Resonance Energy Transfer as an Emerging Strategy for Monitoring Protein-Protein Interactions *In Vivo*: BRET *vs.* FRET

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Protein-protein interactions are known to play an important role in a variety of biochemical systems. To date, thousands of protein-protein interactions have been identified by using the conventional two-hybrid system, but this method is limited in that the interaction must occur in the yeast nucleus. This means interactions that strictly depend upon cell-type specific processing and/or compartmentalization will not be detected. Therefore, a number of new methods have been developed recently that rely on reconstitution of biochemical function *in vivo*, such as fluorescence resonance energy transfer (FRET), protein mass spectrometry, or evanescent wave.[1] Among those methods, the resonance energy transfer techniques have potential advantages for assaying protein-protein interactions in living cells and in real time. In this article, we will describe a new resonance energy transfer method.

Fluorescence Resonance Energy Transfer (FRET)

Fluorescence Resonance Energy Transfer (FRET)[2,3] is a well-established phenomenon that has found limited use in cellular microscopy. When two fluorophores (the "donor" and the "acceptor") with overlapping emission/absorption spectra are within ~50 Å of one another and their transition dipoles are appropriately oriented, the donor fluorophore is able to transfer its excited-state energy to the acceptor fluorophore. Therefore, if appropriate fluorophores are linked to proteins that might interact with each other, the proximity of these candidate interactors could be measured by determining if fluorescence resonance energy is transferred from the donor to the acceptor. Thus, the presence or absence of FRET acts as a "molecular yardstick."

The discovery and development of green fluorescent protein (GFP) and its mutants made possible their use as FRET donors and acceptors.[4-10] Genetically fusing GFP derivatives to the candidate proteins enabled the detection of protein-protein proximity in real time in living cells of the organisms from which the proteins were originally obtained.[7,8] In those studies, blue fluorescent protein (BFP) was used as the donor fluorophore and GFP was the acceptor. As mentioned above, the efficiency of the resonance transfer depends upon the spectral overlap of the fluorophores, their relative orientation, as well as the distance between the donor and acceptor fluorophores. By targeting the fusion proteins to specific compartments, this FRET-based assay can also allow protein interactions to be observed within cellular compartments *in vivo*, as has been shown for mitochondria and nuclei.[7,8] However, because FRET demands that the donor fluorophore be excited by illumination, the practical usefulness of FRET can be limited because of the concomitant results of excitation: photobleaching, autofluorescence, and direct excitation of the acceptor fluorophore. Furthermore, some tissues might be easily damaged by the excitation light or might be photoresponsive (e.g., retina).

Bioluminescence Resonance Energy Transfer (BRET)

Recently, we developed a bioluminescence resonance energy transfer (BRET) system for assaying protein-protein interactions that incorporates the attractive advantages of the FRET assay while avoiding the problems associated with fluorescence excitation.[11] In BRET, the donor fluorophore of the FRET pair is replaced by a luciferase, in which bioluminescence from the luciferase in the presence of a substrate excites the acceptor fluorophore through the same resonance energy transfer mechanisms as FRET.

The bioluminescent *Renilla* luciferase (RLUC; MW = 35 kD) was chosen as the donor luciferase in our BRET because its emission spectrum is similar to the cyan mutant of *Aequorea* GFP ($\lambda_{max} \approx 480$ nm) which has been shown to exhibit FRET with the acceptor fluorophore EYFP, which is a yellow-emitting GFP mutant.[6] The excitation peak of EYFP (513 nm) does not perfectly match to the emission peak of RLUC, but the emission spectrum of RLUC is sufficiently broad that it provides good excitation of EYFP. The spectral overlap between RLUC and EYFP is similar to that of EYFP and the enhanced cyan mutant of GFP, ECFP, which yields a critical Förster radius (R_0) for FRET of ~50 Å.[7] Thus, we would expect significant BRET between RLUC and EYFP, with an R_0 for BRET of ~50 Å. The fluorescence emission of EYFP is yellow, peaking at 527 nm, which is distinct from the RLUC emission peak. Furthermore, the

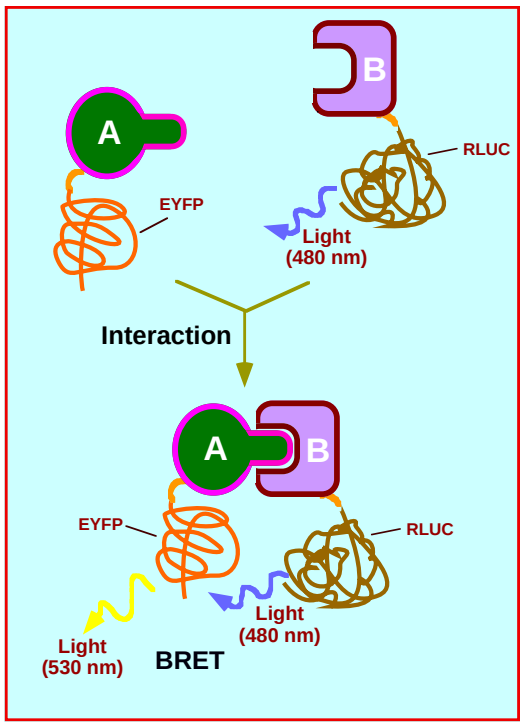

Figure 1. A diagram of bioluminescence resonance energy transfer (BRET) as for a protein-protein interaction assay. One protein of interest (B) is genetically fused to the donor luciferase RLUC, and the other candidate protein (A) is fused to the acceptor fluorophore EYFP. Interaction between the two fusion proteins can bring RLUC and EYFP close enough for BRET to occur, with an emission of longer wavelength light.

substrate for RLUC, coelenterazine, is a hydrophobic molecule that is able to permeate cell membranes, and RLUC and EYFP do not naturally interact with each other.

In the BRET assay of protein interactions, RLUC is genetically fused to one candidate protein, and EYFP is fused to another protein of interest that perhaps interacts with the first protein. If RLUC and EYFP can be brought close enough for resonance energy transfer to occur, the bioluminescence energy generated by RLUC can be transferred to EYFP, which then emits yellow light (Figure 1). In the BRET assay for protein interaction, this resonance transfer can occur between RLUC/EYFP fusion proteins that interact. If there is no interaction between the two proteins of interest, RLUC and EYFP will be too far apart for significant transfer and only the blue-emitting spectrum of RLUC will be detected. Thus, protein-protein interactions can be monitored both *in vivo* and *in vitro* by detecting the emission spectrum and quantifying the emission ratio at 530nm/480nm.

BRET between RLUC and EYFP was first demonstrated in control experiments in which RLUC was fused directly to EYFP through a linkage of 11 amino acids.[11] The luminescence profile of the *E. coli* cells expressing the RLUC::EYFP fusion construct yielded a bimodal spectrum, with one peak centered at 480 nm (as for RLUC), and a new peak centered at 527 nm (as for EYFP fluorescence).[11] This result suggests that a significant proportion of the RLUC energy is transferred to EYFP and emitted at the characteristic wavelength of EYFP. This indicates that RLUC/EYFP could be an effective combination to apply in a protein-protein interaction assay.

Application of BRET to Clock Proteins

To test BRET as a protein-protein assay, we chose the proteins encoded by circadian (daily) clock genes from cyanobacteria and fused them to RLUC or EYFP, respectively. In cyanobacteria, the *kaiABC* gene cluster encodes three proteins, KaiA (MW = 32.6 kD), KaiB (MW = 11.4 kD), and KaiC (MW = 58 kD) that are essential for circadian clock function.[12] Iwasaki *et al.* have used the yeast two-hybrid and *in vitro* binding assays to discover that Kai proteins interact in various ways, such as formation of KaiB-KaiB homodimers.[13] First, we tried the N-terminal fusions of KaiB to RLUC and to EYFP. The luminescence spectra of *E. coli* expressing these fusions showed a second peak in the cells expressing both RLUC::KaiB and EYFP::KaiB (Figure 2, right side). This spectrum is similar to that depicted for the fusion protein RLUC::EYFP.[11]

We further tested all possible combinations of KaiB fusions with RLUC or EYFP, including N- vs. N-, N- vs. C-, C- vs. N-, as well as C- vs. C-terminal fusions. All of these combinations of the KaiB's fusion proteins showed BRET (unpublished data). KaiB interactions was also observed *in vitro* by BRET.[11] To demonstrate that this bimodal spectrum does not occur nonspecifically, we used KaiA as a control, in which EYFP was fused to a slightly truncated KaiA. The luminescence spectra of *E. coli* co-expressing EYFP::KaiA with RLUC::KaiB did not exhibit the second luminescence peak, indicating no interaction occurred between KaiA and KaiB. Our results, therefore, strongly suggest that interaction among KaiB molecules either in N-terminal or C-terminal fusions to the donor luciferase or the acceptor fluorophore has brought the RLUC and EYFP into close proximity such that energy transfer occurs for ~50% of the RLUC luminescence. Thus, BRET supports the data from the yeast two-hybrid assay[13] demonstrating that the clock protein KaiB self associates to form multimers.

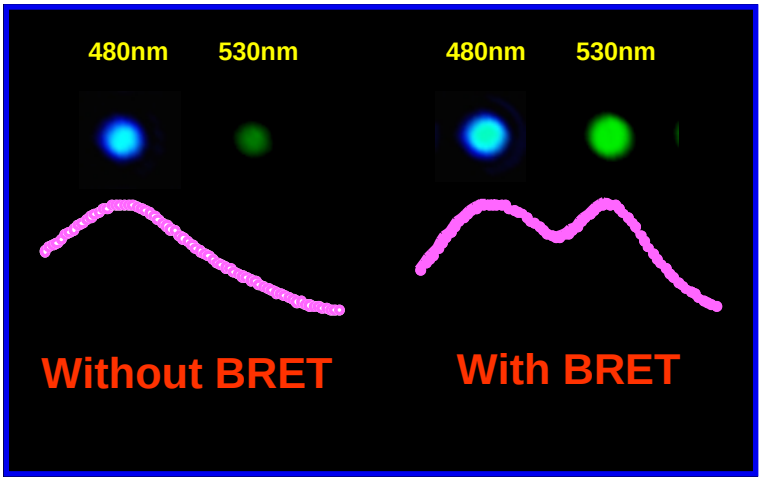

Figure 2. Comparison of complete BRET spectra using a fluorescence spectrophotometer with camera images of *E.coli* cells. Top, cultures imaged with a CCD-camera through filters transmitting light of 480 nm or 530 nm from the transformed *E. coli* strains co-expressing fusion proteins exhibiting BRET on the right side (RLUC::KaiB and EYFP::KaiB) or fusion proteins that are not exhibiting BRET on the left side (RLUC::KaiB and EYFP::KaiA). Bottom, luminescence emission spectra measured continously from 440 nm to 580 nm for the same strains.

In the experiments described above, the extent of BRET was determined by measuring emission spectra.[11] For applications such as microscopic imaging and high-throughput screening, it would be more convenient to measure the ratio of luminescence intensities at two fixed wavelengths, e.g., 480 nm and 530 nm. Ratio imaging has the advantage of automatically correcting for differences in overall levels of expression of RLUC and EYFP fusion proteins. The top portion of Figure 2 is the images of *E. coli* cultures expressing fusion proteins that either exhibit (right) or do not exhibit (left) BRET. These images of liquid *E. coli* cultures (5 microliter cultures) were collected using a charge-coupled device (CCD) camera through interference bandpass filters centered at 480 nm and 530 nm, respectively. In the cultures co-expressing the interacting combination of RLUC::KaiB with EYFP::KaiB, the amount of light emitted at 480 nm and 530 nm are roughly equal, as would be predicted from the spectra depicted (Figure 2, right). In contrast, in the cultures containing a non-interacting combination of RLUC::KaiB with EYFP::KaiA, there is much less light emitted at 530 nm than at 480 nm (Figure 2, left). As we reported previously, the extent of BRET can be quantified according to the 530nm/480nm ratios of luminescence intensity in the image.[11] Thus, the 530nm/480nm ratios can apparently be used to evaluate BRET and thereby infer if protein-protein interaction has occurred.

New Tools for BRET

Very recently, several new tools for BRET have appeared that may prove useful. The first is that there is a new luciferase that was isolated from *Gaussia* that has an emission spectrum like that of RLUC but whose MW is 20 kD (available from Prolume Ltd.). Like RLUC, this luciferase uses coelenterazine as a substrate. This luciferase may have all the advantages of RLUC, but by virtue of its smaller size, better allow native interactions without steric hinderance in fusion proteins. Another tool of potential advantage is a new fluorescent protein isolated from anthozoans.[14] These proteins that are remote homologs of GFP form a new group of fluorescent tags. One of these proteins has a much longer wavelength than any other fluorescent protein yet isolated, with an excitation spectrum peaking at 558 nm and a sharp emission spectrum peaking at 583 nm. The excitation spectrum is broad enough that a luciferase like RLUC might excite it. The advantage of this fluorescent protein is that its emission spectrum is sufficiently red-shifted that the separation between BRET and non-BRET luminescence is much greater than with YFP, and hence quantification of BRET could be more accurate. This red fluorescent protein is now available from Clontech as "DsRed."

A Potential Screening System

Based on these data, we proposed[11] a relatively simple scheme for designing an *in vivo* library screening system for protein-protein interaction through BRET (Figure 3). By measuring the light emission collected through interference filters, the 530 nm/480 nm luminescence ratio of *E. coli* (or yeast) colonies expressing a "bait" protein fused to RLUC and a library of "prey" molecules fused to EYFP (or *vice versa*) could be measured. It would be possible to screen colonies of bacteria or yeast on agar plates using a camera imaging system. On the other hand, a photomultiplier-based instrument designed to measure luminescence of liquid cultures in 96-well plates could be adapted to

high-throughput BRET screening by insertion of switchable 480 or 530 nm filters in front of the photomultiplier tube. Colonies that show high light intensity (i.e. bright colonies) at 530 nm or exhibit an above-background ratio of the 530 nm/480 nm could be selected and the "prey" DNA sequence further characterized. Thus, an efficient BRET screening system could be practical by using an appropriate instrument.

Advantages of Resonance Energy Transfer Techniques (BRET and FRET)

The features of BRET or FRET techniques offer some attractive advantages over other current assays for protein-protein interactions, especially the yeast two-hybrid method, which is currently the most popular method. For instance, BRET or FRET methods can be applied to determine whether the interaction changes with time because the measurement is noninvasive. BRET/FRET is suitable to assay the protein-protein interactions in different subcellular compartments or specific organelles of the native cells, as has already been shown to work for FRET.[7,8] In particular, the yeast nucleus may be a poor place for some compatible proteins to meet. This advantage of BRET/FRET could be particularly useful in the case of interacting membrane proteins for which assays are limited with other traditional methods. BRET or FRET also may be used to reveal interactions that depend upon cell-type specific post-translational modifications that do not occur in yeast and therefore can not be assayed by the yeast-two hybrid method. By using cell-type specific promoters and/or fusion to targeting-sequences, the GFP-based BRET or FRET indicators can be observed specifically in the cell-type and subcellular location of choice. Moreover, BRET/FRET assays could be adopted to monitor the dynamic processes of protein-protein interactions *in vivo*, such as intracellular signaling.

No Technique is Perfect

As with any technique, however, the resonance energy transfer methods have some limitations. For example, the efficiency of both BRET and FRET is dependent on proper orientation of the donor and acceptor dipoles. Conformational states of the fusion proteins may fix the dipoles into a geometry that is unfavorable for energy transfer. Further, because the fluorophore/luciferase tags are fused to ends of the potentially interacting molecules, it is possible that some parts of the candidate molecules are interacting without allowing the fluorophore/luciferase tags to be close enough for energy transfer to occur. Consequently, two proteins might interact in a way that is blind to the FRET/BRET technique. In other words, a negative result with a resonance transfer technique does not prove non-interaction. In such a case, testing different combinations of N-terminal and C-terminal fusions in the BRET/FRET assays could help to determine the optimal orientation in which candidate proteins interact.

The luciferase/fluorescent protein tags that are fused to the candidate interacting proteins could interfere with the interaction by steric hinderance (this problem is true for the yeast two-hybrid assay as well). Therefore, the smaller the tags, the less likely will be the hindrance. This is the reason why the *Gaussia* luciferase might prove to be superior to RLUC. Furthermore, these luciferase/fluorescent tags might cause inactive or incorrectly folded fusion proteins. For example,the bulkiness of the GFP (and its derivatives) cylinders (20 x 30 Å) have been shown to impede correct folding of fusion proteins.[10]

Another consideration in the use of GFP variants as fluorophore tags is that the slow kinetics of GFP turnover may hamper measuring the kinetics of interaction (whereas *Renilla* luciferase does not suffer these same disadvantages in turnover rate). New GFPs are available that have been engineered to be less stable (ref. 15 and Clontech's d2EGFP) and the re-engineering of BRET fluorophores to be less stable could be useful in temporal studies. Moreover, the pH-sensitivity of some of the GFPs might restrict their application to subcellular areas with a higher pH value. However, this limitation can be overcome by utilizing the mutants that are less sensitive to pH.[16]

BRET Versus FRET

BRET has potential advantages over FRET because it does not require the use of excitation illumination. BRET should be superior for cells that are either photo-responsive (e.g., retina or any photoreceptive tissue) or damaged by the wavelength of light used to excite FRET. Cells that have significant auto-fluorescence would also be better assayed by BRET than by FRET. Moreover, while photobleaching of the fluorophores can be a serious limitation of FRET, it is irrelevant to BRET. BRET assay requires a substrate for the luciferase. In the case of RLUC, coelenterazine is the substrate. Coelenterazine is hydrophobic and can permeate all the cell types we have tested, including bacteria (*E. coli* and cyanobacteria), yeast, *Chlamydomonas*,[17] plant seedlings and calli,[18] and animal cells in culture. Other workers have found that coelenterazine can permeate the cytoplasm of human 293 cells,[19] the cytoplasm of primary neuronal and HeLa cells,[20] the nucleus of COS7 cell cultures,[21] the mitochondria of endothelial cells,[22] and the ER of arterial cells.[23]

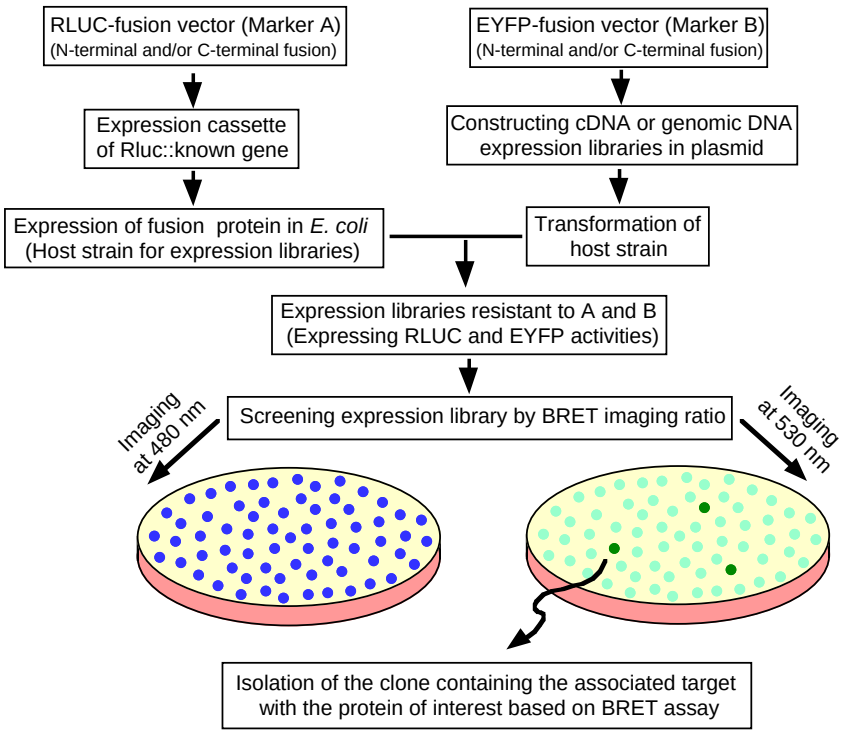

Figure 3. Schematic diagram of a screening system for protein-protein interaction using ratio or visual imaging of BRET.

In addition, FRET may be prone to complications due to simultaneous excitation of both donor and acceptor fluorophores. Specifically, even with monochromatic laser excitation, it is impossible with the current generation of fluorescent proteins to excite only the donor without exciting the acceptor fluorophore to some degree. In contrast, because BRET does not involve optical excitation, all the light emitted by the fluorophore must result from resonance transfer. Therefore, BRET is theoretically superior to FRET for quantifying resonance transfer.

The major limitation that BRET suffers in comparison to FRET is that its luminescence may often be too dim to accurately measure 530/480 nm ratios if the researcher does not have access to a very sensitive light-measuring apparatus. Manufacturers are continuously developing improved instrumentation for measuring low-light levels, and these improvements in technology will undoubtably aid the further development of BRET assays of real-time protein-protein interactions in living organisms.

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