Real Time Measurement of Fast Kinetic BRET Assays with POLARstar OPTIMA

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Introduction

Bioluminescence **R**esonance **E**nergy **T**ransfer (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 BRET²™ 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
- ☐ BRET filter set,
  PN 009-102; BMG LABTECH, Germany
- ☐ Luminescence 384 top optic, optimized for 384 injection,
  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

Experimental

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 or 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:
   Wells A10-D12: 15 µL of BRET² buffer

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

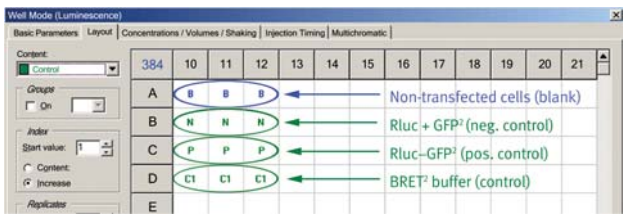

Fig. 1: Layout for the BRET² demo kit assay.

3. Automated injection of DBC and measurement:
   Insert the prepared plate in the instrument and fill the injector with DBC solution. Enter for wells 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.

   Wells 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.

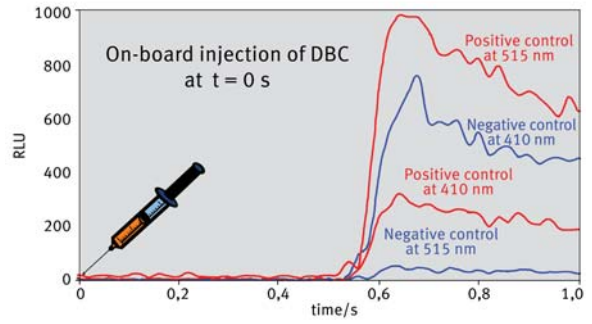

Fig. 2: Resonance energy transfer is obvious for the positive control. No BRET occurs for the negative control.

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

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

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.

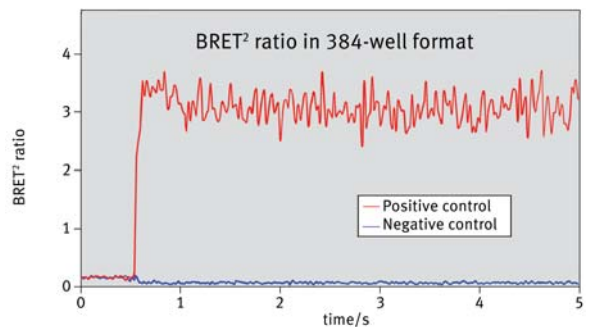

Fig. 3: Calculated ratios of the positive and negative controls.

Instantly after the injection of DBC the ratio of the positive control reaches a high level and remains constant during the measurement. On the other hand the ratio of the negative control stays constant on a lower level and a clear discrimination between theses controls is given by a calculated factor of around 50. 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 a spectral resolution of around 50 nm.[3]

Conclusion

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 real measurement at two wavelengths is required.

The capability of 50 measurements per second makes the POLARstar OPTIMA perfectly suited for sophisticated HTS applications.

References

1. Xu Y, Piston DW, Johnson CH. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc Natl Acad Sci USA 1999; **96**:151-6.

2. Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M. Detection of ß$_2$-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci USA 2000; **97**:3684-9.

3. Mahajan NP, Linder K, Berry G, Gordon GW, Heim R, Herman B. Bcl-2 and Bax interactions in mitochondria probed with green fluorescent protein and fluorescence resonance energy transfer. Nat Biotechnol 1998; **16**:547-52.

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