Simultaneous Dual Emission Detection for Fast Kinetic BRET Assays

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- Simultaneous detection of two wavelengths
- 50 measurements per second for fast kinetics and HTS
- On-board reagent injectors for 384-well plate format
- A factor of 50 between positive and negative controls

Introduction

Bioluminescence Resonance Energy Transfer (BRET) is a system of choice for monitoring intermolecular interactions *in vivo*. BRET is an advanced, non-destructive, cell-based assay technology that is perfectly suited for proteomics applications, including receptor research and the mapping of signal transduction pathways. The assay is based on non-radiative energy transfer between fusion proteins containing Renilla luciferase (Rluc) and e.g. Yellow Fluorescent Protein (YFP).[1,2] The BRET signal is generated by the oxidation of a coelenterazine derivative substrate.

For this application note the BRET²™ demo kit has been used to prove the feasibility of performing a BRET assay on the POLARstar OPTIMA microplate reader. The BRET demo kit applies the cell-permeable and non-toxic coelenterazine derivative substrate DeepBlueC™ (DBC) and a mutant of the Green Fluorescent Protein (GFP²) as acceptor. These compounds show improved spectral resolution and sensitivity over earlier variants.

Materials

All materials were obtained through normal distribution channels from the manufacturer stated.

- POLARstar OPTIMA, PN 413-201; BMG LABTECH, Germany
- BRET²™ filter set, PN 009-102; BMG LABTECH, Germany
- Luminescence 384 top optic, optimized for 384 injection from top, PN 11-322; BMG LABTECH Germany
- BRET²™ demo kit, Cat.# 6310556; PE Life Sciences Inc., Canada
- White 384-well OptiPlate™, Cat.# 6007290;PE Life Sciences Inc., USA

Experimentals

A description for the development of BRET²™ protein-protein interaction assays is included with the demo kit. The following section focuses on the microplate reader settings recommended in the assay protocol.

BRET²™ demo kit reagents:

- Non-transfected CHO cell extracts
- Negative control (Rluc + GFP² not fused together)
- Positive control (Rluc-GFP² fused together)
- DeepBlueC™
- BRET²™ assay buffer

POLARstar OPTIMA settings:

Reader setup for simultaneous dual luminescence well mode detection are listed below:

- Dual emission: activated
- No. of multichromatics: 1
- 1st emission filter: 410-80 nm
- 2nd emission filter: 515-30 nm
- Gain for both PMTs: 3800
- Measurement interval time: 0.02 s
- No. of intervals: 50
- Injection start time: 0 s
- Pump speed: 260 µL/s
- OPTIMA software version: 1.30-0 and higher

Simultaneous emission detection at two channels with highest possible resolution of 0.02 s for every data point.

Assay protocol (white 384-well plate):

1. Addition of BRET²™ assay buffer:
   A10-D12: 15 µL of BRET²™ buffer
2. Addition of 10 µL of each cell extracts (Fig.1):
   A10-A12: Non-transfected cells (blank)
   B10-B12: Neg. BRET²™ control (Rluc + GFP²)
   C10-C12: Pos. BRET²™ control (Rluc-GFP²)
   D10-D12: BRET²™ assay buffer

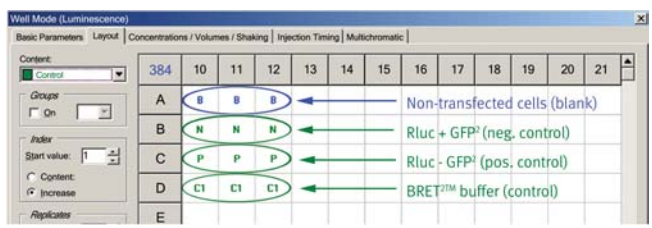

Fig. 1: Layout for the BRET²™ demo kit assay

3. Automated injection of DBC and measurement:
   Insert the prepared plate in the instrument and fill the injector with DBC solution. Enter for A10-D12 (B, N, P, C1) 25 µL in the "Concentrations/Volumes/Shaking" window for "Volume 1". Set the injection start time to 0 s and start the measurement.

   A10-D12: Injection of 25 µL of DBC at 10 µM

On-board reagent injectors allow the measurement of high throughput assays and fast kinetic signals. The data from the measurement was evaluated using the OPTIMA Evaluation Software package (1.30 or higher).

Results and Discussion

When the donor and acceptor are in close proximity, the energy resulting from the catalytic degradation of the DBC is transferred from Rluc to GFP² which will then emit fluorescence at its characteristic wavelength.
The kinetic curves (raw data - blank) of the negative control are shown in Fig.2 for both channels. The low values of the 515 nm channel indicate that no resonance energy transfer occurred. Whereas the positive control shows reduced values at the 410 nm and elevated values at the 515 nm channel due to the BRET effect.

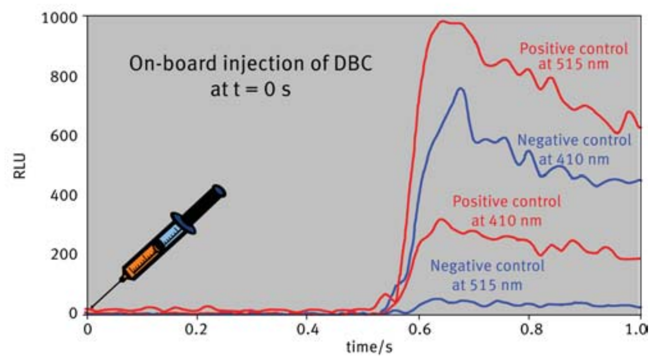

Fig. 2: Resonance energy transfer is obvious for the positive control. No BRET occurs for the negative control.

The calculated BRET ratio indicates the occurrence of protein-protein interaction *in vivo*. This type of detection eliminates data variability caused by fluctuations in light output which can be found with variations e.g. in assay volume, cell types, number of cells per well and/or signal decay across the plate.

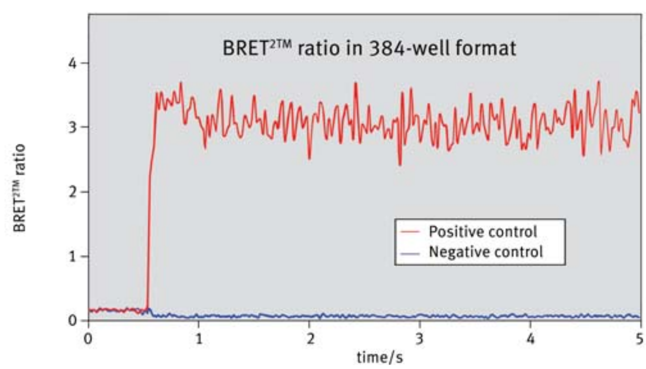

Fig. 3: Ratio of negative and positive control over time

In Fig.3 the blank corrected BRET²™ ratios for both, negative and positive control, are shown and were determined as:

$$\text{BRET}^{2\text{TM}}\text{ ratio} = \frac{(\text{Emission at 515 nm - emission at 515 nm of non-transfected cells})}{(\text{Emission at 410 nm - emission at 410 nm of non-transfected cells})}$$

In the OPTIMA Evaluation Software the calculation corresponds to the quotient of “Raw data – blank” for channel 2 (515 nm) and channel 1 (410 nm) of an arbitrarily selected range of the emission curves. The signal for negative and positive control here reveals a value of around 0.06 and 3.3 respectively, which leads to a factor of around 50 and a clear discrimination between these controls.

The high factor between these controls is caused by the artificial fusion construct of the positive control (Rluc-GFP²) resulting in an extremely high BRET. Real assay samples will presumably result in lower ratios.

Nevertheless BRET assays show no photo-bleaching or photo-isomerization of the donor protein, or auto-fluorescence from cells or microplates which can be caused by incident excitation light and therefore has potential advantages over similar FRET assays. Furthermore the large spectral resolution between donor and emission peaks in BRET²™ (115 nm) greatly improves the signal to background ratio over traditionally used BRET and FRET technologies that typically have only an 50 nm spectral resolution.[3]

In conclusion, ratiometrically quantifiable fast kinetic BRET²™ assays on the POLARstar OPTIMA have been successfully demonstrated. The POLARstar OPTIMA’s internal reagent injectors for 384-well plate format combined with high-end simultaneous dual emission detection offer a unique advantage for fast kinetic assays where simultaneous emission detection at two wavelengths is required. The capability of 50 measurements per second makes the POLARstar OPTIMA perfectly suited for sophisticated HTS applications.

References

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