Bioluminescence Resonance Energy Transfer (BRET²™)
Principle, Applications and Products

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Introduction

BRET² (Bioluminescence Resonance Energy Transfer) 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. BRET is based on energy transfer between fusion proteins containing Renilla luciferase (Rluc) and a mutant of the Green Fluorescent Protein (GFP²). The BRET² signal is generated by the oxidation of DeepBlueC™, a coelenterazine derivative that maximizes spectral resolution for superior sensitivity. This homogeneous assay technology provides a simple, robust and versatile platform with applications in basic academic as well as applied research.

We describe here the BRET² assay technology, its countless applications, and compare BRET² to existing technologies.

BRET² Principle

BRET² assay technology is based on the efficient Resonance Energy Transfer (RET) between a bioluminescent donor moiety and a fluorescent acceptor moiety (Figure 1). RET was first described in the 1940’s by Förster (1) and is characterized by the radiationless transfer of excited state energy from a donor to an acceptor molecule. From a practical point of view, Förster and others (2-4) have shown that energy transfer efficiency is highly dependent on the distance between the donor and acceptor moieties and their relative orientation with respect to each other. In most RET-based assays, the typical effective distance between the donor and acceptor is 10 to 100 angstroms (Å). This range correlates well with most biological interactions thus making RET an excellent tool for monitoring macromolecular interactions. Examples of RET-based technologies are Fluorescence Resonance Energy Transfer (FRET) and Bioluminescence Resonance Energy Transfer (BRET).

BRET is a naturally occurring phenomenon (6) and differs from FRET in that it uses a luciferase as the donor. BRET² uses a luciferase (Rluc) isolated from the sea pansy Renilla reniformis (5) as the donor and the proprietary coelenterazine substrate DeepBlueC (DBC). In the presence of oxygen, Rluc catalyzes the transformation of DBC into coelenteramide with concomitant light emission peaking at 395 nm (blue light). When a suitable acceptor is in close proximity, the blue light energy is captured by RET. The acceptor in BRET² is a GFP variant (GFP²) that is engineered to maximally absorb the energy emitted by the Rluc/DBC reaction. Excitation of GFP² by RET results in an emission of green light at 510 nm. Energy transfer efficiencies between Rluc/DBC and GFP² are determined ratiometrically by dividing the acceptor emission intensity by the donor emission intensity. This ratiometric measurement is referred to as the BRET² signal and reflects the proximity of Rluc to GFP².

Figure 1. BRET² Principle

RET occurs when part of the energy (radiationless: dipole-dipole resonance) from DeepBlueC (DBC)-bound Rluc is transferred to GFP², which in turn, emits green light. If Rluc and GFP² are not in close proximity (upper panel), energy is not efficiently transferred and only the blue light emitted by the Rluc/DBC reaction is detected. When Rluc and GFP² are brought into close proximity (lower panel), energy is efficiently transferred from DBC-bound Rluc to GFP² resulting in the production of green light from GFP². The BRET² signal is measured by dividing the amount of green light by the amount of blue light.
BRET and BRET²

The original BRET (7) system uses Rluc as the donor, the h derivative of coelenterazine as its substrate and a yellow fluorescent protein (YFP) as the acceptor (7-8). In this generation, Rluc and YFP emit light between 475-480 nm and 525-530 nm, respectively, resulting in a poor spectral resolution (difference between donor and acceptor emissions) of 45-55 nm. Also, Rluc produces a broad emission peak that substantially overlaps the YFP emission (Figure 2). The Rluc emission therefore “contaminates” the YFP emission contributing to a high YFP background and thus decreasing the signal to background ratio of the system.

The new generation BRET² uses a proprietary coelenterazine derivative called DeepBlueC that displays special spectral properties when oxidized by Rluc. Indeed, the reaction produces light at 395 nm, a much shorter wavelength than that of the original BRET system. GFP² has an excitation spectrum that is adapted to this wavelength and, upon energy absorption, re-emits light at 510 nm thus providing a broad spectral resolution between the donor and acceptor emission (~115 nm; see Figure 2). This large spectral resolution permits the selection of filters that absorb the entire bandwidth of the donor and acceptor emission peaks thereby increasing the robustness of the detection.

BRET² Features

The BRET² assay technology offers many advantages over existing technologies. BRET², as opposed to FRET, does not require the excitation of the donor with an external light source. Therefore, BRET² does not suffer from problems usually associated with FRET-based technologies such as autofluorescence, light scattering, photobleaching and/or photoisomerization of the donor moiety or photodamage to cells. Also, the absence of contamination of the light output by the incident light results in a very low background in BRET² assays, thereby permitting the detection of smaller variations in the BRET² signal as compared to GFP-based FRET.

The BRET² signal is a ratiometric measurement. This type of detection eliminates data variability caused by fluctuations in light output due to variations in assay volume, cell types, number of cells per well and/or signal decay across a plate.

Finally, the coelenterazine derivative DeepBlueC used in BRET² is membrane permeable and non-toxic, which makes BRET² an ideal assay technology for live cell assays. DeepBlueC penetrates the cell membrane in seconds to activate Rluc emission.

BRET² Configurations

BRET² is versatile and offers the possibility of using the system in two different configurations. Figure 1 shows a BRET² protein-protein interaction assay configuration where both Rluc and GFP² are expressed as fusion proteins (Protein A and Protein B) that are expected to interact. Changes in the interaction, modulated for example by ligands or compounds, can be monitored by a change in the ratio of green (GFP²) to blue (Rluc) light emission. Since this assay configuration monitors protein-protein interactions, different expression levels of each partner have to be tested in order to obtain an optimal signal. This can be easily accomplished by varying the amount of plasmid DNA used in transfection experiments (see below).

BRET² assays can also be carried out in a biosensor configuration where Rluc is directly fused to GFP² with a peptide linker (Figure 3). The linker is designed to include regulatory sequences that are recognized and modified by cellular proteins. Such modifications can result in a variation of the distance between Rluc and GFP² or their relative orientation, or both, which ultimately modifies the BRET² signal. Examples of regulatory sequences are: phosphorylation sites, protease sites and docking sites for proteins.

Figure 2. Comparison of BRET and BRET² Emission Spectra

The BRET² positive control GFP²-Rluc fusion or its BRET counterpart (EYFP-Rluc) was expressed in CHO cells. BRET² and BRET emission spectra were recorded using a spectrofluorometer with the light source turned off (Fluorolog-3 from Instruments SA) after addition of DeepBlueC and coelenterazine h derivative, respectively.

Figure 3. Biosensor Configuration

In this configuration, the GFP² coding sequence is genetically fused to the Rluc coding sequence by a linker. The linker may contain regulatory sequences such as a protease or phosphorylation site, which are recognized and modified by intracellular proteins. Structural modification of the linker changes the distance between GFP² and Rluc or their relative orientation, or both, resulting in a change in the BRET² signal.
BRET² Applications

The BRET² assay technology has been used successfully for a wide range of assay types including the interaction of circadian clock proteins in E. coli (7), the activation and dimerization of G-protein coupled receptors in mammalian cells (8-9), and many others (unpublished and 10) such as:

- Receptor dimerization
- GPCR-β arrestin assay for monitoring G-protein coupled receptor activity
- Tyrosine kinase receptor activation
- Ca²⁺ detection
- cAMP detection
- Apoptosis assay
- Kinase activity
- Protease activity

Basically, any assay for intracellular signalling involving protein-protein interactions can be adapted for BRET² detection.

Some Simple Steps for the Successful Development of BRET² Protein-Protein Interaction Assays

As a first step in the development of a BRET² assay to study protein-protein interactions, three questions must be addressed:

What fusion construct should be used?

Are the fusion constructs functional and are they efficiently expressed upon transfection?

What is the optimal expression level of the two fusion constructs needed to maximize the BRET² signal?

1. Engineering Expression Constructs

The generation of four vectors for each protein should be considered to test the optimal orientation (N- vs. C- terminal) and nature (GFP² vs Rluc) of the tagging protein, since both the orientation and the composition of the fusion protein can affect protein expression and/or activity (Table 1 below).

2. Testing of Functionality and Expression Levels of the Fusion Constructs

The functionality of each fusion construct can be confirmed by testing the enzymatic activity of the target protein (if possible) and by measuring GFP² fluorescence levels or Rluc activity. Also, because expression of fusion proteins can vary depending on the construct used, it is recommended to carry out preliminary transfection experiments to determine the amount of DNA needed for the BRET² assays.

Relative expression levels of Rluc fusion proteins are determined by measuring luminescence levels in presence of coelenterazine h derivative. Relative expression levels of GFP² fusion proteins are determined by measuring fluorescence levels by exciting at 425 nm and examining emission signal at 515 nm. In addition, subcellular localization of GFP² fusion proteins can be determined by fluorescence microscopy.

3. Determination of the Optimal Protein Expression Levels

The optimal amount of each fusion construct to be used in co-transfection experiments will depend on factors such as the affinity of the two interacting domains, the levels of endogenous proteins and the levels at which each construct is expressed. For this reason, a simple 1 to 1 DNA ratio (for example, 5 µg of plasmid of each construct) may not give the best results. It is therefore recommended to carry out a transfection matrix experiment in which cells are transfected with various ratios of each construct and used for BRET² signal measurements. It should be kept in mind that the best BRET² signal is obtained when each Rluc fusion protein interacts with a GFP² fusion protein. Excessive Rluc will contribute to a decrease in the BRET² signal and therefore it is generally more desirable to overexpress GFP².

Table 2 shows an example of a transfection matrix.

4. Confirmation of the Transfection Protocol

In parallel, the transfection protocol should be confirmed by transfecting the GFP²-Rluc(h) positive control vector (cat. #6310030) in the cells of interest and measuring the BRET² signal.

5. Assessment of Various Cell Lines (if possible) to Fine-tune the Assay

Different cell lines should be tested as some could be a better choice to express specific proteins, provided that they can be transfected at high efficiency.

Detection of BRET² Signals

Instrument

The selection of an instrument capable of reading BRET² is of great importance. The instrument must feature sequential dual luminescence measurements: one for the donor (395 nm) and one for the acceptor (510 nm) for each microplate well. Therefore, donor and acceptor light output must be taken consecutively or simultaneously before moving to the next microplate well. The Fusion™ Universal Microplate Analyzer was developed with the right specifications for monitoring BRET² assays.

Filters

Packard BioScience Company provides BRET² optimized filters with center wavelengths at 410 nm with 80 nm bandpass and 515 nm with 30 nm bandpass for use with the Fusion universal microplate analyzer.

BRET² Demo Kit

It is recommended that the BRET² Demo Kit (cat. #6310556) be ordered and used to test the feasibility of performing a BRET² assay on alternate instruments that meet the above specifications.

Table 1: GFP² and Rluc Fusion Construct Combinations

<table>
<thead>
<tr>
<th>Interaction</th>
<th>N-terminal GFP² Fusion Protein</th>
<th>C-terminal GFP² Fusion Protein</th>
<th>N-terminal Rluc Fusion Protein</th>
<th>C-terminal Rluc Fusion Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protein A</td>
<td>Protein B</td>
<td>Protein B</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein B</td>
</tr>
<tr>
<td>3</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein B</td>
</tr>
<tr>
<td>4</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein B</td>
</tr>
<tr>
<td>5</td>
<td>Protein B</td>
<td>Protein B</td>
<td>Protein A</td>
<td>Protein B</td>
</tr>
<tr>
<td>6</td>
<td>Protein B</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein B</td>
</tr>
<tr>
<td>7</td>
<td>Protein B</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein B</td>
</tr>
<tr>
<td>8</td>
<td>Protein B</td>
<td>Protein A</td>
<td>Protein A</td>
<td>Protein B</td>
</tr>
</tbody>
</table>
Table 2: Example of a Transfection Matrix used for the Development of BRET² β-arrestin Assays.

Transfections were done using LipofectAMINE 2000 in HEK-293T cells seeded in 100-mm dishes. Numbers in parenthesis show the ratio of GFP²-β-arrestin2 fusion construct to GPCR-Rluc construct.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>GPCR - Rluc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µg</td>
</tr>
<tr>
<td>0 µg β-arrestin2</td>
<td>1</td>
</tr>
<tr>
<td>3 µg</td>
<td>2</td>
</tr>
<tr>
<td>10 µg</td>
<td>3</td>
</tr>
<tr>
<td>40 µg</td>
<td>5</td>
</tr>
</tbody>
</table>

BRET² Products

The BRET² product offering includes generic and specialized expression vectors, cell lines, demo kit as well as the proprietary coelenterazine derivative DeepBlueC.

Generic Vectors

Rluc Fusion Protein Expression Vectors

These vectors encode either the native or the codon humanized (h) form of the Rluc gene. A Multiple Cloning Site (MCS) is located upstream (-N series vectors) or downstream (-C series vector) of Rluc so proteins or protein domains can be fused to either the N- or C-terminus of Rluc, respectively. Rluc is placed under the control of the cytomegalovirus (CMV) promoter for a high constitutive expression in a variety of cells. Each Rluc Fusion Protein Expression Vector kit includes three plasmids (one for each possible reading frame).

pRluc-N1, -N2, -N3 (Cat. # 6310200)
pRluc-C1, -C2, -C3 (Cat. # 6310210)
pRluc(h)-N1, -N2, -N3 (Cat. # 6310220)
pRluc(h)-C1, -C2, -C3 (Cat. # 6310230)

GFP² Fusion Protein Expression Vectors

GFP² is an improved wild type GFP possessing higher cellular solubility and stability when expressed in mammalian cells. GFP² has also been codon humanized to improve its expression in mammalian cells. A valine residue has been engineered after the initiation codon to help create a perfect Kozak consensus resulting in higher expression levels. Finally, a mutation at position 64 (F64L, GFP wild type numbering) has been introduced to increase protein folding and stability at 37°C. These vectors are also offered as N- (-N series vectors) or C-terminus (-C series vectors) GFP² fusion constructs. Each GFP² Fusion Protein Expression Vector Kit also includes three plasmids (one for each possible reading frame).

pGFP²-N1, -N2, -N3 (Cat. # 6310240)
pGFP²-C1, -C2, -C3 (Cat. # 6310250)

BRET² Positive Control

A positive control vector expressing the GFP²-Rluc(h) fusion protein is also available. Once expressed, the fusion protein generates a high and robust BRET² signal that can easily be detected.

pGFP²-Rluc(h) (Cat. # 6310030)

BioSensor pGFP²-MCS-Rluc(h) Vector

Structurally, this vector is similar to the positive control but has a MCS in between the GFP² and the Rluc(h) genes for insertion of regulatory sequences such as protease or kinase sites.

pGFP²-MCS-Rluc(h) (Cat. # 6310051)
Specialized Vectors and Cell Lines

GPCR Assay Kit

Based on the interaction between agonist-stimulated receptor and arrestin proteins, BioSignal Packard offers a BRET²/Arrestin assay kit to monitor GPCR activation. For this assay, the receptor is fused to Renilla luciferase to generate a receptor-Rluc(h) fusion protein and the human β-arrestin2 is fused to GFP² generating GFP²-β-arrestin2. Any receptor can be fused to Rluc using the pRluc-N vector kit (cat. # 6310220). However, BioSignal Packard offers the V₂ Vasopressin receptor fused to Rluc to be used as a positive control. Upon interaction of the β-arrestin2 moiety with the phosphorylated chimeric V₂-Rluc(h) receptor, Rluc is brought into close proximity with GFP² and a BRET² signal can be detected.

V₂ Vasopressin/β-arrestin2 vector kit (Cat. # 6310301)
pGFP²-β-arrestin2 vector (Cat. # 6310176)
pV₂ Vasopressin receptor-Rluc(h) vector (Cat. # 6310193)

A GFP²-β-arrestin2 cell line can be made using a HEK 293-derived cell line expressing the GFP²-β-arrestin2 fusion protein under the control of an ecdysone-inducible promoter. This system allows for the regulated expression of the fusion protein, which can be achieved by incubation of the cells with the ecdysone analog ponasterone A.

A GFP²-β-arrestin2 cell line can then be used to monitor the activation of various transiently expressed GPCRs in BRET²/arrestin assays. In addition, using a 293/GFP²-β-arrestin2 cell line as a recipient, double-stable lines co-expressing GPCRs fused to Rluc (GPCR-Rluc) can be made for the pharmacological characterization of compounds (agonists and antagonists) in BRET²/arrestin assays. The BRET²/arrestin assay can be used to characterize ligand-GPCR interactions for known GPCRs and can be applied to ligand identification for orphan receptors.

Caspase-3 BioSensor Vector

Apoptosis or Programmed Cell Death (PCD) is a regulated and structured process by which a cell self-destructs into membrane encapsulated particles called apoptotic bodies that are rapidly phagocytosed and degraded. This mechanism involves the activation of proteases (caspases), which are elements of a signaling cascade ending in the activation of effectors responsible for the degradation of cellular proteins and genomic DNA. In the BRET² Caspase Assay, the linker region between GFP² and Rluc(h) in the GFP²-linker-Rluc(h) fusion construct is a caspase-3 consensus sequence (DEVD in a single amino acid letter code). When this construct is transfected in cells containing caspase-3 (e.g. HeLa cells) the DEVD linker is cleaved and, the energy transfer is disrupted (see Figure 5).

pGFP²-DEVD-Rluc(h)-Caspase-3 biosensor (Cat. # 6310066)

DeepBlueC and Other Products

DeepBlueC

When oxidized by Rluc, the proprietary coelenterazine derivative DeepBlueC produces light at 395 nm as opposed to 450-480 for wild type coelenterazine. This unique spectral characteristic makes DeepBlueC the optimal Rluc substrate for GFP² in BRET² assays. The blue-shifted emission spectrum of DeepBlueC makes BRET² assays possible and offers a large resolution between donor and acceptor emission peaks (115 nm) when used in conjunction with GFP² (BRET²-adapted GFP) as opposed to the much lower resolution found in FRET assays (typically ~50 nm).

DeepBlueC (5 X 50 µg) (Cat. # 6310100F)
DeepBlueC (5 X 200 µg) (Cat. # 6310101M)

BRET² Demo Kit

BioSignal Packard offers a demo kit to demonstrate the BRET² energy transfer or to determine if an instrument is suitable to detect the BRET² signal. The demo kit contains CHO cell extracts expressing positive (GPCR-Rluc(h) fused together) and negative controls (GFP² + Rluc(h); not fused together), buffer and DeepBlueC.

BRET² demo kit (Cat. # 6310556)

Figure 5. BRET² Caspase Assay

In the BRET² Caspase Assay, the linker region between GFP² and Rluc(h) in the GFP²-linker-Rluc(h) fusion construct is a caspase-3 consensus peptide sequence (DEVD in a single amino acid letter code). When this construct is transfected in cells that contain caspase-3 (e.g. HeLa cells) the DEVD linker is cleaved and the energy transfer is disrupted.
Conclusion

With the initial draft of the human genome now available, the daunting task of identifying and characterizing the encoded proteins is now at hand with academic, government and industrial research facilities all playing a role in this burgeoning field of proteomics. Various technologies have emerged to fulfill complementary roles in this complex task. High throughput mass spectrometry and X-ray crystallography have opened the door to rapidly identify and structurally characterize proteins. In combination with the time-honored tools of cell biology, these instrument-based techniques offer researchers the opportunity to map the proteome of organelles. However, these technologies do not provide simple methods for the precise identification of protein partners in vivo.

BRET2 provides the ideal assay platform for cell-based proteomics by offering the possibility of studying complex protein-protein interactions in cells. BRET2 allows the quantitation of protein-protein interactions and signaling events in live cells without the need for an excitation light source, which eliminates problems associated with FRET-based assays such as photobleaching and high background signals due to the fluorescence of endogenous intracellular proteins. The Rluc and GFP2 fusion proteins can themselves be used as markers to assure appropriate expression levels and subcellular localization of the fusion proteins being examined. Finally the large spectral resolution between donor and emission peaks greatly improves the signal to noise ratio over traditionally used BRET and FRET technologies.

The advent of microplate-based light-detection instruments such as the Fusion Universal Microplate Analyzer and highly sensitive camera-based plate-readers has made screening over traditionally used BRET and FRET technologies.

References


Related Publications

BRET2 Poster Presentations:

Joly E. et al. BRET2™: Bioluminescence Resonance Energy Transfer. A Novel Assay Technology for the Measurement of Signal Transduction Pathways in Intact Cells. (Reference S3411)

Houle B. et al. Bioluminescence Resonance Energy Transfer (BRET2™): A Powerful Platform to Study G Protein-Coupled Receptor (GPCR) Activation in Intact Cells. (Reference S3280)

Normand C. et al. BRET2: Bioluminescence Resonance Energy Transfer, a novel assay technology to examine G Protein Coupled Receptor Activation in Intact Cells. (Reference S3441)

Bertrand L. et al. BRET2 Assay in Stable Recombinant Cells: A Platform to Screen for compounds that Interact with G Protein-Coupled Receptors (GPCRs). (Reference S3516)

BRET2 Publication: