Investigation of a Novel, High-throughput Method for Real-time Measurement of β-arrestin Recruitment using a Live-cell Luminescence Based Assay System

P. Brescia[1], P. Banks[1], A Paguio[2], B Binkowski[2] and A. Landreman[2]

[1]BioTek Instruments, Inc. | Winooski, VT | USA • [2]Promega Corporation | Madison, WI | USA

Abstract

GPCRs and their involvement in cellular signaling remain a focal point of concerted efforts into the investigation of 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 a variety of diseases such as cancer. The ability to perform live-cell measurements of these interactions remove many of the limitations of other approaches by providing real-time, kinetic results. The development of the NanoLuc® Binary Technology (NanoBiT®) in combination with Nano-Glo® Live Cell Reagent, allows real-time measurement of PPI dynamics in living cells using a simple bioluminescent readout. Here we show the ability to perform a GPCR: β-arrestin2 recruitment assay in a high-throughput, 1536-well microplate format using automated liquid handling. The NanoBiT CX3CR1/ARRB2 stable cell line was used to demonstrate this PPI when activated by addition of CX3CL1 ligand (fractalkine). A dose response titration of CX3CL1, as well as Z'-factor determination to assess assay performance, are demonstrated here.

NanoBiT Overview

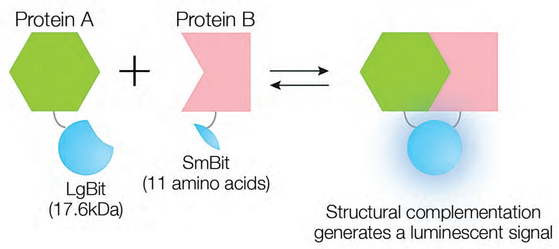

Figure 1. The NanoBiT® assay. LgBiT and SmBiT are fused to proteins A & B. A:B interaction facilitates LgBiT:SmBiT interaction, generating a bright luminescent enzyme. Non-lytic assay format allows real-time measurements of protein interaction dynamics for 1-2 hours.

BioTek Instrumentation

Figure 2. Synergy™ Neo2 Multi-Mode Microplate Reader. The Synergy Neo2 Multi-Mode Microplate Reader is designed for speed and ultra high performance, incorporating BioTek's patented Hybrid Technology™, with its independent optical paths that ensure uncompromised performance. Continuously variable bandwidth quadruple monochromators, sensitive high transmission filter-based optics and up to 4 PMTs provide ultra-fast measurements with excellent results. Advanced environment controls, including availabl CO_2/O_2 control, incubation to 65 °C and variable shaking support live cell assays; cell-based detection is optimized with direct bottom illumination.

Figure 3. MultiFlo FX™ Multimode 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 new, 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.

NanoBiT Procedure

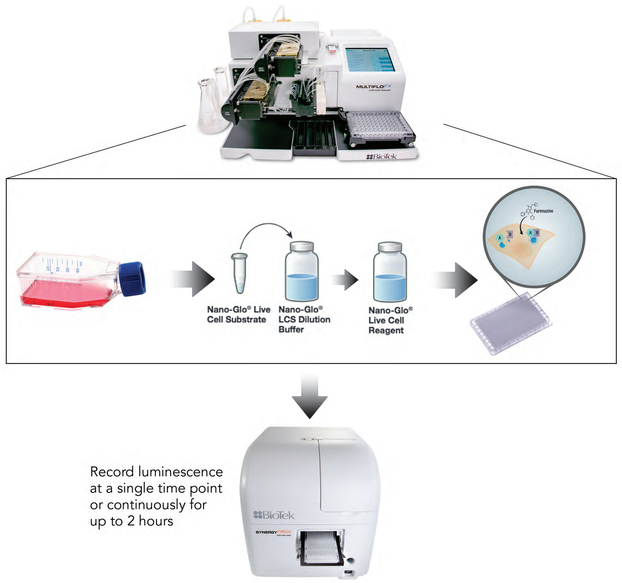

Figure 4. NanoBiT® Assay workflow.

Materials and Methods

NanoBiT® CX3CR1/ARRB2 cell line, HEK293 (PN:CS208104A, Promega Corp.) were cultured using standard tissue culture methods as per the manufacturers' recommendations. Blastocidin (PN:A11139-03, ThermoFisher) at 5µg/mL was used to maintain selective pressure.

The cells were harvested at 80-90% confluence using TrypLE dissociation reagent (PN:12605036, Fisher Scientific) with gentle handling.

The cells were collected by centrifugation and resuspended at the desired cell density in OptiMEM 1 (OM1) (PN:11058-021, ThermoFisher) for serum starvation during incubation at 37 °C, 5% CO_2 for 4-6 hours.

Nano-Glo Live Cell Reagent (PN: N2012, Promega Corp.) was prepared as a 5X stock as per the manufacturers' recommendations and added to the cell suspension with mixing.

Cells were plated in a volume of 5 µL at a concentration of 600,000 cells/mL resulting in 3,000 cells/well using the MultiFlo FX.

Baseline luminescence measurements were taken for ~10 minutes at ambient temperature using a Neo2.

Fractalkine was prepared as an 8-point 1:3 serial dilution series in OM1 at 2x concentration and added as 8 replicates in equal 5 µL volume.

Luminescence measurements were taken using the minimal read time interval (~ every 90 seconds) for 60 minutes.

Synergy Neo2 Reader Settings

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

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

- The Synergy Neo2 was fitted with a 1536-well aperture (3.5 mm)
- The read mode selected was Luminescence in the Top position to take advantage of the high-sensitivity luminescence fiber-optic light-guide
- Photo-Multiplier Tube (PMT) gain setting was optimized and set to 135.
- The integration times was set to 0.2 seconds which showed sufficient signal-to-background.
- Read height was set to 8 mm for use with a 1536-well plate.

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 36 replicate data points for negative and positive control wells using vehicle alone and the highest concentrating of fractalkine from the dose response titration, 0 and 30 nM, respectively.

GPCR Activation Pathway

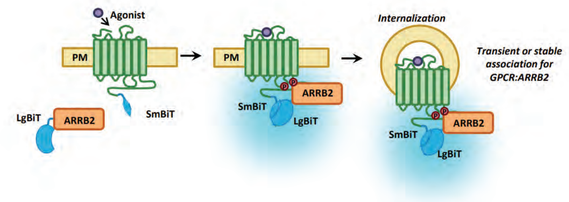

Figure 5. NanoBiT® CX3CR1/ARRB2 cell line. Stable expression of CX3CR1/ARRB2 in HEK293 cells allow the real-time investigation of GPCR :β-arrestin2 recruitment.

Fractalkine Titration and Z'-Factor

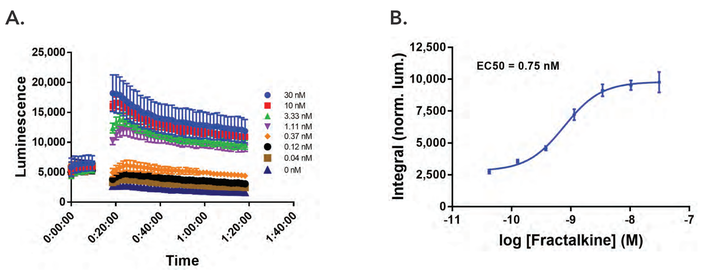

Figure 6. Agonist titration. (A) Raw kinetic luminescence data. **(B)** Fractalkine titration dose response curve based on integral of area under the kinetic curve post fractalkine addition.

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

Table 3. EC50 Value. The EC_{50} value for the agonist Fractalkine was determined in a high-throughput, 1536-well assay format.

- Cells were plated as 8 replicates for each agonist concentration in a 1536-well, solid white microplate.
- The luminescence baseline signal was measured on the Synergy Neo2 for ~10 mins. prior to agonist addition.
- The plate was removed and an 8-point, 1:3 serial dilution series of the CX3CR1 agonist fractalkine, including a zero compound point, was added in an equal volume using the MultiFlo FX.
- The luminescence signal was read kinetically for 60 minutes immediately following agonist addition.
- The EC_{50} concentration was determined using a four-parameter dose-response curve fit in Prism (GraphPad Software, Inc., La Jolla, CA)(Figure 6).
- An EC_{50} value of 0.75 nM correlate well with previously published data (Table 3).

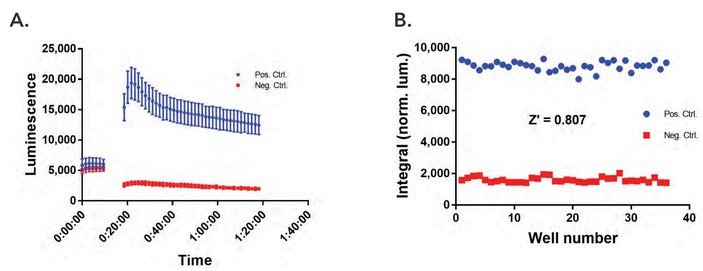

Figure 7. Z' Factor determination. (A) Raw kinetic luminescence data. **(B)** Control measurement based on integral of area under the kinetic curve post fractalkine addition.

Z'-Factor	
1536-well assay	0.83

Table 4. Z'- Factor.

- The Z factor was calculated using 36 replicate measurement of +/- fractalkine (30 nM).
- The assay resulted in Z' factor of 0.83 indicative of very robust assay performance with low variability.

Conclusions

1. The live-cell NanoBiT® assay allowed for the investigation of dynamic receptor recruitment within a biologically relevant environment in a format amenable to high-throughput screening efforts.
2. A dose response of fractalkine was performed using CX3CR1-LgBiT:SmBiT-AARB2 in a HEK293 background resulted in a EC_{50} = 0.75 which correlates with previous reported values.
3. The Synergy Neo2 provides rapid detection which is necessary for high-throughput assay formats. Read time was < 8 min for 1536-wells with the above reader parameters while still providing a highly robust assay (z' = 0.83).
4. The combination of assay and instrumentation provide an ideal solution for high-throughput detection of protein:protein interactions.