Application Note

Drug Discovery and Development

Agilent

Trusted Answers

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

Authors

Peter J. Brescia, MSc and
Peter Banks, PhD
Agilent Technologies, Inc.
Winooski, VT, USA

Aileen Paguio,
Brock Binkowski, and
Amy Landreman,
Promega Corporation
Madison, WI, USA

Abstract

Characterizing protein:protein interactions (PPI) in relation to G protein-coupled receptor (GPCR) signaling 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 cancer.[1]

Kinetic live cell measurements of these interactions capture important real-time information that may be otherwise missed using endpoint measurements. NanoLuc Binary Technology (NanoBiT) from Promega Corporation (Madison, WI) 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.

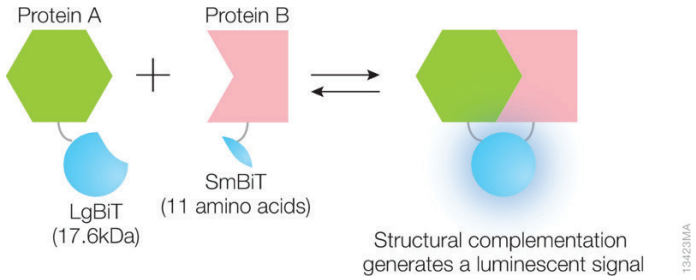

Figure 1. NanoBiT assay overview.

In this application note, specialized NanoBit CX3CR1/ARRB2 cells, derived from the stable expression of CX3CR1/ARRB2 in HEK293 cells, and activated by CX3CL1 ligand (fractalkine) addition, are used to demonstrate the GPCR: β-arrestin2 recruitment assay (Figure 2). The assay is performed in 1,536-well microplate format, using an automated liquid handler and the luminescence mode of a multimode reader. A dose-response titration of CX3CL1, as well as z'-factor determination, are used to assess automated assay performance.

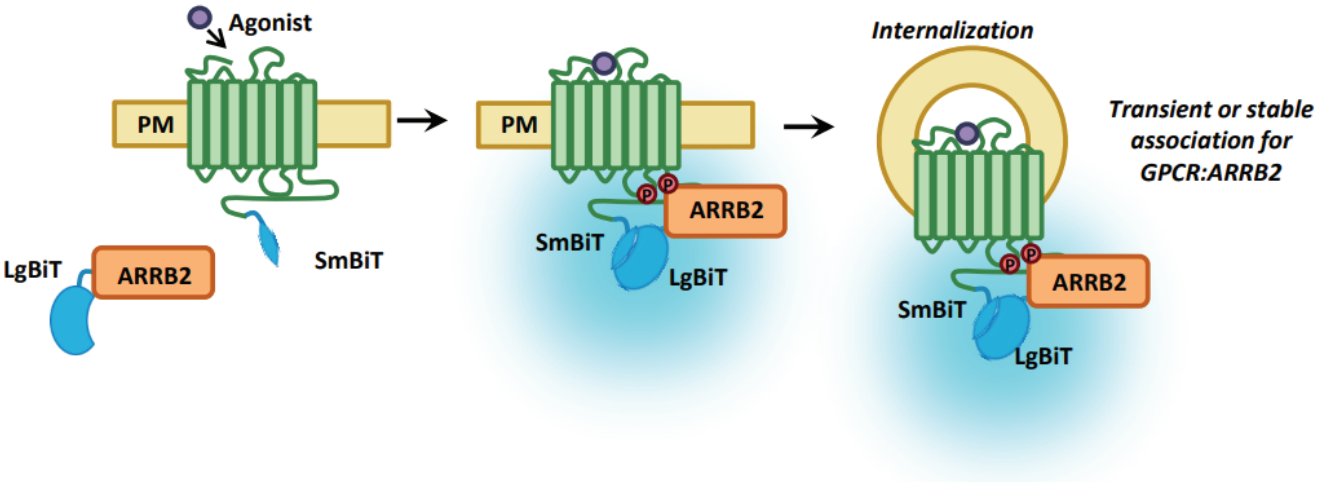

Figure 2. NanoBit CX3CR1/ARRB2 cells. NanoBit CX3CR1/ARRB2 cells, derived from the stable expression of CX3CR1/ARRB2 in HEK cells, allow the real-time investigation of GPCR: β-arrestin2 recruitment.

Materials and methods

Materials

Cells and reagents

NanoBit CX3CR1/ARRB2 cells (part number CS208104A) and the Nano-Glo Live Cell Assay System (part number 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 (part number A11139-03), TrypLE Express Enzyme (1x), phenol red, and Opti-MEM reduced serum medium (part number 11058-021) were purchased from Thermo Fisher Scientific (Waltham, MA). Recombinant human fractalkine (CX3CL1, part number 300-31) was obtained from PeproTech, Inc. (Rocky Hill, NJ).

Agilent BioTek Synergy Neo2 hybrid multimode reader

The Agilent BioTek Synergy Neo2 hybrid multimode reader is designed for speed and ultra-high performance, incorporating Agilent BioTek 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 CO_2/O_2 control, incubation to 65 °C and variable shaking, support live cell assays while cell-based detection is optimized with direct bottom illumination.

Agilent BioTek MultiFlo FX multimode dispenser

Agilent BioTek MultiFlo FX is an automated multimode reagent dispenser for 6- to 1536-well microplates. MultiFlo FX incorporates several unique technologies in its modular design, such as Parallel Dispense, Random Access Dispense (RAD), and the patented Automated Media Exchange (AMX) 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 Agilent BioTek BioSpa 8 automated incubator and an Agilent BioTek imager or multimode reader, for complete workflow automation for many cell imaging and biochemical applications.

Methods

Cell preparation

NanoBiT CX3CR1/ARRB2 and HEK293 cell lines 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 to 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% CO_2 for four to six 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 1,536-well, solid white microplate in a volume of 5 µL at a concentration of 600,000 cells/mL resulting in 3,000 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.

Table 1. Agilent BioTek 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.

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

The known CX3CR1 agonist, fractalkine, was prepared as an eight-point 1:3 serial dilution series ranging from 30 to 0 nM in Opti-MEM media at 2x concentration and added as eight replicates in an equal 5 µL volume using the MultiFlo FX. Luminescence measurements were taken using the 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 and discussion

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 data (Table 2).[2]

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).

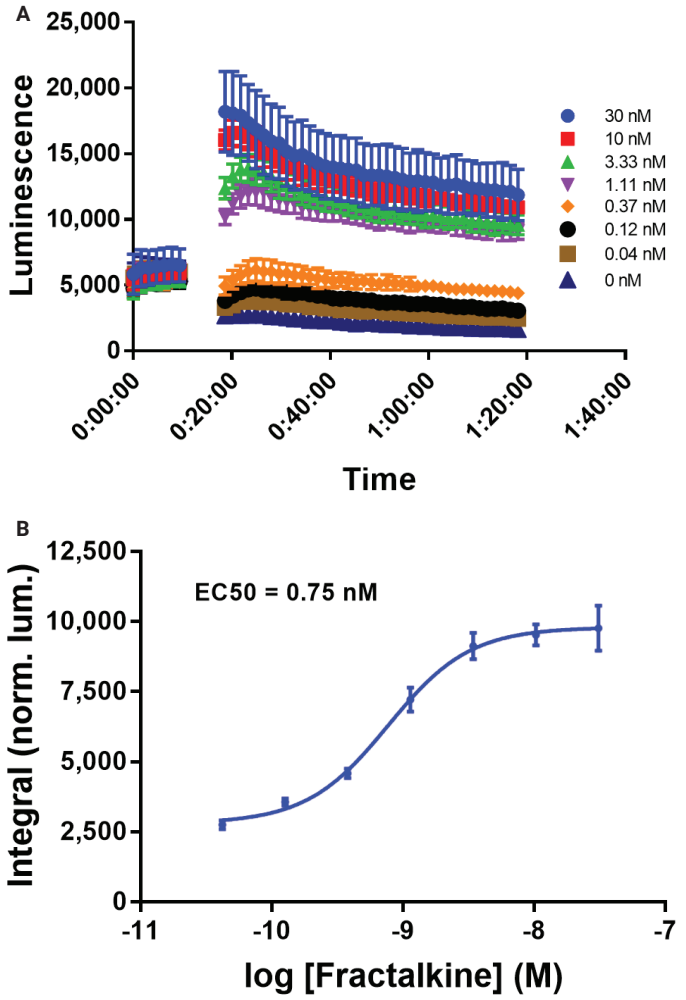

Figure 3. Agonist titration. (A) Raw kinetic luminescence data. (B) Fractalkine titration dose-response curve based on integral of area under the kinetic curve post fractalkine addition.

Table 2. EC_{50} value for the agonist fractalkine was determined in a high-throughput 1,536-well assay format.

	Fractalkine EC_{50} (nM)
1,536-Well Assay	0.75

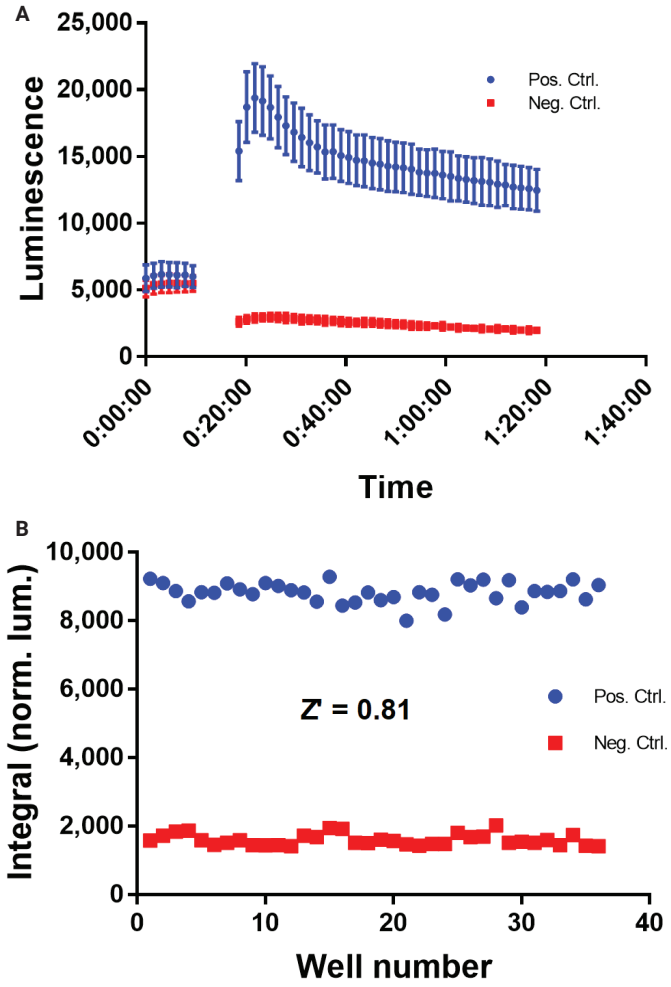

Figure 4. Z'-factor determination. (A) Raw kinetic luminescence data. (B) Control measurement based on integral of area under the kinetic curve post fractalkine addition.

Table 3. Z'-factor value was determined in a high-throughput 1,536-well assay format.

Z'-Factor	
1,536-Well Assay	0.81

Conclusion

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 EC_{50} value of 0.75 nM, which correlates with previous reported values. The Agilent BioTek Synergy Neo2 hybrid multimode reader provides rapid detection, which is necessary for high-throughput assay formats. Read time was less than eight minutes for 1,536-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. *et al.* 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).

www.agilent.com/lifesciences/biotek

RA44412.5361689815

Printed in the USA, April 8, 2022
5994-3398EN

Agilent
Trusted Answers