Chapter 5

Use of BRET to Study Protein–Protein Interactions In Vitro and In Vivo

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Abstract

Application of bioluminescence resonance energy transfer (BRET) assay has been of special value in measuring dynamic events such as protein–protein interactions (PPIs) in vitro *or* in vivo. It was only in the late 1990s the BRET assay using RLuc-YFP was introduced for biological research showing its use in determining interaction of two proteins involved in circadian rhythm. Several inherent attributes such as rapid and fairly sensitive ratiometric measurements, assessment of PPI irrespective of protein location in cellular compartment, and cost-effectiveness consenting to high-throughput assay development make BRET a popular genetic reporter-based assay for PPI studies. In BRET-based screening, within a defined proximity range of 10–100 Å, excited state energy of the luminescence molecule can excite the acceptor fluorophore in the form of resonance energy transfer, causing it to emit at its characteristic emission wavelength. Based on this principle, several such donor–acceptor pairs, using the *Renilla* luciferase or its mutants as donor and either GFP2, YFP, mOrange, TagRFP, or TurboFP as acceptor, have been reported for use.

In recent years, BRET-related research has become significantly versatile in the assay format and its applicability by adopting the assay on multiple detection devices such as small-animal optical imaging platform or bioluminescence microscope. Beyond the scope of quantitative measurement of PPIs and protein dimerization, molecular optical imaging applications based on BRET assays have broadened its scope for screening of pharmacological compounds by unifying in vitro, *live cell*, *and* in vivo animal/plant measurement all on one platform. Taking examples from the literature, this chapter contributes to in-depth methodological details on how to perform in vitro and in vivo BRET experiments, and illustrates its advantages as a single-format assay.

Key words Bioluminescence resonance energy transfer, Protein–protein interactions, Cell-based assay, Luciferase, Fluorescent proteins, Optical imaging

1 Introduction

Protein–protein interactions form the key molecular process in a biological system and drive almost all the cellular and molecular functions like cell division, cell signaling, immune responses, and response to environmental stimuli. For a better understanding of how cellular functions are regulated, noninvasive measurement of protein–protein

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interactions in a live cell environment is important. To do so, a sensitive and real-time method that can qualitatively as well as quantitatively measure dynamic events in an unperturbed condition is of high demand. Conventional methods used so far to study protein–protein interactions, like chromatography, co-immunoprecipitation, tandem affinity purification, phage display, and chemical cross-linking [1], are unable to provide a direct insight into macromolecular interactions in live cells maintaining the spatial-temporal information intact. The shortcomings of the above approaches have in part been overcome by newer reporter gene-based strategies like inducible yeast two-hybrid systems, bimolecular fluorescence complementation (BiFC), fluorescence resonance energy transfer (FRET), and bioluminescence resonance energy transfer (BRET)—which can provide visual perception to what is happening to proteins inside the cell and in their native environment.

BRET is based on the principle of Forster resonance energy transfer in which the transfer of resonance energy from excited bioluminescent molecule (called donor) to a fluorescent molecule (called acceptor) forms the basis of detection. In the presence of an appropriate substrate, the bioluminescent reporter protein conjugated to one of the proteins of interest oxidizes the substrate reaching it to the excited state. The excited substrate then releases energy, which is taken up by the fluorescent molecule conjugated to the second protein of interest, when the proximity range is achieved by their interaction. The excited fluorophore then emits the characteristic light at a longer wavelength. The nonradiative transfer of energy between donor and acceptor can take place only when the two molecules of interest are in close proximity, i.e., 1–10 nm of distance, which is a distance for true protein–protein interactions in the physiological and biological environment [2]. Hence a positive BRET signal is an actual interpretation to a true protein interaction (*see* Fig. 1). At the same time, however, absence of BRET signal does not necessarily mean that the two proteins are not interacting, rather their interaction simply failed to achieve the necessary proximity [3]. To produce an efficient BRET output signal, the selection and design of BRET partners should fulfill the following conditions: (1) the distance between the donor and acceptor molecule should be less than 10 nm; (2) spectral overlap between the donor emission and acceptor excitation peak wavelength; (3) relative orientation of donor and the acceptor molecule, i.e., either N-terminus or C-terminus localization in which the dipoles of donor and acceptor are aligned in a way that there is maximum transfer of resonance energy through nonradiative dipole–dipole coupling; and (4) donor quantum output; the higher the donor quantum output the better will be the nonradiative transfer of energy to acceptor and the minimum will be the energy loss due to decay [2]. Inside the cell there are thousands of transient and nonspecific interactions taking place. To differentiate

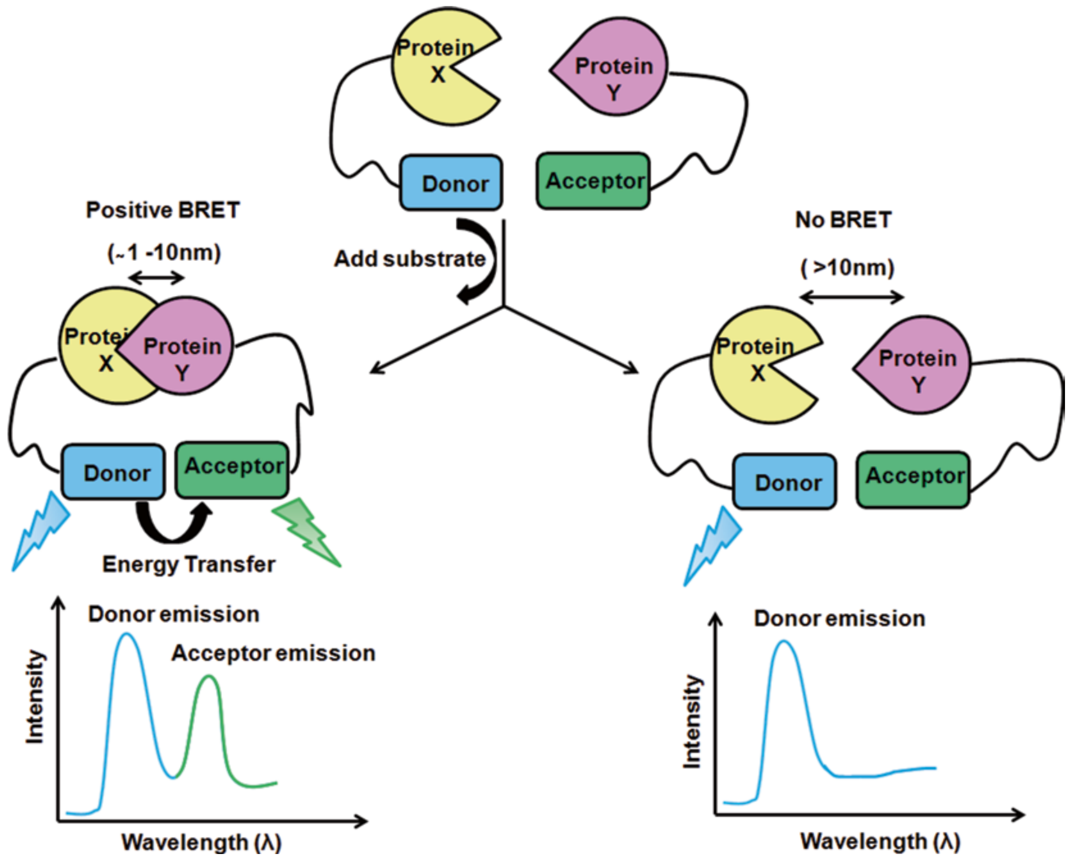


Fig. 1 Diagrammatic representation of a typical BRET assay for determining protein–protein interactions. The protein candidates *X* and *Y* can be tagged with donor and acceptor. If only the two proteins of interest achieve the proximity distance (1–10 nm), BRET occurs in the presence of donor-specific substrate. The transferred resonance energy excites the acceptor fluorophore which then emits at its characteristic wavelength indicating positive interaction of *X* and *Y*. Light signals emitted by both donor and acceptor can be measured by suitable band-pass filters and can be represented ratiometrically as acceptor/donor signal output. If the protein *X* and *Y* fail to achieve the required proximity distance, then only the donor output is obtained

between specific and nonspecific interactions, various formats of BRET assay like donor saturation assay, competition assay, and dilution assay with appropriate positive and negative controls can be performed [4]. These assays not only provide evidence of the specificity of the protein–protein interaction, but can also be extended to study the oligomerization state of receptors [5].

BRET has successfully emerged as a potential, advanced, and noninvasive tool to study a wide variety of assays like protein–protein interactions (e.g., cyanobacterial clock protein-KaiB and light-regulatory basic leucine zipper (bZip) transcription factor-HY5) [6]; oligomerization study of receptors (e.g., GPCRs, receptor tyrosine kinases, and cytokine receptors) [7]; mapping signal transduction pathway; studying protein posttranslational modifications

such as ubiquitination [8], sumoylation [9], phosphorylation [10], and acetylation [11]; and monitoring protease activity in live cells [12]. With the advancements made over the years, various modifications in BRET components have enhanced the overall sensitivity and specificity of the method. The short half-life and low stability of Rluc were overcome by introducing a series of point mutations in the enzyme sequence leading to the generation of Rluc 8.6. This mutant version of Rluc has greater stability and red-shifted emission spectrum which makes it a more appropriate donor for animal imaging [13]. Apart from Rluc, other luciferase enzymes such as *Gaussia* luciferase (19.9 KDa), *Vargula* luciferase (62 KDa), and *Oplophorus* luciferase (18 KDa) have been evaluated for use as alternative BRET assays [14–17]. Modified version of coelenterazine substrate like ViviRen™ and EnduRen™ offer brighter and extended signal output [18, 19]. New acceptor fluorophores like TurboRFP635, mOrange, and mCherry have excitation and emission at higher wavelength and hence serve as invaluable elements in expanding BRET application to in vivo animal imaging [20, 21]. Using new version of acceptor, donor, substrate, and instrumentations (BLI microscopy, IVIS), researchers have extended the protein–protein interaction study from in vitro to single cell, and even tissue-scale in vivo imaging both in plants and animals.

2 Materials

2.1 Construction of Fusion Proteins

1. cDNAs for proteins of interest.
2. cDNA for complementary BRET donor and acceptor. Donors for all common BRET systems used so far are *Renilla* luciferase or its mutants: Rluc for $BRET^1$ [22] and $BRET^2$, Rluc2 or Rluc8 for $BRET^3$ [21, 23], and Rluc8.6 for $BRET^8$ [24]. Acceptor for $BRET^1$ is YFP/EYFP, $BRET^2$ is GFP^2, $BRET^3$ is mOrange, and $BRET^8$ is TurboRFP635.
3. For mammalian cell experiments, an expression plasmid such as pcDNA3.1 (+) or similar is required.
4. 1× Passive lysis buffer (Promega, USA).
5. Bradford reagent (BioRad, USA).
6. 1× PBS (pH 7.0).

2.2 Cell Culture

1. Cell culture plates: 6-Well clear cell culture plates.
2. Cell culture plates: 96-Well white cell culture plates.
3. Cell culture plates: 96-Well black cell culture plates.
4. Cell line for transfection: 293-T, HT1080, COS7, Hela, or any specific type.

5. Appropriate media for the cell line: Typically, Dulbecco's modified Eagle's medium (DMEM) containing 0.3 mg/ml glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS) or other specific medium recommended for specific cell type.
6. 0.05% Trypsin–0.53 mM ethylenediamine tetraacetic acid (EDTA).
7. Transfection system or reagent: Effectene (Qiagen, USA), Lipofectamine2000 (Life Technologies, USA), or any other suitable system.

2.3 BRET Assay Ingredients

1. BRET assay buffer: Dulbecco's phosphate-buffered saline (DPBS) containing 0.1 g/l $CaCl_2$, 0.1 g/l $MgCl_2 \cdot 6H_2O$, and 1 g/l d-glucose.
2. Media for BRET measurement: DMEM without phenol red containing 0.3 mg/ml glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 10% FBS, and 25 mM HEPES.
3. Preparation and dilution of luciferase substrate: Coelenterazine h (Promega or Biotium) is reconstituted in methanol at a concentration of 1 mg/ml for $BRET^1$, $BRET^3$, and $BRET^8$ and stored as stock solution in −80 °C freezer. For $BRET^2$, coelenterazine 400a (Molecular Imaging Products Company or Biotium) is reconstituted in anhydrous or absolute ethanol and stored as stock solution (*see* **Note 1**). Just before the experiment, dilute the substrate by adding 10 μg of coelenterazine stock per 100 μl of DPBS. If higher concentration of coelenterazine (80–100 μg) is required, directly dissolve coelenterazine powder in 50% ethanol and 50% PEG mix. EnduRen (Promega) at a stock concentration of 60 mM is reconstituted in cell culture-grade dimethylsulfoxide (Sigma) for eBRET, $BRET^3$, and $BRET^8$ measurement. Extensive vortexing up to 10 min and warming to 37 °C are required during reconstitution of EnduRen. EnduRen stocks can be stored at −20 °C protected from light and moisture.
4. Dilution of luciferase substrate in appropriate assay buffer: Typical assay buffer for coelenterazine h and coelenterazine 400a is d-PBS with $CaCl_2$, $MgCl_2$, and d-glucose (Gibco) and the final concentration of the substrates is 5 μM. Enduren is diluted in a final concentration of 30–60 μM in HEPES-buffered DMEM without phenol red at 37 °C (*see* **Note 2**).
5. Ligand or other modulating reagent: Depending on the interaction being assayed, stock and working solutions are to be made and stored as per the manufacturer's recommendation.
6. Selection of antibiotics such as geniticin (100 mg/ml stock concentration), zeocin (100 mg/ml stock concentration), or puromycin (10 mg/ml stock concentration) depending on the marker present on the vector backbone.

2.4 Animals

For studying protein–protein interactions in live animal subjects, the following considerations should be kept in mind: select animals of same strain, sex (sex has to be determined as per the experimental need), weight, and age group (*see* **Note 3**). For conducting animal experiments generally prior permission is required as per the institutional and national animal ethical guidelines.

2.5 Measurement Equipment

1. Standard cell culture facility including class II biological safety cabinet.
2. 37 °C Incubator with 5% CO_2.
3. Fluorometer (Fluoroskan Ascent™) or scanning spectrophotometer with 96-well plate capability.
4. Microplate Luminometer like LUMIstar Optima (BMG Labtech, Germany), Mithras LB 940 (Berthold Technologies, Germany), or several other company brands compatible for performing simultaneous dual-channel photon measurement using donor- and acceptor-specific filter sets can be used. Photon measurements can be done using 0.5–5-s integration time per filter (e.g., for $BRET^8$ 540 nm with 10 band-pass as donor and 630 nm with 10 band-pass filter as acceptor when using Enduren or coelenterazine h as substrate). Simultaneous detection increases accuracy and reduces measurement time, and thus would be suitable for high-throughput screening assays.
5. IVIS Lumina, IVIS200, IVIS Spectrum (Perkin Elmer, USA) equipped with 20 nm band-pass spectral filter sets (typically range varies from 450 to 800 nm) or similar other BRET-compatible brands for live cell or in vivo tissue-scale animal imaging.
6. Dual-View microimager (Optical Insights, Tucson, AZ) with modified electron bombardment-CCD camera: It is an emission splitting system that allows user to acquire spectrally distinct but spatially identical images simultaneously. The microimager consists of a dichroic mirror that can split the image into two distinct wavelengths—above and below 505 nm, and the interference filters allow refinement of the distinction [25].
7. Olympus LV200 luminescent microscope (Olympus America, Inc., New York, USA) with respective donor filter and acceptor filters: It has designed optical elements to enhance collection and transmission of light through the specimen. It can co-image phase contrast, transmitted fluorescence, and bright field with luminescence signal and allows detection of localization and co-localization of the luminescence signals with fluorescence probe in tissues or in cells.

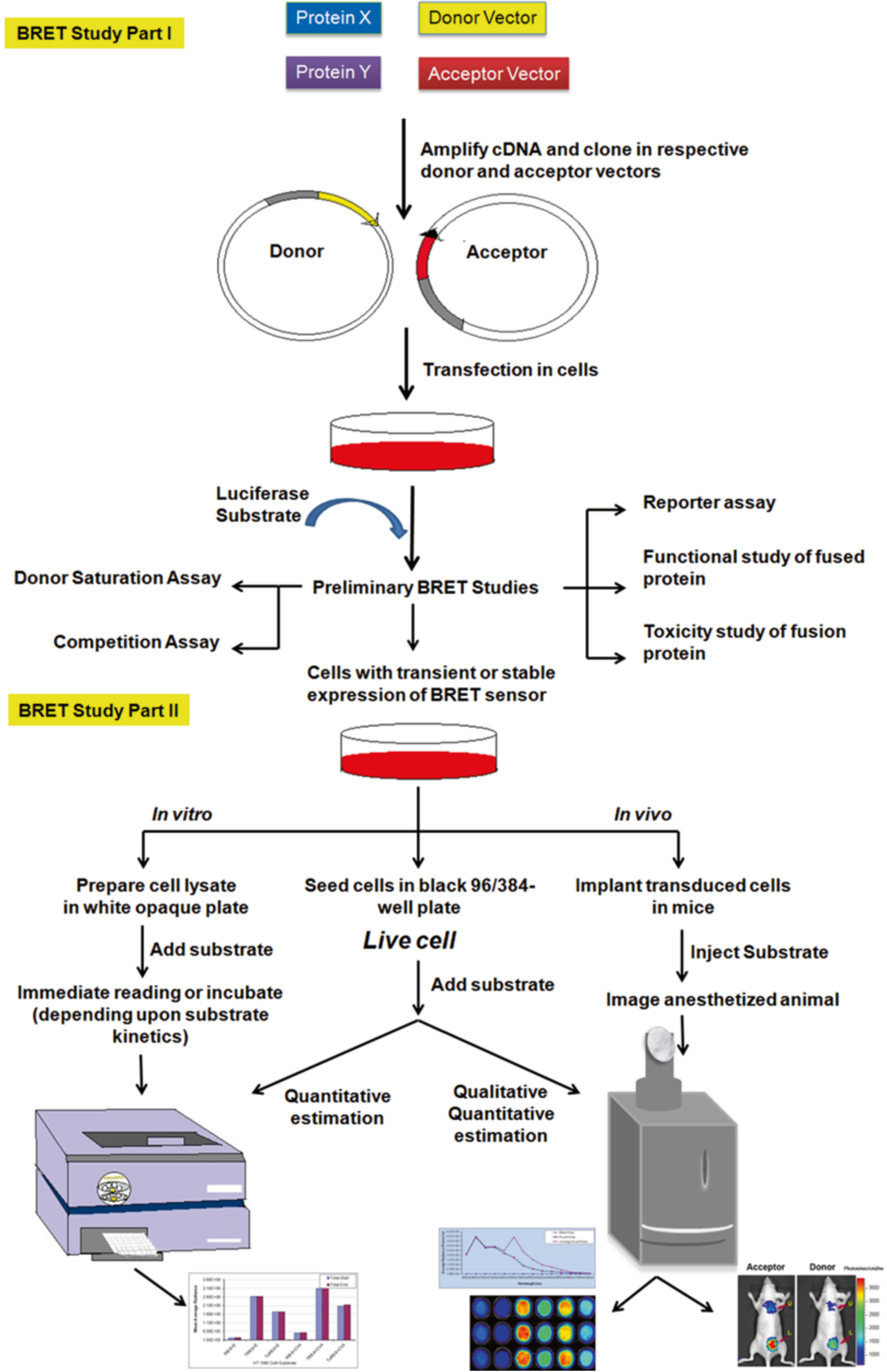
3 Methods

3.1 Basic BRET Vector Design and Optimization

1. First select an appropriate donor (luciferase protein) and acceptor (fluorophore) pair with suitable substrate required (*see* Table 1). In many cases N- or C-terminal fusion vectors are available for cloning and expression in mammalian cells. Make sure that for dual selection, different selection markers (e.g., neomycin or zeomycin or puromycin) are inserted in the donor- and acceptor-containing plasmids (*see* Fig. 2).
2. To make fusion constructs PCR amplify the cDNA of target proteins, e.g., X and Y (where X and Y are intended to be interacting partners), donor (e.g., Rluc8.6), and acceptor proteins (e.g., TurboRFP635), flanked by unique restriction sites required for cloning at MCS of expression vector.
3. Insert the cDNA of target protein in frame with cDNA of donor or acceptor protein. If required separate the two proteins using cDNA for a flexible linker. Presence of a linker between

Table 1
Table highlighting the key features of existing and newly developed BRET assays using *Renilla* luciferase. Modified with permission [24]

Assay	Donor	Acceptor	Substrate	Spectral resolution (nm)	Dynamic range	Efficiency
BRET[1]	RLUC *480 nm* (Improved version using RLUC2/RLUC8)	YFP/EYFP *535 nm*	Clz/Enduren™	55	Small	Moderate
BRET[2]	RLUC *400 nm* (Improved version using RLUC2/RLUC8)	GFP[2] *515 nm*	Clz400/protected Clz400	115	Very large	Moderate
BRET[3]	RLUC8 *480 nm*	mOrange *564 nm*	Clz/EnduRen™	85	Large	Moderate
BRET[4]	RLUC8 *480 nm*	TagRFP *584 nm*	Clz/EnduRen™	104	Large	High
BRET[5]	RLUC8 *515 nm*	TagRFP *584 nm*	Clz -*v*	70	Moderate	Low
BRET[6]	RLUC8.6 *535 nm*	TagRFP *584 nm*	Clz/EnduRen™	50	Large	High
BRET[7]	RLUC8 *480 nm*	TurboFP *635 nm*	Clz-*v*	155	Small	Low
BRET[8]	RLUC8.6 *535 nm*	TurboFP *635 nm*	Clz/EnduRen™	100	Moderate	Moderate


BRET Study Part I
Protein X
Donor Vector
Protein Y
Acceptor Vector
Amplify cDNA and clone in respective donor and acceptor vectors
Donor
Acceptor
Transfection in cells
Luciferase Substrate
Reporter assay
Functional study of fused protein
Donor Saturation Assay
Preliminary BRET Studies
Competition Assay
Toxicity study of fusion protein
Cells with transient or stable expression of BRET sensor
BRET Study Part II
In vitro
In vivo
Prepare cell lysate in white opaque plate
Seed cells in black 96/384-well plate
Implant transduced cells in mice
Live cell
Add substrate
Inject Substrate
Immediate reading or incubate (depending upon substrate kinetics)
Add substrate
Image anesthetized animal
Quantitative estimation
Qualitative Quantitative estimation
Acceptor
Donor

donor and acceptor protein allows the fusion construct to fold properly and minimize the conformational constraints.

4. To ensure generation of fusion protein remove stop codon between the two cDNAs either using site-directed mutagenesis or delete using primer-based amplification.
5. To optimize suitable dipole orientation, while making BRET vectors, prepare all eight plasmid clones to find the best possible combination, i.e., pX-Rluc8.6 + pY-TurboRFP635 and pRluc8.6-X + pTurboRFP635-Y, pY-Rluc8.6 + pX-TurboRFP635 and pRluc8.6-Y + pTurboRFP635-X. An absence of BRET signal does not always mean that the two proteins are not interacting. It is possible that donor and acceptor dipoles are not aligned optimally to allow sufficient transfer of resonance energy. In this case clone donor–acceptor in both the orientation, i.e., N- and C-terminus, and select the appropriate orientation (*see* **Note 4**).

3.1.1 Fusion Construct Validation

1. Co-transfect cells with donor fusion construct and acceptor fusion construct that had the maximum BRET output signal. Simultaneously transfect cells with donor alone and keep a set of untransfected cells as well. Validate the BRET constructs either by preparing cell lysates or using live cells for imaging.
2. Fluorescence and luminescence study should also be done for transfected cells to judge the relative protein expression level. This is of particular significance while doing competition and saturation assay because the fluorescence and luminescence study will confirm that the changes in BRET signal obtained are not the result of the low expression of tagged proteins but the resultant of the assay [4].
3. Make 40–100 μl aliquots of sample/well in a 96-well plate, diluted in 1× PBS. Excite the fluorophore by using laser at specific wavelength, i.e., excitation wavelength of fluorophore in fluorometer/scanning spectrometer/flow cytometer, and collect the emission output signal at respective filter, i.e., emission wavelength of the fluorophore. For background correction of fluorescence, excite the untransfected cells also at the same wavelength followed by collecting the emission at respective filter. For example, TurboRFP635 has excitation maximum (Ex^{MAX}) at 588 nm and emission maximum (Em^{MAX}) at 635 nm.

Fig. 2 Diagrammatic illustration of basic steps involved in establishing BRET assay. BRET study 1 outlines the primary steps starting from vector selection to preliminary BRET methods required prior to in vitro or in vivo experiments. BRET study 2 highlights the different formats in which BRET assay can be performed using cell lysates, live cells, or animal model. For measuring donor and acceptor light output, a microplate reader can be used for experiments where only cell lysates or cultured live cells are involved, whereas CCD-coupled black box imaging device can be adopted for simultaneous quantitative and qualitative measurements at all levels. Bioluminescence imaging (BLI) microscopy or recently developed dual-view microimager with modified electron bombardment-CCD camera can also be used for live cell BRET measurement

4. Luminescence study can be done on same set of samples as used for fluorescence study. Add substrate, diluted in respective assay buffer, to the samples in 96-well plate and take readings in luminometer. Use only luciferase-expressing cell as positive and untransfected cell as negative control to compare luciferase activity. For background correction add substrate to untransfected cells as well and check luminescence signal, if any.
5. Check the functionality of target protein after fusion (as sometimes fusion tags might affect the biological function of the target protein) by performing respective functional assays of the protein, e.g., kinase assay, or localization studies using immunofluorescence/confocal microscopy [26].
6. Western blot should also be done to ensure that the fusion protein is fully translated by checking the size reference in the blot.
7. If one experiences cell death or change in cell growth pattern after transfection of BRET fusion plasmids, a cell viability assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] or trypan blue exclusion assay should be performed.

3.2 BRET for Protein–Protein Interaction Measurement

The technique of BRET has successfully been evolved to study protein–protein interaction in vivo and an increased BRET signal indicates the proximity achieved. However BRET does not provide a direct measure of physical interaction between the two proteins of interest. The same can be validated by various other assay formats like saturation assays, competition assays, and dilution assays [7].

3.2.1 BRET Saturation Assay

BRET saturation assay has successfully been used to demonstrate receptor oligomerization. It provides direct evidence for specific interaction as well as presence of physical contact between the two proteins in question. The saturation assay involves one protein tagged with donor molecule expressed in constant amount and subsequently increasing the amount of other protein tagged with the acceptor molecule. For specific interaction as the concentration of acceptor molecule increases the BRET signal will keep on increasing and attain saturation ($BRET_{max}$) level once all the donor molecules are occupied (hyperbola curve). Beyond $BRET_{max}$ any further increase in acceptor concentration will not enhance the BRET output signal. On contrary if the interaction is not specific or merely the result of random collision between the two proteins, then the BRET signal will continue to increase with increasing acceptor concentration in a quasi-linear fashion [7]. To perform BRET saturation assay, follow the steps below:

1. Seed cells in 6-well plate and culture for 24 h before transfection.
2. Transfect cells using appropriate transfection kit with constant amount of donor plasmid and increasing amount of acceptor

plasmid. In parallel maintain a set of donor-only transfected cells and untransfected cells for background correction.

3. Incubate at 37 °C and 5 % CO_2 for 24 h.
4. 48 h post-transfection, wash cells with 1× PBS (twice) and seed cells in 96-well plate (in duplicate or triplicate) at a density of ~100,000 cells/well for BRET assay.
5. Repeat **steps 3** and **4** of Subheading 3.1.1 for fluorescence and luminescence study.
6. Incubate at 37 °C for another 24 h to allow cells to adhere.
7. Dilute substrate in suitable medium/buffer and add to cells. Either take the BRET reading immediately or incubate cells depending upon the substrate kinetics using luminometer or IVIS.

3.2.2 Competition Assay

The specificity of protein–protein interaction can further be validated by BRET competition assay. In this assay the acceptor-tagged protein and donor-tagged protein are co-expressed along with an untagged protein or in the presence of an inhibitor that competes with one of the tagged proteins for interaction, at a single concentration (generally excess) or in a dose-dependent manner. If the two tagged proteins are specific interacting partners then with increasing concentration of the inhibitor or untagged competing protein the BRET signal will go down. In parallel also transfect an untagged noninteracting protein or a nonspecific inhibitor as a negative control that will not affect the BRET signal as it cannot interact with the tagged proteins [27]. The competition assay has been successfully used to study the oligomerization of GPCR receptors and dimerization of melatonin receptor [28].

1. Seed cells in 6-well plate and culture for 24 h before transfection.
2. Transfect cells using appropriate transfection kit with constant amount of donor plasmid and acceptor plasmid in the ratio of 1:1. In parallel maintain a set of cells transfected with noninteracting untagged protein plasmid at a single dose (excess) or increasing dose, untransfected cells, cells transfected with donor alone, and cells transfected with acceptor alone for background correction.
3. 48 h post-transfection, wash cells with 1× PBS (twice) and seed cells in 96-well plate (in duplicate or triplicate) at a density of ~100,000 cells/well.
4. Before taking BRET reading aliquot 40–100 μl sample and dilute in 1× PBS and 0.1 % BSA for fluorescence and luminescence analysis.
5. Repeat **steps 6** and 7 of Subheading 3.2.1.
6. If an antagonist is to be used in place of untagged interacting protein, after 48 h of transfection incubate ~10,000 cells in 1×

PBS and 0.1% BSA with a single excess dose of the radiolabeled antagonist or with the increasing concentration.

7. To check the nonspecific binding in negative control, treat cells with a radiolabeled nonspecific inhibitor that will not affect the interaction between tagged proteins.
8. Carry out the binding reaction at room temperature for 90 min and then stop the reaction by filtering through the Whatman/glass fiber filter.
9. The linear regression curve can be plotted between fluorescence and luminescence signal and total amount of tagged protein as determined using radiolabeled ligand binding in cells expressing each of the constructs individually.

3.3 In Vitro BRET Measurement from Cell Lysate

1. On day 1, seed cells in 6-well plate using the recommended complete culture medium and incubate at 37 °C, 5% CO_2. Typically there will be 50–80% confluency after 24 h of seeding. For example, 293 T or HT1080 cells are plated out at a density of $1.5–2 \times 10^5$ cells/well in 6-well plate. However, the amount of cells to be seeded will differ depending upon the growth rate, size of the cells, and the transfection kit protocol in use.
2. The following day, co-transfect cells with donor fusion construct and acceptor fusion construct, cloned in best orientation for optimal BRET signal, using suitable transfection reagent in accordance with the manufacturer's protocol. Depending on the form of analysis, a population of cells expressing only donor-labeled proteins at similar expression levels to those co-expressing donor- and acceptor-labeled proteins need to be generated. Also use suitable positive- and negative-control plasmids in parallel wells. Positive controls can be well-established interacting partners while the negative controls can be a biologically inactive mutant of one or both of the two protein partners. For background correction of fluorescence and luminescence, maintain a population of untransfected cells in parallel. Total amount of DNA used in transfection should be constant.
3. If interaction study of the two proteins of interest requires the presence of ligand or any other reagent, pre-treat the cells with the same at an appropriate time after transfection (after 24 h) and before BRET detection. Only vehicle-treated cells will serve as negative control.
4. The optimal expression time for transiently transfected proteins should be established before.
5. 24–48 h after transfection, aspirate medium from the cells and wash twice with 1× PBS. Trypsinize the cells and obtain cell pellet by centrifuging at 400*g*- force for 5 min, 4 °C.
6. Add 1× passive lysis buffer to the cell pellet at a volume approximately equal to thrice of the cell pellet.

7. Vortex to detach the pellet (if frozen, thaw on ice) after addition of lysis buffer and keep on ice for 20 min.
8. Collect supernatant by spinning the tubes at 2000*g*-force for 30 min, 4 °C.
9. Determine the protein concentration by mixing 5 μl of supernatant of each sample with 1 ml of 1× Bradford reagent. Mix well and take absorbance at 595 nm. Calculate the protein concentration using standard curve determined from known protein samples (e.g., BSA).
10. Prepare different dilutions of sample in 1× passive lysis buffer and aliquot 100 μl in 96-well plate. Add coelenterazine (1 μg/well concentration).
11. Take readings immediately on luminometer or microplate reader or IVIS Spectrum/IVIS 200.

3.4 BRET Measurement from Live Cell

Live cell protein–protein interactions are dependent on factors like subcellular localization, posttranslational modifications, and competitive interactions with other cellular partners. Currently used in vitro drug/ligand screening platforms are controlled and artificial, while for in vivo screening the drug/ligand should cross the cell plasma membrane, and reach their target protein in subcellular compartments with enough specificity to compete and interact exclusively with its target minimizing the potential interaction with thousands of other intracellular compounds. Thus the live cell protein–protein interaction studies are advantageous over classical in vitro biochemical analyses like co-immunoprecipitation, co-purification analysis, as in this case the host cell acts as a live cell test tube and allows noninvasive, quantitative, real-time readout of protein interactions in live cells even in single live cell format [21]. The live cell image can be performed in multiple formats as follows:

3.4.1 Live Cell BRET Measurement of Adherent Cells

1. Same as in Subheading 3.3, **steps 1–4**.
2. 24 h post-transfection, trypsinize the cells with trypsin–EDTA.
3. Resuspend cells in HEPES-buffered DMEM without phenol red and split the cells (in triplicate) at a density of 10,000–30,000 cells/100 μl/well in 96-well white cell culture plate, if measurement is being carried out by luminometer or into 96-well black cell culture plate, and if measurement is being carried out by IVIS.
4. Maintain cells at 37 °C, 5 % CO_2, in a humidified incubator for further 24 h before BRET assay, to allow cell attachment. To establish a suitable cell dilution initial titration is required.
5. Carry out the BRET assay in existing phenol red-free medium or replace medium with suitable assay buffer such as DPBS.

6. Remove medium from the cells and add substrate prepared in respective assay buffer. Following addition of coelenterazine h and 400a, BRET is determined immediately, while for EnduRen, incubate cells at 37 °C and 5 % CO_2 in incubator for at least 1 h following addition of substrate and before proceeding for BRET (*see* **Note 5**).
7. Initialize the instrument and place the 96-well black culture plate inside the black box imaging chamber and close the door.
8. On IVIS software (Living Image software) set different parameters like imaging mode as luminescence, exposure time, binning (balances between sensitivity and resolution of the CCD camera, usually set at medium), emission filter (as per the acceptor emission wavelength), field of view (FOV, defines the size of the squares in the alignment grid), and subject height (0.5 cm for plate). Acquire spectral scan of the plate by selecting filters starting from 480 to 800 nm or above as per the acceptor emission wavelength. Acquisition time may vary from seconds to minutes depending upon the reporter and the substrate kinetics. If an interaction has to be monitored at different time intervals, the IVIS instrument has the facility of setting up delay time for spectral scan ranging from seconds to minutes. To acquire image make sure that the "Photograph" and "Overlay" (to obtain a co-registered image) buttons are in on mode. Then go to image setup and select respective emission filters and acquire the sequences by clicking "acquire sequence" button or set time points for spectral scan in delayed kinetic assays.

   If fluorescent needs to be measured, set imaging mode in fluorescent, and the excitation filter is automatically selected based on the wavelength of the emission filter selected from the IVIS System control panel. However, the automatic selection in the IVIS System control panel can be overridden.
9. At first the camera acquires the photograph of the plate followed by the luminescence for the set period of time. As soon as the acquisition is over, a superimposed image of the photograph and pseudocolor luminescence image will appear on the screen.
10. After image acquisition, save the image data to desired location. For data analysis, draw ROI on the target sites; the measured photon values expressed as photons/s/cm^2/sr (steradian: a measure of solid angle) will be displayed in a new window that can be exported to Microsoft Excel for further use and statistical analysis. However, the total photons (photon/s) from a specific ROI can also be used for the analysis.
11. Calculate BRET ratios as described in Subheading 3.6.

3.4.2 Live Cell BRET Measurement of Cell Suspensions

1. Same as in Subheading 3.3, **steps 1–4**.
2. Typically 48 h after transfection, detach the cells using trypsin–EDTA.

3. Resuspend cells in suitable BRET assay buffer at required dilution and plate in 96-well white cell culture plate or 96-well black cell culture plate depending on the instrument used.
4. Same as in Subheading 3.4.1, **steps 7–11**.

3.4.3 BRET Kinetic Measurement

General substrates for Rluc-based BRET assays are coelenterazine h or DeepBlueC™. However these substrates have major drawbacks like less stability in aqueous solution at physiological temperature (37 °C) and enhanced autofluorescence in the presence of serum that limits its use for assays with prolonged kinetics. A new or modified version of coelenterazine, EnduRen™, has been produced that can be activated only and after being acted upon by cellular esterase in live cells to produce free coelenterazine h. Once coelenterazine h is produced it can interact with the respective donor molecule to produce the BRET output signal. This version of BRET measurement of dynamic events was named as eBRET or extended BRET that utilizes EnduRen™ as luciferase substrate (*see* **Note 6**). The method provides potential advantage of monitoring protein–protein interaction in live cells under physiological conditions for prolonged hours without significant depletion of the output signal [18].

The IVIS Spectrum/IVIS 200 optical imaging systems provides option of performing BRET assay using sequential mode wherein image sequences can be collected at delayed time frame defined by user. Using this feature, it is possible to capture the kinetics of a protein–protein interaction assay in a real-time manner.

3.4.4 Live Cell Imaging Using BLI Microscope

1. Seed the cells in 6-well plate.
2. Transient transfection or stable cell generation with respective plasmids.
3. Addition of respective ligand and substrate. Allow substrate incubation at 37 °C, 5% CO_2, up to 2 h, if the substrate is EnduRen™.
4. Image cells with an Olympus LV200 luminescent microscope (Olympus America, Inc., Melville, NY, USA) with respective donor filter and acceptor filters.
5. Adjust the acquisition time to resolve the cells clearly.
6. For measuring mean integrated pixel densities on regions of interest software like ImageJ (NIH, Bethesda, MD, USA) can be used.
7. Do the ratiometric calculation of acceptor/donor signal (refer to Subheading 3.6).

3.4.5 Multiplexed BRET Assay Using Spectral Imaging

This can be performed either from live cell or cell lysate. Protocol for assay setup is same as described in Subheading 3.4.2, except that the scan has to be performed using IVIS Spectrum or IVIS200 loaded

with 20 nm spectral band-pass filter ranging from 460 to 800 nm. Using this equipment, it is possible to set a sequential scan using emission filter sets ranging from 480 nm to 700 nm (that is where most of the current BRET donor and acceptor elements emit). Total spectral scan time may vary according to reporter intensity, but generally require 2–5 min.

1. Ideally for spectral scan procedure one should use substrates that are soluble, stable at 37 °C, and protected in live cell to yield stable signal intensity for certain time or during the course of the assay. These properties of substrate allow for BRET spectral studies of cellular functions at extended time scale.
2. To control signal intensity variation, the scan sequence should collect image using total light at the beginning and at the end of the spectral images. It should be assured that the total light output remain unaltered during the spectral scan. Any decrease or increase in the total light output will affect or produce error in the calculation of BRET ratio for protein interaction assay.

3.5 In Vivo BRET Measurement from Animal Model

3.5.1 Cell Implantation

For short-term protein–protein interaction studies either use transiently transfected cells or cells stably expressing the fusion construct: (1) donor alone, (2) positively interacting fusion constructs, and (3) negatively interacting fusion constructs. A maximum of four sites can be used in single-animal model for implanting cells. On average cells between one and five million can be implanted in a single animal.

To study the ligand-dependent protein–protein interaction in living animals, the mice is first injected with suitable concentrations of the ligand (reagent) through tail vein while the control mice receives the same volume of vehicle.

3.5.2 Animal Anaesthesia

Before conducting any animal experiment a project license must be obtained from both the local and national animal ethics committee that ensures that all ethical concerns are addressed prior to conducting animal experiments. All experimentation protocols should be ethical and humane and only a well-trained person should be allowed to handle and experiment on live animals. It is also important that the individual must follow all the local and national guidelines set up for ethical use and care of animals during performing an experiment.

Intraperitoneal (i.p.) injection (40 μl/25 g body weight) of ketamine and xylazine solution at a ratio of 4:1 can be used for anesthetizing mouse. This method can be used for imaging experiments lasting up to 30 min. For experiments where repeated mouse imaging within a day is required, try to use isoflurane gas anesthesia (*see* **Note 7**).

3.5.3 Instrument Setup

In the meantime initialize the IVIS instrument. Adjust different parameters same as in Subheading 3.4.1, **step 8**. For animal imaging set subject height at 1.5 cm. FOV is adjusted at 25 cm^2 for five mice and 10 cm^2 for one mice, and it varies depending upon the number of mice to be scanned at a time.

3.5.4 Substrate Delivery

1. Preferred location: Lateral veins on animal tail are the preferred location for i.v. tail vein injection.
2. Needle used for mice: sterile small 28–30 G, usually used with 1 cc insulin syringe.
3. A dose of 1 mg/kg body weight of coelenterazine is recommended. For example, for a mouse weighing 20 g, inject 200 μl of 1 μg/10 μl to deliver 20 μg of coelenterazine. Higher coelenterazine concentration up to five times (100 μg) can be required for some applications (*see* **Note 8**).
4. Inject the substrate via intravenous (i.v.) tail vein route and image immediately by placing the subject inside the imaging platform (*see* **Note 9**).
5. Injection method: Under deep anaesthesia place the animal on side. Grasp the tail at the distal end. Place the index and middle fingers of the non-dominant hand around the tail above the site of needle insertion (these fingers act as a tourniquet) and the lower part of the tail is held between the thumb and ring fingers below the injection site. Slight opposing pressure is applied with both sets of fingers to straighten and stabilize the tail. Needle should be level-side up and slightly angled when entering the veins. It should be advanced parallel to the vein approximately ½ (~5 mm) of the tail length; protrude the needle into the vein being very careful not to perforate the vein. Draw back on the syringe slightly and look for traces of blood flow into the needle hub indicating that the needle is successfully inserted into the vein. Release pressure before administering the substrate steadily over few seconds into the vein. There should be minimal resistance during injection. Remove the needle and apply gentle compression until bleeding stops and perform scan by placing the animal within the field of view of the imager. Return animals to their cage and observe for 5–10 min to make sure that bleeding has stopped (*see* **Notes 10–12**).

3.5.5 Image Acquisition in Dark Chamber Using Cooled CCD Camera in IVIS

1. Place the anesthetized animal inside the temperature-controlled lighttight black box imaging chamber, either prone or supine, depending on the site of cell implantation or tumor growth and close the door. For example, if the implanted cells are on back, place the animal exposing dorsal side towards the camera

so that the path length of fluorescent light through different tissues or organs is minimized.

2. Acquire, save, and analyze data as described in Subheading 3.4.1, **steps 9–11**.

3.6 BRET Data Analyses

3.6.1 BRET Ratio Calculation and Interpretation

1. BRET ratio is calculated as the "emission through the acceptor wavelength filter" divided by the "emission through the donor wavelength filter." For example, in $BRET^8$ with TurboRFP as acceptor, emission through 630 nm filter over 540 nm filter is to be measured.
2. Measurement of BRET ratio in in vitro and in vivo studies is carried out using the following generalized equation [3]:

$$BRET\ \ ratio = \frac{BL\,emission\,(Acceptorl) - cf \times BL\,emission\,(Donor\,\lambda)}{BL\,emission\,(Donor\,\lambda)}$$

$$Cf = \frac{\mathrm{BL}_{\mathrm{emission}}\,(\mathrm{Acceptor}\ \lambda)_{\mathrm{donor\ only}}}{\mathrm{BL}_{\mathrm{emission}}\,(\mathrm{Donor}\ \lambda)_{\mathrm{donor\ only}}}$$

where BL is the average radiance and *Cf* is the correction factor.

3. To study ligand- (reagent-) induced protein–protein interaction, BRET data are collected prior to addition of ligand (reagent). Then ligand (reagent) is added preferably through an injector (available with luminometer, BMG Labtech), if post-addition early time points (<1 min) are needed. Repeated measurements are taken over a period of time to determine the effect. To provide the control for background signal, vehicle-treated samples are measured in parallel.
4. The BRET ratio measurement for ligand-induced (reagent) interaction involves BRET ratio from both ligand (reagent)-treated and vehicle-treated samples (samples can be cells or cell lysates from cells co-expressing donor and acceptor fusion proteins). BRET ratios of both ligand (reagent)-treated and vehicle-treated samples need to be subtracted first from BRET ratio of untransfected cells. Then subtract the BRET ratio of vehicle-treated samples from the ligand (reagent)-treated samples which gives us the "ligand (reagent)-induced BRET ratio" (*see* **Note 13**).
5. If the ligand treatment results in a negative BRET ratio, it may imply that there are no or weaker interactions and/or more transient interactions than those observed prior to ligand addition. This may be the case of conformational change of the interacting proteins resulting in the greater distance between them or less favorable relative orientation.

6. The BRET signal can be plotted against time to obtain a time kinetics profile from which apparent association (or dissociation) rate constants can be find out.
7. Ratiometric analysis of PPIs by a BRET in tissue background is hindered considerably by higher tissue attenuation for shorter wavelength light as compared to longer wavelength light (especially >600 nm), which is mainly associated with absorption by hemoglobin and myoglobin [29]. To ensure that the BRET ratio remains constant between cultured cells and mice, the imaging results may be analyzed using the double-ratio (DR) method [30], which partially corrects for signal attenuation (*see* **Note 14**).

   The equation for calculating double ratio is as follows:

$$DR = \frac{\dfrac{BL_{emission}\left(\text{Acceptor }\lambda\right)_{BRET} \quad \mu t\left(\text{Acceptor }\lambda\right)}{BL_{emission}\left(\text{Donor }\lambda\right)_{BRET} \quad \mu t\left(\text{Donor }\lambda\right)}}{\dfrac{BL_{emission}\left(\text{Acceptor }\lambda\right)_{\text{donor only}} \quad \mu t\left(\text{Acceptor }\lambda\right)}{BL_{emission}\left(\text{Donor }\lambda\right)_{\text{donor only}} \quad \mu t\left(\text{Donor }\lambda\right)}}$$

   which is independent of μt (total attenuation coefficient).

4 Notes

1. Coelenterazine is prone of self-oxygenation in the presence of light and at higher temperature. Therefore, always store coelenterazine in dark tubes stored at −80 °C freezer. For running use, store at −20 °C.
2. Dilute the substrates in appropriate assay buffers immediately before adding to samples and protect from light. For EnduRen to avoid precipitation, preincubate the assay buffer at 37 °C.
3. Black mice show 10× reduction in bioluminescence and 20× reduction in fluorescence signal. Hence it is recommended to restrict experiments to the use of nude or white fur mice.
4. Apart from dual-vector construction for donor and acceptor proteins, a single-vector format can also be used in which both acceptor and donor proteins are cloned along with the interacting protein partners in a single-vector backbone (e.g., pRluc8.6-X-Y-TurboRFP635 or pRluc8.6-Y-X-TurboRFP635). Previously De A et al. have demonstrated construction of such single-vector (*pCMV-mOrange-FRB-FKBP12-RLuc8*) plasmid [21].
5. Washing is generally not recommended when conducting experiments in live cell format as cells may get washed off from well causing difference in light output. Many cell types are loosely

attached on the plate; hence extreme care should be taken during medium aspiration, if any.

6. Activation of EnduRen™ requires activity of cellular esterases; hence EnduRen™ can only be used for live cell BRET imaging and not for in vitro BRET studies.
7. Anesthetic drugs are highly regulated products, available under licensed prescription only. Stage temperature should be maintained at 37 °C, to avoid drop in the animal body temperature during and after imaging experiments, until the animal returns to conscious state.
8. Coelenterazine is prone to self-oxidation at room temperature and light; thus working solutions must be kept until use in ice and light-protected condition.
9. As coelenterazine has flash time kinetics and signal decays very quickly, a coelenterazine kinetic study should be performed for each animal immediately after substrate administration. Generally tolerated volume of i.v. injection is up to 200 μl of aqueous solution.
10. Great care needs to be taken during the tail vein injection. A successful tail vein flushes out the red color of the vein while pushing the injection and a blood droplet oozes out after the needle is withdrawn. If the vein is missed during injection, the substrate will then go into the surrounding subcutaneous and dermal tissues, resulting in a blanching and bulging at the injection site. If this occurs, carefully withdraw the needle and reattempt injection at a more proximal (towards the mouse's body) location.
11. Body temperature of the animal must be maintained at 37 °C during i.v. injection, preferably by means of a heated stage or heating lamp.
12. Care should be taken not to spill coelenterazine during injection as any spillage of substrate at the site of injection can give rise to strong background signal due to autofluorescence of coelenterazine.
13. For ligand-induced interactions donor-only controls are not needed as vehicle-treated samples will represent the background. Moreover, since BRET-based assays are ratiometric, any variability due to assay volume or cell number variation or time point of measurement is nullified.
14. DR is a dimensionless parameter independent of the total attenuation coefficient assuming that the attenuation coefficient is constant for all mice and identical over the entire thorax area. The DR method provides a depth and number of reporter cell-independent measure of the BRET signal; however, both donor and acceptor signals used to calculate the DRs decrease with tissue depth.

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