

A beginner's guide to bioluminescence resonance energy transfer (BRET)

**Etienne Sauvageau and
Stephane Lefrancois**

(Institut National de la
Recherche Scientifique,
Canada)

The Beginner's Guides are an ongoing series of articles in the magazine, each one covering a key technique and offering the scientifically literate but not necessarily expert audience a background briefing on the underlying science of a technique that is (or will be) widely used in molecular bioscience. The series covers a mixture of techniques, including some that are well established amongst a subset of our readership but not necessarily familiar to those in different specialisms. This Beginner's Guide covers bioluminescence resonance energy transfer (BRET).

In order to understand the function of a protein, biologists use myriad techniques. For example, RNA interference (RNAi) and more recently, clustered regularly interspaced short palindromic repeat (CRISPR) Cas9, have been used to decrease or knock-out (KO) protein expression, respectively. The function of the protein can then be inferred by what goes wrong in the cell or organism. Another useful approach to determine the role of a protein is to identify its interacting partners.

Several protein-protein interaction (PPI) methods exist with various benefits and drawbacks. When trying to determine the interactome of a protein, that is, all proteins that may interact with a protein of interest, people have historically used immunoprecipitation or pull-down methods. One advantage of immunoprecipitation is the isolation of endogenous proteins using antibodies. In pull-down methods, a protein is produced in bacteria or other system, purified using a tag such as GST or His, and then mixed with a cellular lysate. In both these cases, when the protein of interest is isolated, mass spectrometry can be used to identify proteins that interacted with, and were isolated by, the protein of interest. Immunoprecipitation can also be used to investigate a pair of proteins using immunoblotting after isolating the protein of interest, a technique known as co-immunoprecipitation (co-IP). Using co-IP, it is not possible to determine if the interaction is direct or via a third protein. In pull-down methods, both proteins can be translated in bacteria, therefore, it is possible to determine a direct interaction between two proteins.

Although these methods have provided significant information, a main drawback to them is the disruption

of cellular membranes using detergents to generate lysates. This results in the loss of cellular locations where interactions may occur. This could lead to false-positives as proteins normally localized to different cellular compartments could suddenly find each other in the lysate and interact. The presence of detergents can also disrupt weaker interactions, leading to false-negatives. In the pull-down methods, proteins produced in bacteria may not have the same post-translational modifications as in mammalian cells, potentially impacting interactions. Finally, these techniques cannot be used to follow the dynamics (association, dissociation, conformational changes) of PPI in real time in living cells.

Recent advances to overcome these aforementioned drawbacks include proximity-labelling methods such as proximity-based biotin labelling (BioID) and engineered ascorbic acid peroxidase (APEX), which are useful to determine the interactome of a protein of interest. Bioluminescence resonance energy transfer (BRET) is another useful technique to determine the PPI of a pair of proteins of interest. Proximity-labelling techniques and BRET can determine PPIs in live cells with proteins expressed in their native environments. BRET, similar to fluorescence resonance energy transfer (FRET), makes use of a phenomenon known as resonance energy transfer (RET). This phenomenon occurs when the emission spectrum of a donor chromophore overlaps with the excitation spectrum of an acceptor chromophore allowing the non-radiative transfer of energy from the donor, following its excitation, to the acceptor molecule. This transfer of energy results in a decrease of the donor emission and a concomitant

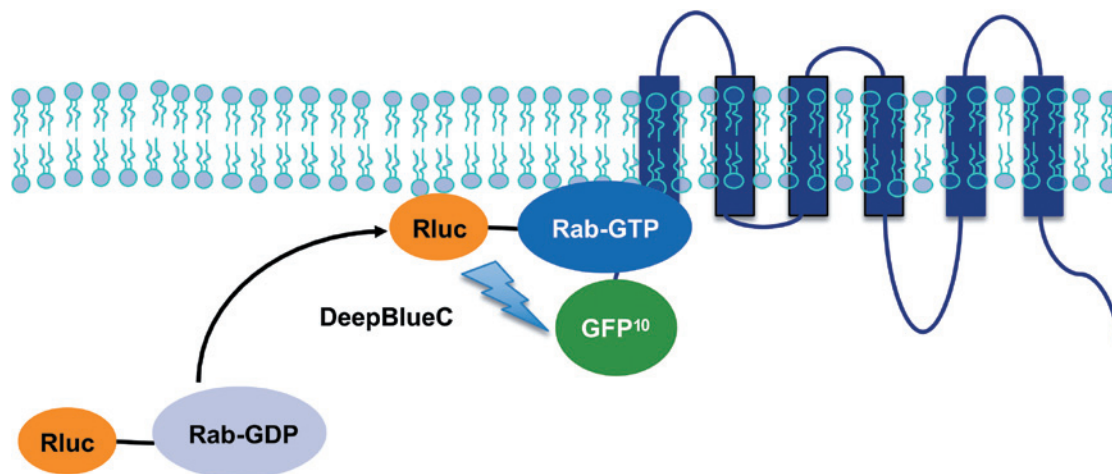


Figure 1. Schematic of a BRET interaction between an integral membrane protein and a Rab GTPase. RLuc-tagged Rab GTPase is recruited to the membrane where once in a GTP-loaded form, can interact with its effector, in this case, an integral membrane protein. The addition of the substrate, DeepBlueC, results in the emission of light by RLuc, which can then excite GFP¹⁰.

increase in the acceptor fluorescence. The RET efficacy is inversely proportional to the sixth power of the distance between the donor and acceptor and will not occur if the two chromophores are separated by more than ~10 nm. As such, the occurrence of RET between chromophores attached to two proteins expressed in living cells can be interpreted as a direct interaction between these proteins, their presence in a multi-protein complex or their concentration in a crowded cellular compartment. In BRET, the donor chromophore is the bioluminescent enzyme *Renilla* luciferase (RLuc), whereas in FRET it is a photoexcitable chromophore (Figure 1).

Since RLuc is excited by the addition of an enzyme substrate instead of an external light beam, as is the case for FRET, the main advantages of BRET over FRET are no photobleaching of the donor and the absence of background emission coming from non-specific cell excitation or direct acceptor excitation, resulting in a higher signal-to-noise ratio. One drawback that must be controlled is the use of protein tags. These methods require proteins to be genetically fused with large tags roughly the size of GFP (26 kilodaltons [kDa]), generally at their N- or C-terminus. It is therefore critical to ensure that the presence of the tag does not alter protein localization, function and expression. BRET also requires exogenous expression of the tagged proteins which could lead to false-positives due to overexpression or improper cellular localization. This problem could be solved using CRISPR/Cas9 to fuse chromophores to proteins of interest under the control of their endogenous promoter, but this can be technically challenging. Nevertheless, in addition to being fast, highly reproducible and sensitive, BRET experiments can monitor dynamics of PPIs in living cells in their native environment, allowing investigators to test hypothesis that are impossible to test using other methods such as co-IP or pull-down assays.

Different types of BRET experiments

Various luciferases, substrates and fluorescent acceptors, possessing different spectral characteristics, can be combined to perform BRET experiments and have different advantages and disadvantages. The original form of BRET, also called BRET¹, used RLuc as a donor, enhanced yellow fluorescent protein (eYFP) as an acceptor and coelenterazine as a substrate. Even though the light signal in BRET¹ is relatively high and stable in time, the significant overlap between the emission spectrum of RLuc and eYFP results in a low signal-to-noise ratio, reducing the sensitivity of the assay.

To circumvent this problem, the peak emission of RLuc can be blue-shifted using a different substrate called coelenterazine 400a (also known as DeepBlueC) and in combination with a different acceptor, GFP² or GFP¹⁰, BRET² experiments with much higher signal-to-noise ratio can be performed. Unfortunately, the light output of coelenterazine 400a is low and decreases rapidly in time, which led to the development of RLuc8, a mutated form of RLuc displaying a significantly brighter and more stable signal, thus alleviating the disadvantages of coelenterazine 400a. RLuc8 is also routinely used in BRET¹ and other forms of BRET experiments.

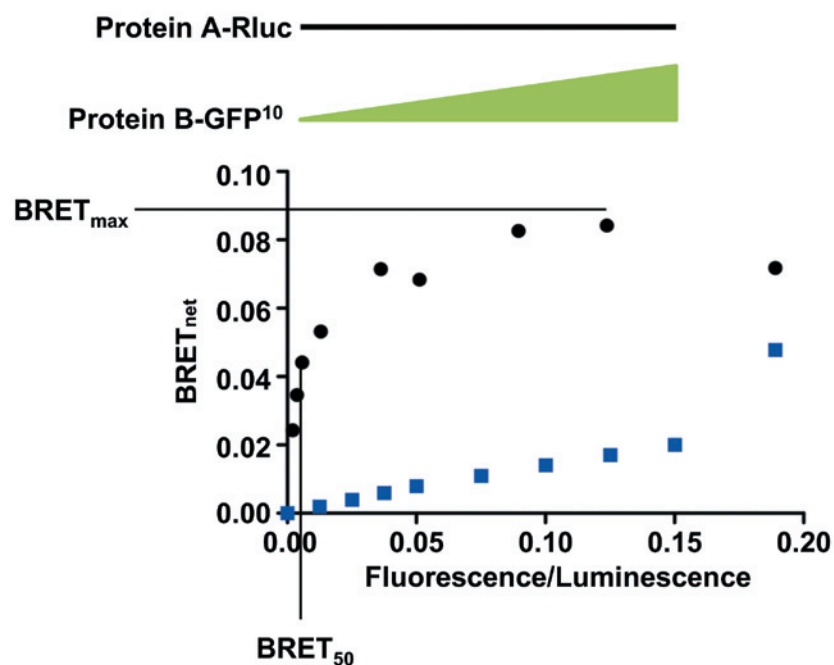
Recently, a red-shifted form of BRET (BRET³) using a RLuc8/mOrange BRET pair displaying higher light emission than BRET¹ and BRET² was developed to allow BRET imaging in animal models. Since one of the drawbacks of BRET is the tagging of proteins with bulky polypeptides such as RLuc (35 kDa), a smaller luciferase called NanoLuc (NLuc) was artificially engineered from the *Oplophorus* luciferase by the company Promega. Significantly smaller (19 kDa) and brighter, this luciferase is increasingly used in BRET experiments. Many other forms of luciferase, substrates and fluorescent acceptors

have and are being developed, the description of which are beyond the scope of this guide.

True interaction or random collisions?

When designing experiments using BRET, one must take into account random collisions versus true interactions. Random collisions occur when two proteins are close enough for RET to occur, such as two proteins in the same membrane compartment, but do not interact. First, proper negative controls should be included in the experiment. Ideally, a similar protein to the one being investigated, localized to the same cellular compartment and expressed at a comparable level should be tested in parallel. Also, BRET experiments should be performed using titration curves. To generate these curves, cells are plated into different wells, usually from 8 to 12, which will provide 8 to 12 points on the curve. Each well is transfected with a constant amount of the donor plasmid (Rluc or similar) and increasing amounts of the acceptor plasmid (GFP¹⁰, eYFP, etc.). Therefore, when measuring the amount of luminescence and fluorescence from the cells, the luminescence should be constant, while the fluorescence intensity should increase. BRET signals are usually expressed as a ratio of the light coming from the acceptor (fluorescence) at peak emission over the light coming from the Rluc at peak emission (luminescence). This BRET ratio is thus independent of the level of expression of the Rluc-tagged protein and by subtracting the BRET ratio obtained when the Rluc-tagged protein is expressed alone, we obtain the BRET_{net}. When plotting

Figure 2. Schematic of BRET titration curves. In order to distinguish from random collisions and true interaction, BRET titration curves are generated by transfecting a constant amount of the Rluc tagged protein, and an increasing amount of the GFP¹⁰ tagged protein. Random collisions will generate a straight line (blue squares), while specific interaction will generate a curve that saturates (black circles). From BRET titration curves, the BRET_{max} and BRET₅₀ can be extrapolated.



the measured BRET_{net} as a function of the fluorescence/luminescence ratio, if the BRET signal comes from random collisions, the signal should increase linearly (Figure 2, blue squares). On the other hand, if a protein pair are interacting, the BRET_{net} increases as a hyperbolic function reaching saturation as the limited amount of donor protein will not be able to bind with all the excess amount of acceptor protein (Figure 2, black circles).

From this curve, two values can be extracted. BRET_{max}, which is the value of the highest BRET_{net} and the BRET₅₀, which is the fluorescence/luminescence ratio at which the curve reaches half of the BRET_{max}. The BRET₅₀ is indicative of the propensity of the two partners to interact. Changes in BRET₅₀ observed following changes in cell homeostasis (pharmacological treatment, KO etc.) can be interpreted as a change in affinity between the interacting pair or a change in cellular localization of one or both proteins affecting their interaction. Additional experiments (microscopy) are often needed to distinguish between those possibilities. In contrast, changes in BRET_{max}, without concomitant alterations of the BRET₅₀, are usually interpreted as changes in conformation of a protein complex.

BRET is not only for PPIs

Although BRET is a powerful tool for determining PPIs and the impact mutations or other proteins have on these interactions, the technique can also be used to study conformational changes in a protein or a protein complex. For example, tagging an integral membrane protein with Rluc at the C-terminal end and GFP¹⁰ at its N-terminal end would result in a BRET signal (Figure 3A). It is then possible to test the effect of a small molecule or another protein on that signal. Here, the BRET_{net} would be read, and a stronger signal would suggest the two ends of the protein coming closer to one another while a weaker signal would be interpreted as the two ends moving further apart. The method can also be used to explore the conformation of a protein complex (Figure 3B). As an example, if a protein complex is in a 'closed' conformation (stronger signal) in the cytosol, recruitment to a membrane compartment could cause it to 'open' (weaker signal). Therefore, experiments could be designed to test whether the protein complex is in the cytosol or on the membrane based on the BRET_{net}. This system could also be used to determine the mechanism of recruitment and complex rearrangement. In order to rule out possible dimerization of a complex as providing the signal, control experiments should be performed by tagging the same protein of a complex with both Rluc and GFP¹⁰ such that one complex would have the donor, while the other complex would have the acceptor.

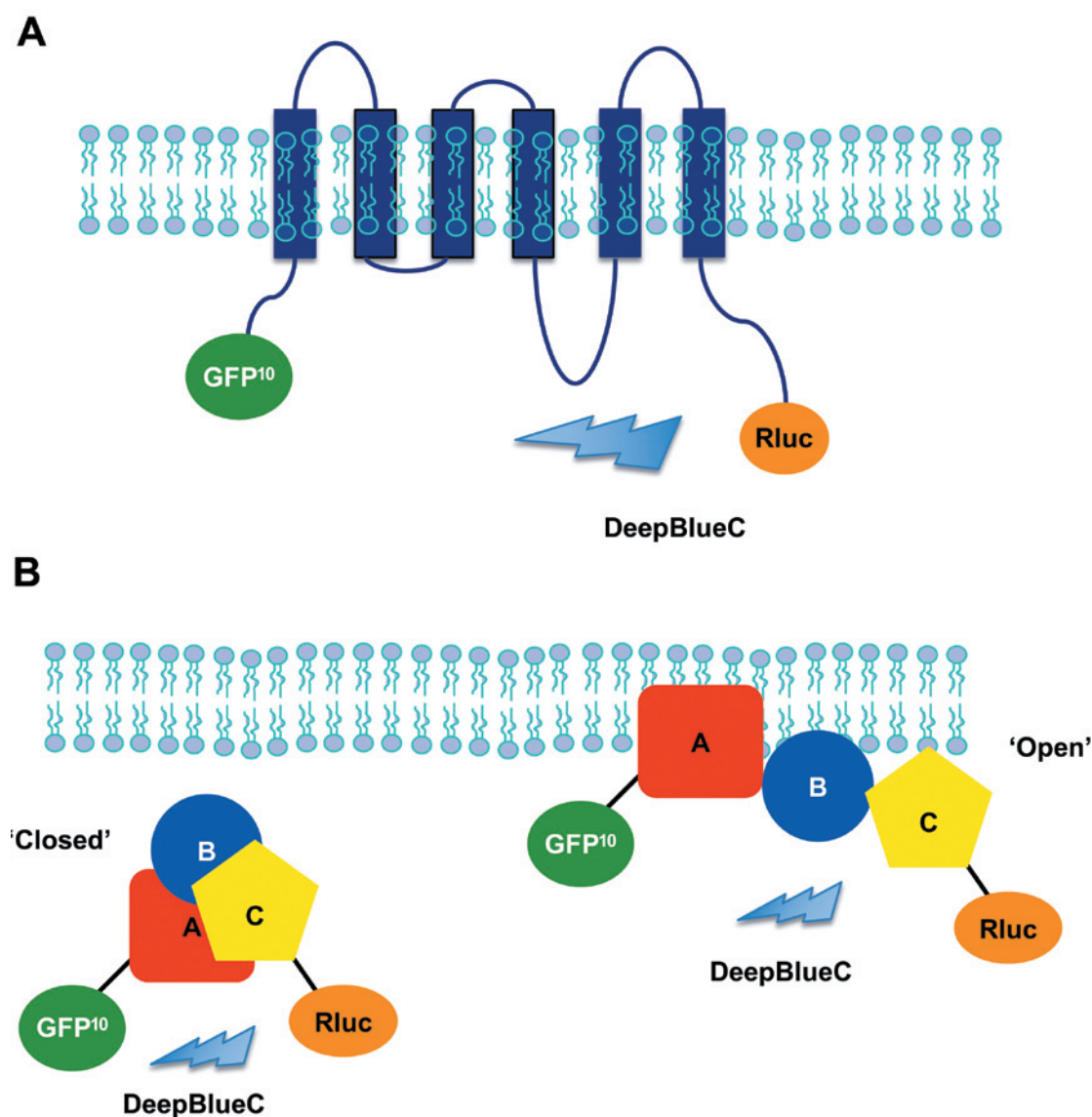


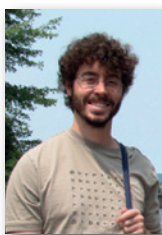
Figure 3. BRET can also be used to determine conformation of a protein or protein complex. **(A)** An integral membrane protein was tagged at its C-terminal end with GFP¹⁰ and with Rluc at its N-terminal end. Measuring the intramolecular BRET, we can determine if a small molecule or another protein can modulate the distance between the two ends. A stronger signal would suggest the two ends getting closer, while a weaker signal would suggest the two ends moving farther apart. **(B)** Two proteins from a protein complex were tagged with GFP¹⁰ (subunit A) or Rluc (subunit C). When the complex is in the cytosol, it is in the 'closed' conformation. When the complex is recruited to membranes, the complex is reorganized into the 'open' conformation. Using BRET, we can determine if the complex is open (further apart, weaker signal) or closed (closer together stronger signal).

Another exciting use of BRET technology is the development of different BRET biosensors to measure cell signaling, protein activation or inhibition. For example, the cAMP EPAC BRET biosensor can be used to monitor the levels of cAMP, an important second messenger in intracellular cell signalling. The protein Epac-2 (exchanged protein directly activated by cAMP) is fused at its N-terminus with Rluc and with eYFP at its C-terminus. Epac2 is a cAMP effector and its conformation changes upon binding to cAMP which leads to an increased distance between Rluc and eYFP and a decrease of the BRET_{net} signal. BRET can also be used to follow the cellular localization of a protein by measuring the BRET between a protein of interest and a specific resident protein of an organelle.

Our experience

Our research group has adopted this technique for a number of reasons. The first, our research has focused on integral membrane proteins which can be difficult to extract from membranes to perform co-IP experiments. BRET enables us to test PPIs with these proteins expressed in their native environment. This has significant advantages; first, proteins with multiple transmembrane domains can maintain their structure. In addition, if other factors such as lipids or other components of the membrane are important for the interaction, they are intact.

Our work also focuses on interactions of small GTPases of the Arf and Rab families with their effectors. These interactions depend of the active status of these small GTPases. Therefore, these interactions are transient and can occur quickly. BRET is well suited for these types of interactions and we have had considerable success using this method.



Dr Etienne Sauvageau is a research associate in the Lefrancois lab at Centre Armand-Frappier Santé Biotechnologie. He obtained his BSc at Université de Sherbrooke followed by graduate studies at Université de Montreal. He joined the Lefrancois group as a postdoctoral fellow and established BRET as a technique in the lab.

Email: etienne.sauvageau@iaf.inrs.cs



Dr Stephane Lefrancois is an Associate Professor at the Institut National de la Recherche Scientifique (INRS). His laboratory is located at the Centre Armand-Frappier Santé Biotechnologie. He obtained his BSc and PhD at McGill University before undertaking post-doctoral training at the NIH in Bethesda, MD. His research interests focus on intracellular trafficking. He was initially recruited to Université de Montreal as an assistant professor prior to moving his lab to INRS.

Email: stephane.lefrancois@iaf.inrs

In summary, we have found BRET to be especially useful when studying the effects of mutations, post-translational modifications and small molecules on PPIs in live cells and in real time. Since BRET is very sensitive and quantifiable, it is a powerful research tool to detect small changes in PPIs that may have significant biological effects and may not be detectable using other techniques. ■

Further reading

Use of BRET in studying protein–protein interactions

- Dacres, H., Michie, M., Wang, J., Pflieger, K.D. and Trowell, S.C. (2012) Effect of enhanced *Renilla* luciferase and fluorescent protein variants on the Förster distance of bioluminescence resonance energy transfer (BRET). *Biochem. Biophys. Res. Commun.* **425**, 625–629
- Ayoub, M.A. and Pflieger, K.D. (2010) Recent advances in bioluminescence resonance energy transfer technologies to study GPCR heteromerization. *Curr. Opin. Pharmacol.* **10**, 44–52
- Gandia, J., Lluís, C., Ferre, S., Franco, R. and Ciruela, F. (2008) Light resonance energy transfer-based methods in the study of G protein-coupled receptor oligomerization. *Bioessays* **30**, 82–89
- Pflieger, K.D. and Eidne, K.A. (2006) Illuminating insights into protein–protein interactions using bioluminescence resonance energy transfer (BRET). *Nat. Methods* **3**, 165–174

Live animal uses

- De, A., Ray, P., Loening, A.M. and Gambhir, S.S. (2009) BRET3: a red-shifted bioluminescence resonance energy transfer (BRET)-based integrated platform for imaging protein–protein interactions from single live cells and living animals. *FASEB J.* **23**, 2702–2709

Uses beyond protein–protein interaction

- Salahpour, A., Espinoza, S., Masri, B., Lam, V., Barak, L.S. and Gainetdinov, R.R. (2012) BRET biosensors to study GPCR biology, pharmacology, and signal transduction. *Front. Endocrinol. (Lausanne)* **3**, 105
- Sauvageau, E., McCormick, P.J. and Lefrancois, S. (2017) *In vivo* monitoring of the recruitment and activation of AP-1 by Arf1. *Sci. Rep.* **7**, 7148
- Hwang, E., Song, J. and Zhang, J. (2019) Integration of nanomaterials and bioluminescence resonance energy transfer techniques for sensing biomolecules. *Biosensors (Basel)* **9**, 42