Investigation of Protein:Protein Interactions (PPI) using a novel Bioluminescence Resonance Energy Transfer (BRET) Proximity-based, High-Throughput Screening Assay

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Abstract

Proteins have been found to play a variety of critical roles in living cells. To better understand how individual proteins function, it is important to be able to elucidate the dynamic interactions occurring within a cellular context. While several methods exist to investigate protein:protein interactions (PPI) in living cells, monitoring proves to be difficult. Here we demonstrate the use of a new PPI system similar in configuration and approach to bioluminescence resonance energy transfer (BRET). The system is a proximity-based assay that relies on the measure of energy transfer from a bioluminescent donor protein to a fluorescently tagged acceptor protein (Figure 1). The optimized blue-shifted donor and red-shifted acceptor pair helps to minimize both assay background due to the biological nature of the sample and from spectral overlap (Figure 2). Preliminary data includes analysis of a donor-acceptor fusion protein for assay and instrumentation optimization as well as investigation of the donor-MDM2 and acceptor-p53 fusion pair in a cell-based system. The MDM2 and p53 fusion protein pair was transiently transformed into HEK293 cells for analysis including Z'-factor and pharmacological studies.

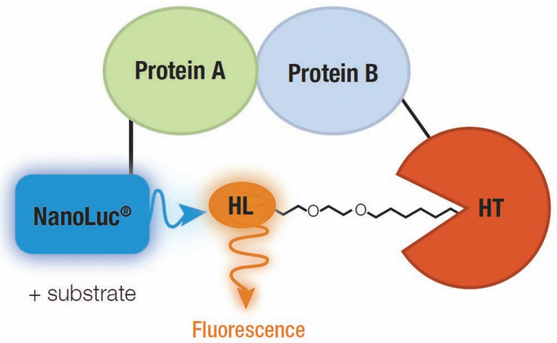

Figure 1 – The NanoBRET™ assay. Diagram of energy transfer from NanoLuc®-Protein A fusion (donor) to a fluorescently labeled HaloTag®-Protein B fusion (acceptor) upon interaction.

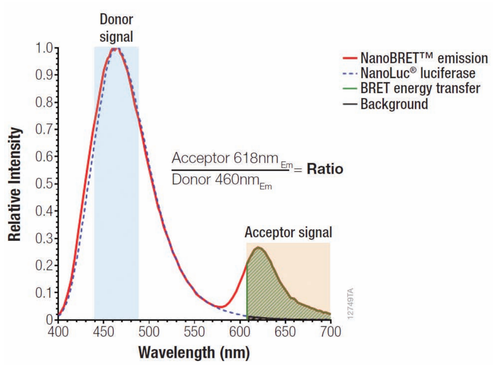

Figure 2 – NanoBRET® Spectra. Spectra of NanoLuc® emission at 460 nm and fluorescent emission at 618 nm of HaloTag® NanoBRET® ligand. NanoBRET® ratio calculation based on donor/acceptor emission.

BioTek Instrumentation

Figure 3 – 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 available 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.

NanoBRET Procedure

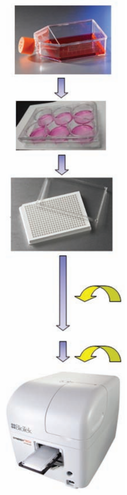

Figure 4. NanoBRET™ Assay workflow.

Materials and Methods

- HEK 293 cells were cultured using standard tissue culture methods.
- HEK 293 cells were harvested at 80-90% confluency and replated into 6-well microplates (P/N:3516, Corning Life Sciences, Tewksbury, MA) @ 800,000 cells/well in 2 mL medium and allowed to attach and recover for 4-6 hours.
- HEK 293 cells were transiently transfected with either the NanoBRET™ Protein:Protein Interaction (PPI) System consisting of the NanoLuc®-MDM2 and p-53-HaloTag® Fusion Vectors or NanoBRET™ Positive Control consisting of a donor-acceptor fusion protein vector (P/N:N1641 and N1581, Promega Corp., Madison, WI).
- Transfections were performed according to the manufacturers recommendations:
  - PPI Control Pair: 2 µg of p-53-HaloTag® Fusion Vector DNA + 0.2 µg NanoLuc®-MDM2 Fusion Vector + 100 µL Opti-MEM® 1 Reduced Serum Medium, no phenol red (P/N: 11058-021,Thermo Fisher Scientific, Waltham, MA)/4% FBS + 8 µL FuGENE® HD Transfection Reagent (P/N: E2311, Promega Corp.).
  - Positive Control: 2 µg of Transfection Carrier DNA + 0.002 µg NanoBRET™ Positive Control Vector diluted in water + 100 µL Opti-MEM® 1 Reduced Serum Medium, no phenol red/4% FBS + 8 µL FuGENE® HD Transfection Reagent.
- Proteins were allowed to express for ~ 20 hours at 37 °C, 5% CO_2
- Cells were harvested and replated into either 96- or 384-well microplates (P/N:3917 and 3570, Corning Life Sciences) either with or without HaloTag® NanoBRET™ 618 Ligand.
- Cells were dispensed in either 100 or 40 µL volume in 96- or 384-well plates, respectively at a density of 200,000 cells/mL for untreated and 90 or 36 µL at 220,000 cells/mL for treated wells.
- Treated well received 10 or 4 µL compound/inhibitor for 96- or 384-well plates, respectively.
- Plates were incubated at 37 °C, 5% CO_2, for a minimum of 4-6 hours to overnight (18-24 hours).
- NanoBRET™ Nano-Glo® Substrate in Opti-MEM I medium was added in a volume of 25 or 10 µL for 96- or 384-well plates, respectively.
- Donor emission (460 nm) and acceptor emission (618 nm) was measured within 10 min. of substrate addition on a Synergy Neo2.

Synergy Neo2 Reader Settings

Synergy Neo2 Read Parameters	
Mode	Lum
Light Path	Dual PMT
Optic Position	Top
Gain (PMT1,PMT2)	135, 135
Intetration Time	0.5 sec
Read Height	6 or 8 mm (96- or 384-well)
Delay	0 msec

Table 1. Synergy Neo2. Synergy Neo2 equipped with dual-PMTs was used with the above settings to simultaneously read both the 450 nm donor signal and 618 nm acceptor signal. Read height was optimized at 6 mm for use with 96-well microplates and 8 mm for 384-well microplates.

- The Synergy Neo2 was fitted with emission filters 450/50 and 610/LP in a dual-emission configuration allowing for simultaneous detection of both donor and acceptor signal.
- Luminescence read mode, using top optics, was selected.
- Photomultiplier Tube (PMT) gain settings were optimized and set to 135 for both PMTs (data not shown).
- Several integration times were tested and optimized and set to 0.5 seconds as discussed below.
- Testing various read heights found optimums at 6 and 8 mm for 96- or 384-well plates, respectively (data not shown).

NanoBRET™ Calculations

$$\frac{618nm_{Em}}{460nm_{Em}} = \text{Raw NanoBRET™ Ratio} = BU$$

$$\frac{618nm_{Em}}{460nm_{Em}} = BU \times 1{,}000 = mBU$$

Mean mBU experimental – Mean mBU no-ligand control = Mean corrected mBU

$$\text{Z factor} = 1 - \left[\frac{(3X\ STDV\ untreated + 3X\ STDV\ treated)}{(Mean\ mBU\ untreated - Mean\ mBU\ treated)}\right]$$

Reader Optimization

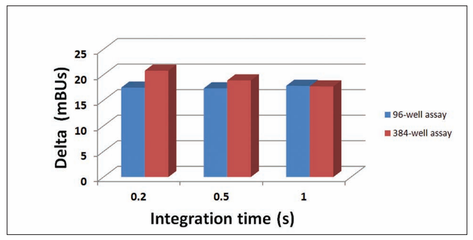

Figure 5. Synergy Neo2 reader settings optimization. The delta between mBU response from treated and untreated cells at various integration times and gain settings were compared to determine optimal reader settings.

- Either duplicate or quadruplicate wells were plated in either 96- and 384-well format, respectively, either with or without (+/-) ligand and were treated with either the specific inhibitor Nutlin-3 at 10 µM or vehicle alone (DMSO).
- The assay was read using various integration times and gain setting (data not shown) to determine optimum assay window and read time.
- The assay window was calculated as the difference between treated and untreated cells.
- It is apparent from Figure 5 that an integration time of 0.5 seconds with gain settings of 135 for both PMTs provide an excellent compromise between read speed and response.

Z-Factor

Z factor	
96-well assay	0.83
384-well assay	0.78

Table 2. Z factor. The Z factor was calculated for each assay format.

- Following transfection the cells were replated in the following manner:
  - 96-well assay format: 10 replicates +/- ligand were either treated with 10 µM Nutlin-3 or treated with vehicle alone (DMSO).
  - 384-well assay format: 48 replicates +/- ligand were either treated with 10 µM Nutlin-3 or treated with vehicle alone (DMSO).
- Following the appropriate incubation period the plate was read on the Synergy Neo2 within 10 mins. of substrate addition.
- The Z factor was calculated for each assay format as previously described.
- Both assay formats resulted in Z factors between 0.5 - 1 indicative of robust assay performance with low variability.

PPI Inhibitor Titration

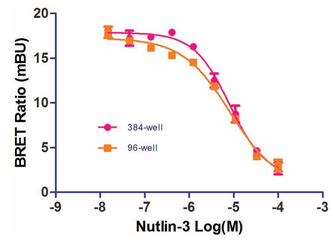

Figure 6. Inhibitor titration.

Nutlin-3 IC_{50} (µM)	
96-well assay	8.4
384-well assay	8.2

Table 3. IC_{50} Values. The IC_{50} values for the inhibitor Nutlin-3 were determined for each assay format.

- Transfected cells were replated as either duplicates or quadruplicates for treatment with each inhibitor concentration in a 96- or 384-well microplate, respectively.
- A 9-point, 1:3 serial dilution series of the specific inhibitor Nutlin-3, including a zero compound point, was added.
- Following the appropriate incubation period the plate was read on the Synergy Neo2 within 10 mins. of substrate addition.
- The IC_{50} concentration was determined using a four-parameter dose-response curve fit in Prism (GraphPad Software, Inc., La Jolla, CA)(Figure 6).
- IC_{50} values of 8.4 and 8.2 µM for 96-well and 384-well assay formats, respectively, correlate well with previously published data1 (Table 3).

Conclusions

1. The live-cell Bioluminescence Resonance Energy Transfer (BRET) assay allowed for the investigation of dynamic protein:protein interactions within a biologically relevant environment.
2. The blue-shifted donor signal and red-shifted acceptor employed provided excellent signal-to-background within a complex *in vivo* environment.
3. The Synergy Neo2 provides rapid detection of multiple signals by simultaneously detecting BRET donor and acceptor emission signals via a dual PMT configuration which is necessary for high-throughput assay formats. Read times were 1m16s and 4m04s for 96 and 384 wells, respectively.
4. The combination of assay and instrumentation provide an ideal solution for high-throughput detection of protein:protein interactions.

[1]Dudgeon, DD., Shinde, SN., Shun, TY., Lazo, JS., Strock, CJ., Giuliano, KA., Taylor, DL., Johnston, PA. and Johnston, PA. (2010) Characterization and optimization of a novel protein:protein interaction biosensor high-content screening assay to identify disruptors of the interactions between p53 and hDM2. Assay Drug Dev Technol, 8, 437-458.