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

Ellaine Abueg¹, P. Bresci¹, P. Banks¹ and A. Landreman²
¹BioTek Instruments, Inc. Winooski, VT USA • ²Promega Corporation Madison, WI USA

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 measurement of energy transfer from a fluorescently labeled 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.

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) at 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 p53-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 manufacturer's recommendations.
- PPI Control Pair: 2 µg of p53-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). 48 µL Fugene HD® Transfection Reagent (P/N: E2211, Promega Corp.).
- Positive Control: 2 µg of Transfection Carrier DNA + 0.002 µg NanoBRET® Positive Control Vector diluted in water + 100 µL OptiMEM® 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% CO₂.
- 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 dispersed 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 plates.
- Plates were incubated at 37 °C, 5% CO₂ for a minimum of 4-6 hours to overnight (18-24 hours).
- NanoBRET® Nano-Glo® Substrate in Opti-MEM 1 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.

Results

The use of the Synergy Neo2 Multi-Mode Microplate Reader with NanoBRET® technology allowed for the simultaneous detection of both donor and acceptor signals. The Synergy Neo2 provides rapid detection of multiple signals by simultaneously detecting BRET donor and acceptor signals, allowing for simultaneous detection of both donor and 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 610/60 to allow 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 to a 0.5 sec as discussed below.

The live-cell Bioluminescence Resonance Energy Transfer (BRET) assay allowed for the investigation of dynamic protein-protein interactions within a biologically relevant environment.

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 1 min and 40 sec for 96- and 394 wells, respectively.
4. The combination of assay and instrumentation provides an ideal solution for high-throughput detection of protein-protein interactions.

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