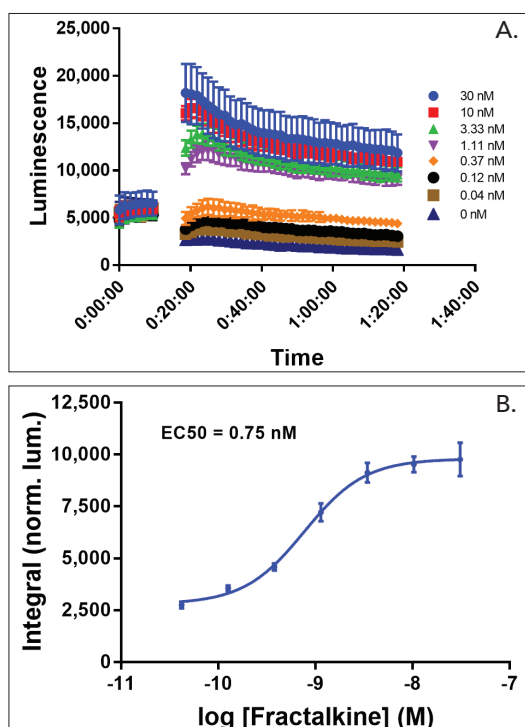


Figure 3. Agonist titration. (A.) Raw kinetic luminescence data. (B.) Fractalkine titration dose response curve based on integral of area under the kinetic curve post fractalkine addition.

Fractalkine EC_{50} (nM)	
1536-well assay	0.75

Table 2. EC_{50} value for the agonist fractalkine was determined in a high-throughput, 1536-well assay format.

Z'-Factor Determination

The Z'-Factor was calculated using 36 replicate measurement of +/- fractalkine at the 30 nM concentration (Figure 4). The assay resulted in a Z'-Factor of 0.81, which is indicative of very robust assay performance with low variability (Table 3).

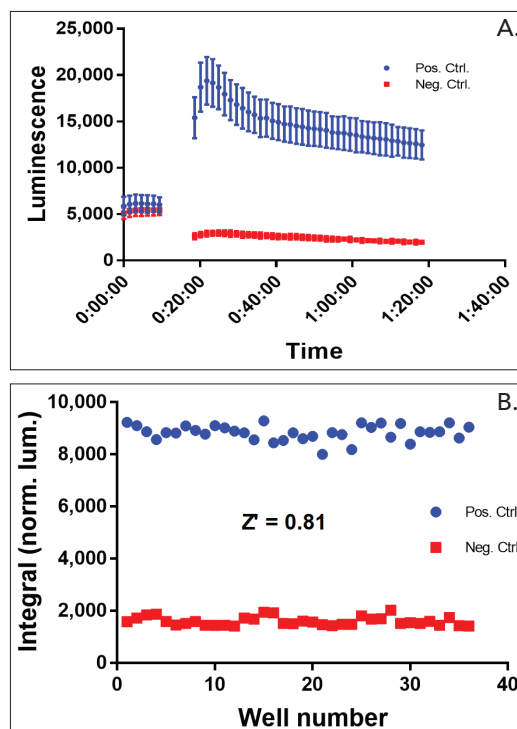


Figure 4. Z'-Factor determination. (A.) Raw kinetic luminescence data. (B.) Control measurement based on integral of area under the kinetic curve post fractalkine addition.

Z'-Factor	
1536-well assay	0.81

Table 3. Z'-factor value was determined in a high-throughput, 1536-well assay format.

Conclusions

The live-cell NanoBiT assay allows for the investigation of dynamic receptor recruitment within a biologically relevant environment in a format amenable to high-throughput screening efforts. A dose response of fractalkine performed using CX3CR1-LgBiT:SmBiT-AARB2 in a HEK293 background resulted in an EC₅₀ value of 0.75 nM which correlates with previous reported values. The Synergy™ Neo2 provides rapid detection which is necessary for high-throughput assay formats. Read time was less than 8 minutes for 1536-wells while still providing a highly robust assay, with a Z'-factor of 0.81. The combination of assay and instrumentation provide an ideal solution for high-throughput detection of protein:protein interactions.

References

1. Peterson, Y.K.; Luttrell, L.M. The Diverse Roles of Arrestin Scaffolds in G Protein-Coupled Receptor Signaling. *Pharmacol Rev.* **2017**, *69*(3), 256-297.
2. Paguio, A.; Butler, B.; Hartnett, J.; Slater, M.; Binkowski, B. Novel Cloning Vectors for Stably Expressing NanoBiT® Fusion Proteins. [Online], November 2018; Promega Web site. <https://promega.media/-/media/files/resources/posters/300-500/bibit-cell-lines-nanoluc-luciferase-poster.pdf> (accessed March 8, 2019).