Basic Considerations for Bioluminescence Resonance Energy Transfer (BRET) Assays for G-protein coupled receptor protein interactions in living cells

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Introduction

BRET has been successfully applied to the study of GPCR homo- and heterodimerization as well as receptor/β-arrestin interactions involved in receptor desensitisation and trafficking in mammalian cells (review, 1). The main advantage of BRET is that it allows protein-protein interactions to be monitored in real-time, in their correct location, in live cells. Due to the highly hydrophobic nature and cellular localisation of GPCRs, conventional techniques for measuring protein-protein interactions (i.e. co-immunoprecipitation and yeast 2-hybrid screening) have significant drawbacks. BRET is a simple, rapid homogeneous assay that may overcome some of these limitations. to 7TM receptors is a universal process the assay can be used as a screening assay for virtually all 7TM receptors independent of their signaling pathway.

BRET Principle

BRET is a naturally occurring phenomenon, an example of which is the non-radiative transfer of energy occurring between aequorin and green fluorescent protein (GFP), in the jellyfish, *Aequoria*. BRET interactions can be studied using fusion proteins which are tagged with either a bioluminescent donor such as the luciferase enzyme (Rluc) from the sea pansy, *Renilla*, or a fluorescent acceptor, such as enhanced green or enhanced yellow fluorescent protein (EGFP or EYFP). When the interaction partners are co-expressed in cells, energy is

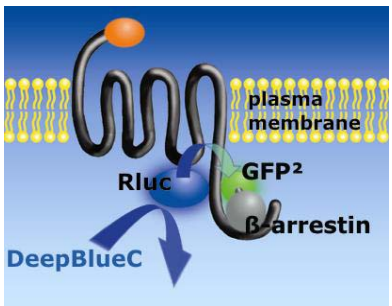

Figure 1: BRET principle

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transferred in the presence of the substrate, coelenterazine, from Rluc to EYFP and light emitted only if the proteins are in close enough proximity (within 100Å) (2). The critical distance dependence between donor and acceptor molecules makes BRET an ideal technique to study receptor-protein interactions involved in all aspects of GPCR function and their modulation by agonists and antagonists. Similarly, fluorescence resonance energy transfer (FRET), which involves the transfer of energy between fluorescent donor and acceptor molecules, also represents a tool for the detection of GPCR-protein interactions and has been used in conjunction with imaging techniques to monitor GPCR interactions both spatially and temporally. Unlike FRET, its derivative BRET avoids the need for excitation, thus circumventing the problems associated with autofluorescence, photobleaching and cell damage. Furthermore, the lower background fluorescence associated with BRET makes it a sensitive technique enabling the detection of low level or weak protein interactions (3). FRET also represents a tool for the detection of GPCR-protein interactions and has been used in conjunction with imaging techniques to monitor GPCR interactions both spatially and temporally. A significant advancement in the study of protein-protein interactions will be represented by single-cell BRET imaging to determine the cellular localization of the protein interaction.

Application of BRET

In order to apply BRET to the study of receptor interactions, fusion proteins are co-expressed in cells and if the two fusion molecules are in close enough proximity, then light is transferred from Rluc (480nm) to EYFP. This results in an excitation and emission of light at the characteristic wavelength of 530nm, with the degree of BRET quantified as a ratio of light emitted at 530nm over 480nm. Initially BRET was performed in *E.coli* to study the interaction of light-sensitive circadian clock proteins (2). Several other groups have now used BRET to study protein interactions between GPCRs in mammalian cells, including receptor homodimerization (4-9) and heterodimerization (10-13). Interactions between GPCRs and intracellular proteins required for receptor function have also been monitored using BRET, such as receptor/β-arrestin interactions involved in receptor desensitisation and internalisation (4,5,13). Thus, in theory BRET could represent a powerful tool with which to study the complex array of receptor interactions critical for GPCR functions ranging from ligand binding to receptor signalling, desensitisation and trafficking.

Mithras LB 940

The Mithras LB 940 is a multimode plate reader with a unique optical design (DOPS – Dedicated Optical Path System) to ensure optimized performance for the detection technologies implied. These are luminescence, BRET, fluorescence (top and bottom reading), absorbance, fluorescence polarization and AlphaScreen™. In addition accessory options, e.g. reagent injectors, temperature control and cooled PMT detection units are available.

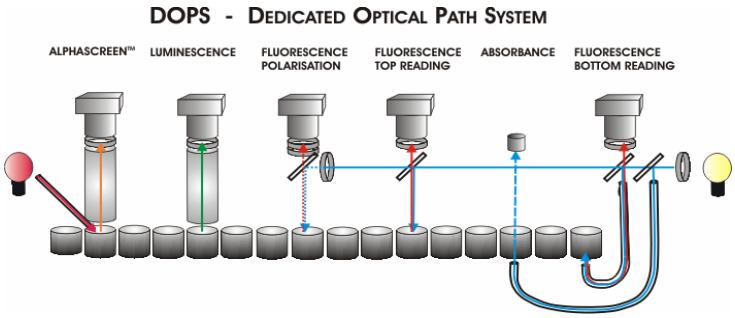

Figure 2: Dedicated Optical Path System and Mithras LB 940

Methods

Performing a BRET assay to investigate a potential protein-protein interaction involves several steps: (i) generation of each of the two proteins of interest genetically fused with either Rluc or EYFP at either the N- or C-terminus, (ii) co-expression of the two BRET fusion proteins in mammalian cells and (iii) detection of the BRET signal. This method can be applied to study any protein-protein interaction in mammalian cells and is not limited to investigation of interactions involving GPCRs.

Generation of BRET fusion constructs

Constructs encoding BRET fusion proteins consist of the following:

1. Positive control BRET fusion construct for expression of the Rluc-EYFP fusion protein (see note 2).
2. Negative control BRET constructs, e.g. pRluc, pEYFP for expression of Rluc and EYFP alone; control GPCRs (other than the GPCR of interest) and other proteins fused to either Rluc or EYFP (see note 3). In our studies we have utilised gonadotropin releasing hormone (GnRH) thyrotropin-releasing hormone (TRH) and β2adrenergic receptors and these were C-terminally tagged with either Rluc or EYFP (see note 3).
3. BRET donor construct of the first protein of interest (for expression of the GPCR that is C-terminally (GPCR/Rluc) or N-terminally (Rluc/GPCR) tagged with Rluc (see note 4-6).
4. BRET acceptor construct of the second protein of interest (for expression of the GPCR that is C-terminally (GPCR/EYFP) or N-terminally (EYFP/GPCR) tagged with EYFP (see notes 4-6).

Transfection and co-expression of BRET fusion constructs

1. Transfect mammalian cells (i.e. COS-1 cells) which were plated out the day prior to transfection at a density of 2 x 10^5 cells/well per 6-well plate) using transfection reagent according to manufacturer's instructions (see note 7).
2. Transfect cells with GPCR/Rluc fusion cDNA alone, or co-transfect with GPCR/Rluc fusion and GPCR/EYFP fusion cDNA (see note 8). Rluc and Rluc-EYFP BRET control cDNA should also be transfected as a positive control for the experiment. Negative control transfections should also be included, for example other GPCRs fused to EYFP or Rluc to determine non-specificity of BRET signal (see notes 3 & 8).

BRET assay

1. At 48 h post-transfection, cells are detached using phosphate buffered saline (PBS)/0.05% trypsin, washed twice in PBS and resuspended in 500µl PBS. For each transfection approximately 100,000 cells are analysed by flow cytometry for EYFP expression, or by using the fluoresence mode of the LB940 (See note 6). For BRET, 40µl cell suspension (approximately 20,000 cells) is distributed into each well of a 96-well plate.

2. Inject 10 µl of coelenterazine (freshly diluted to 25 µM) to each well to obtain a final concentration of 5 µM and immediately read using the Mithras LB940, (see notes 9, 10) with the heating block preset to required temperature (i.e. 37C).

3. To test the effect of an agonist or antagonist (or another reagent/chemical/treatment) on the interaction and hence on the BRET signal, the reagents can be pre-incubated prior to the addition of coelenterazine. Alternatively, coelenterazine can be added, a control 'untreated' reading taken, then up to three reagents added via the injectors, and the effect on the BRET ratio assessed over time. (See Fig 3)

4. Integrated readings are taken for 10 sec collecting light filtered through two filters, each with a different wavelength range.

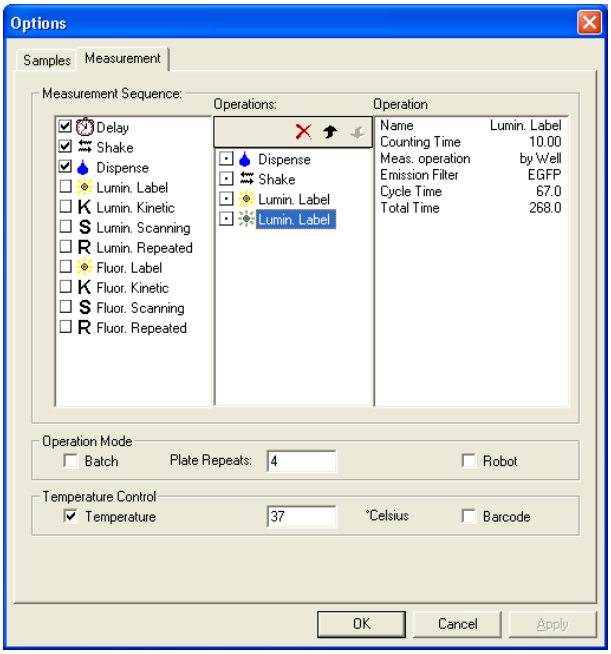

Figure 1: Read options for measuring BRET signals. The first lumin label operation has the Rluc filter selected, the secong has the EYFP filter selected.

The BRET ratio is then calculated from these two reads according to the following calculation:

$$\text{BRET ratio} = \frac{\text{emission at 530nm}}{\text{emission at 480nm}}$$

5. Data is then represented as a normalized BRET ratio, defined as the BRET ratio for co-expressed Rluc and EYFP constructs normalized against the BRET ratio for the Rluc expression construct alone in the same experiment.

Notes

1. The form of coelenterazine used in the BRET assay is important. Several different forms of coelenterazine exist, each resulting in peak light emissions of slightly different wavelengths. In order for energy transfer to occur, the emission spectrum of the donor (Rluc) has to significantly overlap with the excitation/absorption spectrum of the energy acceptor (EYFP). In addition, the emission spectra of donor and acceptor have to be distinct enough to allow separate measurement of emission from each molecule with minimal overlap. With these considerations in mind, and using Rluc and EYFP as donor and acceptor molecules respectively, the *h* form of coelenterazine was employed in the BRET assays. Coelenterazine is dissolved in methanol to prepare a stock solution (500µM) which is stored at –20^{o}C protected from light. Just before use, the coelenterazine is diluted in BRET assay buffer to a final concentration of 25 µM and wrapped in foil to protect it from light.

2. To confirm that a BRET signal can be obtained under the experimental conditions used, a BRET positive control should be used. A direct fusion of the Rluc and EYFP proteins separated by an amino acid linker is a good BRET positive control. This can be prepared by cloning the Rluc coding region without its stop codon upstream and in-frame with the EYFP coding sequence. Following addition of coelenterazine to cells expressing the Rluc-EYFP fusion, light emitted from Rluc is directly transferred to EYFP resulting in a high BRET signal compare to the BRET ratio obtained for Rluc and EYFP or Rluc alone (1).

3. It is important to include negative controls in BRET assays when monitoring receptor-receptor, or receptor-protein interactions. These include the expression of receptors tagged with either Rluc or EYFP with untagged EYFP or Rluc, respectively, to determine if the BRET signal is merely due to overexpression of Rluc and EYFP in the same cell and thus resulting from non-specific interactions. To assess the specificity of the receptor-protein BRET signal, additional negative controls using other tagged receptors should also be performed, with these control tagged receptors expressed at similar levels to the receptors of interest. In addition, untagged proteins can be co-transfected along with the donor and acceptor BRET fusions to disrupt the interaction thereby reducing the

BRET signal. This approach was adopted to assess the specificity of the BRET signal obtained upon co-expression of TRHRs tagged with either Rluc or EYFP (5).

4. To determine if an interaction occurred between TRHRs (homo- oligomerization), the TRHR was C-terminally tagged with either Rluc or EYFP to generate TRHR/Rluc or TRHR/EYFP (5).

5. The absence of a BRET signal does not necessarily imply the lack of an interaction, but instead could mean that the donor and acceptor tags are not in an orientation that would favour energy transfer. This highlights the importance of performing BRET with Rluc or EYFP fusion partner proteins of interest constructed with tags at either the N- or C-terminus. Using this approach, the combination that allows the interaction of the two partner proteins with their respective donor and acceptor fusion molecules with the optimal orientation/distance to give a sensitive BRET signal, can be determined. The insertion of an amino acid linker sequence between the protein of interest and the fusion EYFP or Rluc may also be useful. When performing BRET to detect receptor-protein interactions, receptors are usually tagged at the C-terminus rather than the N-terminus to increase the chance of correct folding and membrane localization of the receptor and to reduce interference with ligand binding.

The functionality of BRET fusion proteins, compared to the untagged protein should be assessed, when possible, prior to its use in a BRET assay. Addition of either the Rluc or EYFP to the receptor or protein of interest may disrupt the expression, folding, localization and/or function of the protein and before assessing the relevance of potential receptor interactions it is important to ascertain whether the function of tagged proteins is compromised or not. To assess the functionality of BRET receptor fusion constructs, receptor binding and signalling assays were performed on the receptor/Rluc and receptor/EYFP fusions in comparison with untagged receptor (5). The identity of the receptor interacting protein (e.g. β-arrestin) will determine which assays should be performed to assess whether function is retained for a particular protein of interest. In the case where β-arrestin was the protein of interest, functionality of β-arrestin/EYFP or Rluc fusions were assessed using i) receptor internalisation assays to measure their ability to promote GPCR internalisation and ii) confocal microscopy to monitor ligand-dependent translocation of β-arrestin/EYFP (5). Forty-eight h following transfection, levels of expressed Rluc tagged protein can be assessed by measuring luminescence using the luminescence mode of the Mithras LB 940 plate reader (see Figure 2A), while levels of expressed EYFP tagged protein can be determined using either the fluorescent mode of the Mithras LB 940 plate reader (Figure 2B) or by fluorescence-activated flow cytometry.

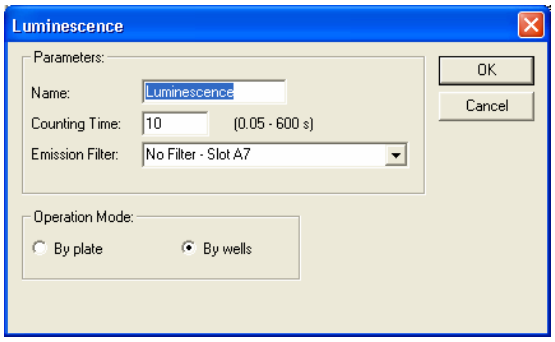

Figure 2A: settings for luminescence mode for measuring Rluc expression. Double-click on 'lumin label' in the left pane of the 'Measurement' tab in the 'Read options' dialog box.

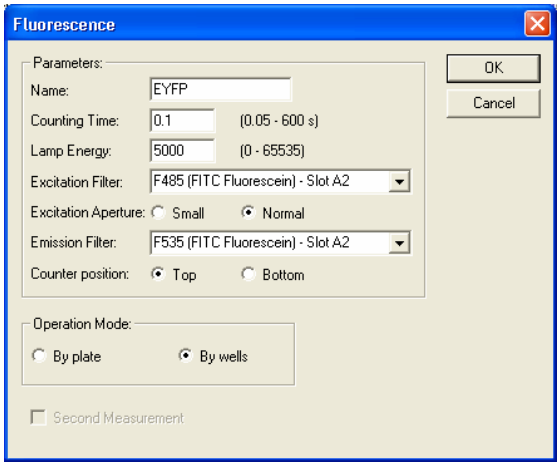

Figure 2B: Settings for fluorescent mode, for EYFP expression. Double-click on 'fluor label' in the left pane of the 'Measurement' tab in the 'Read options' dialog box.

7. COS-1 cells have been used in this BRET assay protocol, however BRET can be performed in any cell type able to undergo transfection. A variety of transfection reagents and techniques can be used to transfect cells with cDNA constructs. In our hands, consistent results were obtained using Polyfect transfection reagent (QIAGEN). However, the transfection reagent used may vary with the cell type chosen.

8. When performing BRET assays, the levels of Rluc compared to EYFP fusion proteins expressed is important. Different amounts of Rluc and EYFP fusion cDNAs should be transfected and assayed to determine the conditions necessary to obtain an optimal BRET signal. Extreme overexpression of fusion proteins is not recommended as this may increase the risk of non-specific BRET signals, hence negative controls such as other Rluc and EYFP tagged proteins expressed at similar levels to the proteins of interest should be included. Certain interactions however (i.e. low affinity interactions), may require higher levels of protein expression to allow detection. In addition to the total amount of cDNA transfected for protein expression, the ratio of donor to acceptor protein expressed is important and the optimal ratio needs to be empirically determined for each protein-protein interaction studied. By titrating the quantity of EYFP fusion protein expression,

whilst keeping the expression of the Rluc fusion constant, the $BRET_{50}$ (the EYFP fusion protein expression as measured by degree of fluorescence, at which the BRET value is half maximal) can be determined as a relative approximate measure of interaction affinities (14). Over-expression of GPCRs in heterologous systems can potentially lead to the generation of artefactual aggregation due the highly hydrophobic nature of these seven transmembrane receptors and non-specific BRET signals (10). Performing BRET at low levels of receptor expression, at or below physiological levels of receptor, may help to prevent non-specific BRET signals. However, protein-protein interactions may vary in their relative affinities and thus different levels of protein expression, and different ratios of donor and acceptor molecules, may be required in order to detect a BRET signal indicative of an interaction.

9. The main feature of an instrument that can detect BRET signals is the ability to sequentially filter, then measure, light emission over two separate wavelength ranges. Several multi-functional plate readers are currently available that can perform this task, however they suffer from limited sensitivity due to compromises in optical systems, the Mithras in contrast is a multifunctional plate reader with multiple dedicated optical systems offering the sensitivity needed to measure BRET signals. (picture, DOPs?) We have collaborated with Berthold Technologies in the development of a 96-well plate reader with the ability to measure BRET signals with high sensitivity, the Mithras LB940 (Berthold Technologies, Inc.). Due to abovementioned problems encountered with extreme over-expression of proteins, it is important that BRET can be performed and signals detected using low levels of protein expression i.e. near/or below physiological levels. Therefore, an instrument capable of detecting low BRET signals to reduce the presence of non-specific BRET signals resulting from high expression levels of BRET fusion proteins is highly desirable.

10. Upon addition of coelenterazine to the cell suspension, light emission should be immediately measured as the luciferase reaction displays rapid decay kinetics. This should ideally be performed by injecting the coelenterazine with the on-board reagent injectors of the instrument.

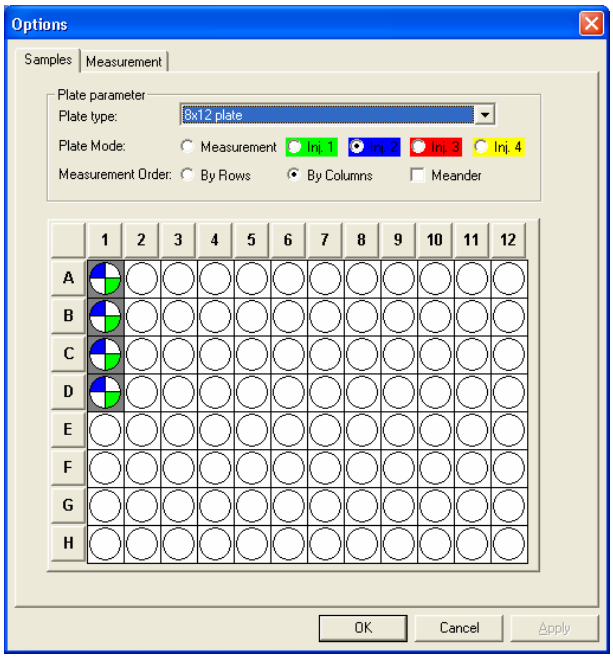

Figure 3A: Selection of wells for injections with substrate and treatment. The 4 injectors allow more than one treatment to be added to different wells as desired.

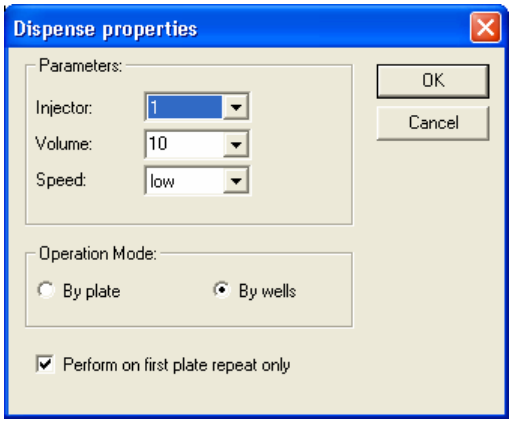

Figure 3B: After selecting wells for injectors, click on the measurement tab, double click on 'dispense' in the left pane and set the properties.

Repeated reads are taken on all samples, as the BRET signal takes several seconds to equilibrate following the addition of coelenterazine. Also, by performing repeated reads following the addition of substrate, the stability of the interaction can be monitored over time. This may be particularly important when monitoring the effect that various agonists or antagonists may have on receptor-protein interactions. By performing repeated reads, a BRET kinetic analysis is being performed essentially allowing an interaction to be monitored in real-time. Although the actual amount of light emitted decreases over time, the BRET ratio of the positive control Rluc-EYFP BRET fusion remains constant for at least 30min after the addition of coelenterazine.

Conclusion

BRET is a novel resonance energy transfer technique that represents a powerful tool for the detection and analysis of a wide range of protein-protein interactions and provides significant advantages over currently used methodologies. It represents a robust, homogeneous live-cell assay system that has been successfully applied to the study of receptor interactions and can be easily adapted for the study of any protein-protein interaction.

Materials

- Black and White Isoplate 96-well (25/pack) Perkin Elmer Cat #1450-581
- PBS Powder 10 x 1L Gibco (Invitrogen) Cat # 21600-010
- Coelenterazine (h form) 250ug Molecular Probes Cat # C-6780
- EYFP vectors – Clontech
  pEYFP-C1 Cat # 6005-1
  pEYFP-N1 Cat # 6006-1
- Rluc vector – Promega
  PRL-CMV Cat # E2261

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