Protocol

TD-P Revision 3.0

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BRET (Bioluminescence Resonance Energy Transfer) Method

Adapted from S.W. Gersting, et al. (2012)

Introduction

Biological processes are complex and determined by networks of interactions, called interactome networks, which include protein-protein interactions. The protein interactome is elemental to cellular function, but our full understanding of it is incomplete. One assay that has helped us begin to map this interactome is the Bioluminescence Resonance Energy Transfer (BRET) assay. First developed to study interactions of circadian proteins, BRET allows for the bioluminescent monitoring of protein-protein interactions in living cells. During this assay, a protein of interest is fused to an energy donor protein (bioluminescence enzyme), while the other protein of interest is fused to an energy acceptor protein (a fluorescent protein). These two fused complexes are expressed in cells and upon addition of donor protein substrate, along with interaction of the proteins of interest, the donor protein is oxidized and the energy is transferred to the acceptor protein. Then, the acceptor protein gives off light that can be measured (Figure 1). There are several versions of this assay. Here, we describe a protocol focusing on the use of first generation BRET, where *Renilla* luciferase (Rluc) is the donor protein and YFP is the acceptor protein. In addition, Coelenterazine or Coelenterazine 400a as the Rluc-specific substrate (donor protein substrate).

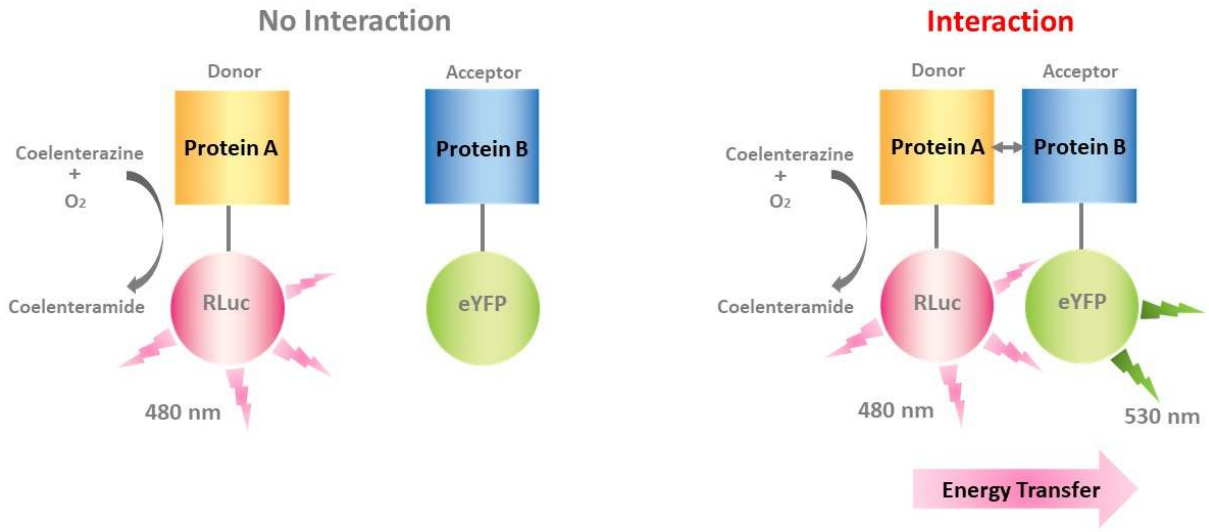

Figure 1. Detection of interaction by the BRET method.

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Materials

Cell culture

- RPMI 1640 Medium with L-glutamine **with** phenol red (GoldBio Catalog # P-117) supplemented with 10% fetal bovine serum and a 1% antibiotic-antimycotic solution containing:
  - 100 U/ml penicillin (GoldBio Catalog # P-304)
  - 0.1 mg/ml, streptomycin (GoldBio Catalog # S-150)
  - 0.25 µg/ml amphotericin B (GoldBio Catalog # A-560)
- Solution of 0.125% trypsin (GoldBio Catalog # T-161) and 0.05% EDTA (GoldBio Catalog # E-210).
- Dulbecco's PBS (1x) without calcium or magnesium (GoldBio Catalog # P-271). Each PBS tablet prepares 100 ml of a 1x PBS solution.
- Cell culture vessel
- COS7 cells

Transfection

- 96-well electroporation system
- Electroporation buffer and cuvettes
- White-wall 96-well plates, clear bottom, TC-treated
- RPMI 1640 Medium with L-glutamine **without** phenol red, supplemented with 10% fetal bovine serum and a 1% antibiotic-antimycotic solution containing:
  - 100 U/ml penicillin (GoldBio Catalog # P-304)
  - 0.1 mg/ml, streptomycin (GoldBio Catalog # S-150)
  - 0.25 µg/ml amphotericin B (GoldBio Catalog # A-560)
- Plasmids:
  - BRET expression constructs based on plasmids for eukaryotic expression coding for proteins of interest that are N- and C-terminally fused to Rluc or YFP, respectively.
  - A plasmid coding for a YFP-Rluc fusion protein (positive control).
  - Plasmid coding for YFP only (correction factor).

Detection of BRET signals

- Coelenterazine native ((Coelenterazine (GoldBio Catalog # CZ) or Coelenterazine 400a (GoldBio Catalog # C-320)) dissolved in methanol (1 mg/ml) and stored in aliquots at -80°C for long term stability. Protected from light.
- *Renilla* luciferase assay buffer consisting of: NaCl 64.28 g/L, Na_2EDTA (GoldBio Catalog # E-210) 0.82 g/L, KH_2PO_4 29.92 g/L, BSA (GoldBio Catalog # A-420) 0.44 g/L. Sterile-filtered and stored at 4°C.
- Dulbecco's PBS (1x) without calcium or magnesium (GoldBio Catalog # P-271).

- Luminescence multi-well plate reader equipped with two filters for simultaneous detection of the emission of Rluc (475 ± 30 nm) and YFP (535 ± 30 nm).

Method

A 96-well electroporation system is used here to enable large-scale transfection of cells at high-efficiency.

Cell culture

1. To prepare a sufficient number of cells, culture COS7 cells under monolayer conditions in a cell culture vessel. Seed ten million COS7 cells into one vessel and culture in the RPMI 1640 medium at 37°C and 5% CO_2 for 1 week.

> **Note: BRET experiments can be performed in all primary and stable adherent and suspension cell lines, allowing for efficient transfection and high expression of the BRET constructs.**
>
> **Note: Transfection conditions have to be optimized for each cell line. Optimization of nucleofection as described here may also be required for COS7 cells of different sources than the cell line used in this protocol.**

2. The day of transfection, detach the cells by trypsinization and determine the cell number in a conventional Neubauer counting chamber. Usually a total of 240 million COS7 cells can be harvested.

> **Note: COS7 cells should be used for transfection up to a maximum of 20 cell passages.**

Transfection

1. For electroporation, use a 96-well nucleofection device, which results in sufficient transfection efficiency and an adequate throughput.

> **Note: A high efficiency transfection system is recommended. Electroporation has proven to provide transfection efficiencies of 80-90% for many different cell lines. However, alternative transfection methods may be equally useful. Please note that for 96-well live cell BRET measurement, a minimal transfection efficiency of about 50% is required.**

2. Add 2 x 10^5 COS7 cells, 1 µg of plasmid DNA and 20 µl of 96-well nucleofector solution per well.

3. To analyze binary protein-protein interactions by BRET, cotransfect BRET expression vectors coding for the two proteins of interest at a ratio of 3:1 of acceptor protein (YFP) to donor protein (Rluc) constructs.

   Note: For binary protein-protein interaction, a sequential increase in the ratio of proteins carrying the YFP tag over proteins carrying the Rluc tag results in hyperbolic behavior of the BRET ratios. A plateau ($BRET_{max}$) is reached when all donor proteins are saturated with the energy acceptor. In contrast, in the case of nonspecific interactions resulting from random collision, the "bystander" BRET signal increases almost linearly with increasing acceptor to donor ratios, making BRET saturation experiments a suitable tool to distinguish positive and false positive interactions. A relative binding affinity index can be determined by the use of the YFP to Rluc ratio (acceptor/donor ratio) at half-maximal BRET ($BRET_{50}$).

4. Test each protein pair in duplicate and perform two independent experiments.

   Note: Tag orientation of proteins can influence the BRET signal or the protein-protein interaction itself. The strategy used here to circumvent this specific problem is the variation of N- and C-terminal fusion constructs for both proteins of interest, resulting in eight possible tag combinations for every tested protein pair.

5. Perform several control experiments (in triplicate) for every plate. A plasmid coding for a YFP-Rluc fusion protein serves as a positive control and always gives similar intra-assay results (~1.0). As a device-specific negative control, co-transfect a construct expressing the Rluc-tagged protein of interest with a YFP construct in the absence of protein of interest. Measure the BRET ratio, which is used as a correction factor (cf) by subtracting from every BRET pair. Here, the light emission detected in the acceptor channel (535 nm) predominantly results from a bleed through of donor emission that is specific for the filter set used. In addition, a background control with non-transfected cells is included to ensure stable assay conditions.

6. Prepare DNA in a sterile 96-well V-bottom plate or conventional sterile PCR-stripes with 0.65 µg for the donor construct and 1.95 µg for the acceptor construct (0.25 and 0.75 µg, respectively, multiply by 2 for duplicates and an additional dead volume factor of 1.3).

7. Centrifuge the required total number of cells (2 x 10^5 COS7 cells multiplied by the respective number of wells and the dead volume factor of 1.3) at 200 g for 5 minutes, at 37°C, and resuspend in the corresponding volume of prewarmed (37°C) electroporation

buffer (20 µl solution multiplied by the respective number of wells and the dead volume factor of 1.3).

Note: The number of wells for each plate can be calculated by the following equation: [(n x 8) x 2] + 9, where n is the number of protein pairs tested, multiplied by eight tag combinations for each protein pair, multiplied by 2 for duplicates and the addition of 9 for the wells for control experiments.

8. Add 52 µl of the cell suspension (20 µl multiplied by 2 for duplicates and the dead volume factor of 1.3) to the prepared DNA and mix by pipetting up and down.

9. Transfer the DNA-cell solution mix (20 µl) to the wells of the 96-well nucleofection plate in duplicates for each sample.

10. For electroporation using the nucleofection system, the appropriate program for COS7 cells (DSMZ ACC 60) is FP-100.

Note: Optimization of nucleofection is required for different types of cells and different electroporation systems.

11. After transfection, add prewarmed RPMI 1640 **without** phenol red (80 µl) to each well and mix thoroughly.

12. Prepare a clear bottom white 96-well plate with prewarmed 150 µl of RPMI media without phenol red per well.

13. Transfer 50 µl of each well (from step 11) to each well of the 96-well white plate prepared in step 12.

Note: To achieve high luminescence signals, particularly when luciferase signals are low due to low protein expression, it is recommended to use white 96-well plates. However, the application of white-wall clear bottom plates provides the advantage of using cell microscopy to determine density and viability of cultured cells well as the possibility for fluorescence imaging to analyze subcellular distribution of YFP-tagged proteins.

14. Incubate the cells at 37°C and 5% CO_2.

Note: When Rluc signals are low, longer incubation periods following transfection (up to 48

hours) and different temperature conditions (e.g., 30°C) may be helpful to achieve sufficient protein expression.

Detection of BRET Signals

1. BRET signals can be detected 24 hours after transfection.

   Note: If Rluc signals are low, see the note for step 12 of the Transfection section.

2. To prepare plates for BRET measurement, aspirate the culture medium (170 µl) and place the plate into the luminescence plate reader.

   Note: For BRET experiments performed in suspension cells, particular attention should be given to accidental elimination of cells while preparing plates for the BRET measurement. A crucial step is the aspiration of culture medium prior to the addition of the luciferase substrate. One possible approach is to centrifuge the plate and to subsequently take off the medium carefully. Alternatively, cells can be cultured in a total of 150 µl medium per well and coelenterazine at higher concentration can be added directly to each well. To allow for sufficient dilution of the media and the added coelenterazine, plates should be shaken in the plate reader while injecting the coelenterazine.

3. Coelenterazine solution has to be prepared at least 15 minutes before the measurement. To prepare coelenterazine solution for the measurement of one plate, add 127 µl of coelenterazine native suspended in methanol to 1 ml of *Renilla* luciferase assay buffer to obtain a 300µM solution. Immediately prior to the start of measurement, dilute 1.1 ml of the 300µM solution with 6.6 ml PBS (equivalent to the total volume for one plate: 70 µl/well x 96 wells + 1 ml dead volume for priming the injection pump).

4. After washing the injection pump with pure water, prime the pump with coelenterazine solution.

5. For BRET measurement, a protocol has to be set up, starting with a sequential injection of 70 µl of the coelenterazine solution to each well (resulting in a concentration of 30µM), followed by an incubation time of 2 minutes. Then, detect signals using the dual emission option at 485 nm (Rluc-signal) and 535 nm (BRET-signal) over 60 seconds with a total of 60 intervals.

Note: Depending on the number of wells that are to be measured, an interlaced sequence of injection, incubation, and detection as well as a reduction of the detection period up to a minimum of 10 seconds, may be required to reduce total measurement time and thereby cell stress.

Calculations

In this section we discuss the steps to determine the BRET ratios, which allow for approximate quantification of the orientation of proteins based on luminosity.

1. To allow data evaluation, Rluc signals at 485 nm for transfected cells should exceed the interval of the mean value and the nine-fold standard error of the nontransfected background control.

2. The BRET-ratio is calculated based on this equation:

$$R = \frac{I_A}{I_D} - cf$$

Where:
R – is the BRET Ratio
I_A – is the intensity of acceptor luminescence emission at 535 nm
I_D – is the intensity of donor luminescence emission at 485 nm
cf – is a correction factor ($\frac{BRET_{control}}{Rluc_{control}}$) with the control being the cotransfection of donor fusion proteins with YFP in the absence of the second protein of interest.

3. As a positive control, the YFP-Rluc fusion protein should result in BRET ratios around 1.0.

4. A positive interaction of two investigated protein pairs is assumed, if at least one out of eight tested tag combinations results in a BRET ratio above a method-specific threshold of 0.1.

Note: The method-specific threshold of 0.1 was determined in a laboratory based on a supervised approach. Using the settings reported in these instructions or in comparable setups assumes that the threshold is transferable. In the evaluation of large datasets, however, we recommend redetermining the threshold based on a reference data set consisting of well-documented pairs of interacting human proteins and randomly chosen protein pairs.

Associated Products

- Coelenterazine (GoldBio Catalog # CZ)
  and
  Coelenterazine 400a (GoldBio Catalog # C-320)

- Phenol Red (GoldBio Catalog # P-117)
- Penicillin (GoldBio Catalog # P-304)
- Streptomycin (GoldBio Catalog # S-150)
- Amphotericin B (GoldBio Catalog # A-560)
- Trypsin (0.125%)
- EDTA (0.05%) (GoldBio Catalog # E-210)
- GoldBio PBS Tablets (GoldBio Catalog # P-271)
- Na_2EDTA (GoldBio Catalog # E-210)
- Amphotericin B (GoldBio Catalog # A-560)
- Trypsin (0.125%)
- EDTA (0.05%) (GoldBio Catalog # E-210)
- GoldBio PBS Tablets (GoldBio Catalog # P-271)
- Na_2EDTA (GoldBio Catalog # E-210)
- BSA (GoldBio Catalog # A-420)

References

Gersting, S. W., Lotz-Havla, A. S., and Muntau, A. C. (2012). Bioluminescence Resonance Energy Transfer: An Emerging Tool for the Detection of Protein–Protein Interaction in Living Cells. In *Functional Genomics* (pp. 253-263). Springer New York.

Xu, Y., Piston, D. W., & Johnson, C. H. (1999). A bioluminescence resonance energy transfer (BRET) system: Application to interacting circadian clock proteins. *Proceedings of the National Academy of Sciences, 96*(1), 151-156. Doi:10.1073/pnas.96.1.151.