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Review Article

Protein-protein interactions of mitochondrial-associated protein via bioluminescence resonance energy transfer

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Protein-protein interactions are essential biological reactions occurring at inter- and intra-cellular levels. The analysis of their mechanism is generally required in order link to understand their various cellular functions. Bioluminescence resonance energy transfer (BRET), which is based on an enzymatic activity of luciferase, is a useful tool for investigating protein-protein interactions in live cells. The combination of the BRET system and biomolecular fluorescence complementation (BiFC) would provide us a better understanding of the hetero-oligomeric structural states of protein complexes. In this review, we discuss the application of BRET to the protein-protein interactions of mitochondrial-associated proteins and discuss its physiological relevance.

Key words: antiviral innate immunity, BRET, mitochondria, protein-protein interactions, signal transduction

Dilemma of analyzing interactions between organelle-bounded membrane proteins

When we investigate an interaction between proteins of interest, there are various experimental approaches are generally available, such as surface plasmon resonance (SPR), calorimetry, immunoprecipitation, or yeast two-hybrid assays all of which are frequently used in biochemical and cell biological studies. As a matter of fact, there are several merits and demerits for selecting these methods, and thus we should carefully consider their experimental environments and ensure that the situation properties fit the purpose of the study. Particularly, when we focus on membrane-embedded proteins such as G protein-coupled receptor (GPCR), cell surface receptors, or organelle-anchored/associated proteins as the targets of our studies, great care must be taken in the preparation of sensitive proteins so that they are maintained with intact structures, topologies and functions, and in many cases such preparation requires high levels of skill and labor [1]. This is why many researchers tend to use various kinds of living cells, including yeast or tissue culture, in order to investigate an interactome, especially if the targets include membrane-associated materials.

Thanks to rapid progress in the field of bio-imaging studies that widely utilize fluorescent proteins such as green fluorescent protein (GFP) or its variants, these molecules are well applied to the monitoring of intermolecular interaction analysis as reporters, and one example is the fluorescence resonance energy transfer (FRET) technique [2,3]. The biggest advantage of using this system is that we can monitor interaction events of interest *in vivo* without considering the preparation issue mentioned above. However, there are other issues that we must regard with caution in the use of the FRET system. FRET requires an external light source when fluorescent proteins are excited, and this excitatory light often results in photochemically inducing damage of the cell materials. Another minor issue is that photochemically induced autofluorescence of intracellular proteins, increasing the background noise in the analysis of data based on the signal-to-noise (S/N) ratio. In order to resolve this dilemma of bio-imaging analysis, a new approach applicable to protein-protein interactions is desired.

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Bioluminescence resonance energy transfer (BRET)

Bioluminescence resonance energy transfer (BRET) has been developed to address the several concerns mentioned above. BRET is a powerful and ideal tool for evaluating protein-protein interactions, especially in live cells. Many well-written reviews on BRET have been published [4,5], so here we summarize some highlights of the system. The basic difference between BRET and FRET is that the BRET utilizes a natural resonance energy transfer process that occurs as a result of enzymatic activity via luciferase (e.g., *Renilla* luciferase; Rluc) with its substrate (coelenterazine) as a donor instead of using an illumination light of fluorescent protein excitation in FRET (Fig. 1). As a result, BRET provides a high S/N ratio that comes from the luminescence detection, and is preferable for analyzing protein-protein interactions under physiologic conditions. There are several combinations of donor (Rluc, Nanoluc, or *Gaussia* luc) and acceptor (GFP, GFP^2, or YFP) molecules are available, but Rluc-YFP is the main pair used in the BRET assay (Fig. 1). Further, using a mutant Rluc (Rluc8) as the donor probe would yield a several-fold improvement in light output, resulting in a much greater S/N ratio [6].

Applying the BRET assay in a tissue culture experiment, we first need to construct two plasmids that encode Rluc protein as a donor and a variant of green fluorescent protein (enhanced GFP or YFP) as the acceptor, and both reporter genes must be fused with the target genes, respectively [4]. It should be noted that the coordination of these fusion tags at their N- or C-termini with the targets has to carefully consider its effect not only on biological activity but also protein expression [7]. Once the plasmids are constructed, cultured cells are co-transfected with the recombinant Rluc- and YFP-tagged plasmids according to the manufacturer's protocol. Within the cell, if the expressed donor and acceptor molecules are ideally adjacent in a comparable range of biological macromolecular complexes such as those ≤10 nm, the optimal BRET signal can be obtained in the presence of coelenterazine after oxidation of the substrate that resulted in energy transfer between donor emission and acceptor

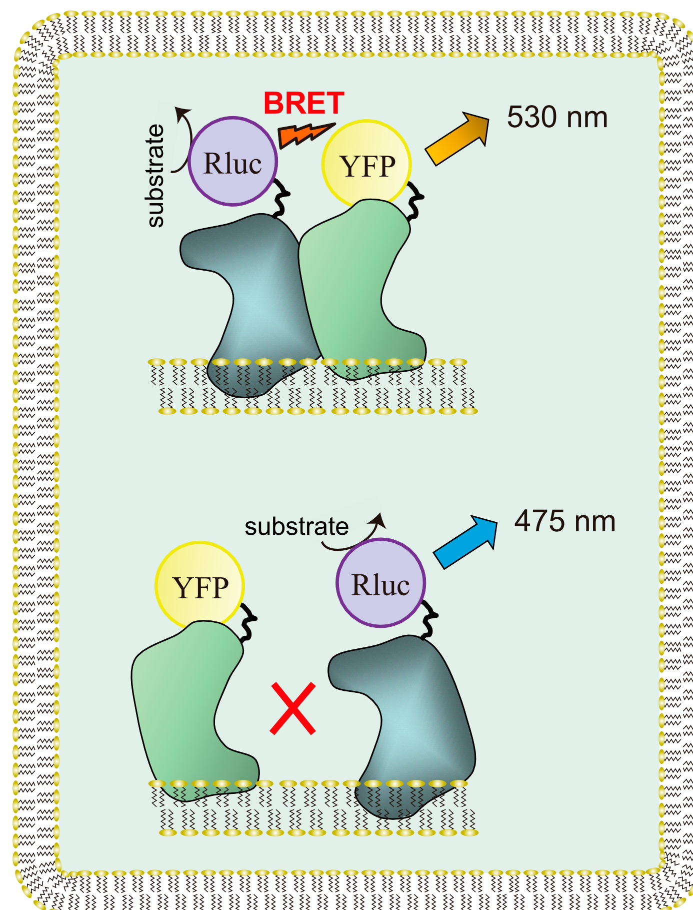

Figure 1 Schematic diagram of BRET by Rluc and YFP. When the two proteins interact and provide a comparable range of biological macromolecular complexes (upper panel), the donor (Rluc) and acceptor (YFP) fluorophores are brought into close proximity and energy is efficiently transferred (BRET) from the donor to the acceptor molecules after substrate oxidation. BRET signal cannot to be monitored if there is no interaction between two proteins and only blue light is emitted by Rluc/substrate oxidation (lower panel).

excitation (Fig. 1; top panel). Up to this point, BRET has successfully been used for the studies of GPCR in order to investigate the protein-protein interactions in living cells [8–10].

Protein-protein interactions of mitochondrial outer membrane protein via BRET analysis

Mitochondria, compartmentalized by two membrane bilayers [outer (OM) and inner (IM) membranes], play various essential roles in cell function and are known to act as central hubs for multiple signal transductions [11,12]. Recent research has revealed that mitochondria are also involved in cellular innate antiviral immunity in vertebrates, particularly mammals [13,14]. In the immune pathway, cytoplasmic viral-derived double-stranded RNA (dsRNA) is recognized by retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and ultimately activates intracellular signaling cascades that result in transcriptional activation (NF-κB and interferon regulatory factor 3), leading finally to the killing of infectious viruses [14]. A mitochondrial OM protein, the mitochondrial antiviral signaling (MAVS) [13], acts as an adaptor molecule downstream of RLR, and its earlier interactions between RLRs and MAVS would trigger the immune signaling pathway (Fig. 2A). It has been proposed that MAVS undergoes its multistep structural changes from inactive to active conformation post the RLR translocates at the OM to interact with MAVS. However, these processes are transient populated states during the signaling, so it has been so difficult to reconstitute the structural changes *in vitro* by using recombinant expressed proteins.

The BRET system has provided important insight into the structural transition of MAVS between inactive and active states in live cells [15,16]. The interaction between MAVS molecules containing either Rluc- or YFP-fusion tags at their N-termini was beautifully monitored via a BRET saturation assay (Fig. 2B; closed squares). Baril *et al.* have revealed that this MAVS-MAVS homotypic oligomerization would be essential for inducing the downstream transcriptional activations, and they proposed that RLR-MAVS interaction induces MAVS activation [15]. Indeed, another group supported the result that RLR recruitment to the MAVS on the surface of OM enhanced the self-oligomerization of MAVS [17]. By combining biomolecular fluorescence complementation (BiFC; non-fluorescent N-terminal and C-terminal fragments of YFP fused with MAVS) (also refer bottom panel of Fig. 2D) with the BRET system, it has been further demonstrated that activated MAVS conformation is highly ordered oligomers as expected to be more than three molecules on the mitochondrial surface [16]. Those specificities of the observed MAVS-MAVS interactions via BRET were also tested by the presence of mitofusin 2 (Mfn2), an inhibitor of the RLR pathway [18] showing that Mfn2 dose-dependently decreased the BRET intensity of MAVS oligomerization because of steric hindrance (Fig. 2C).

Viral protein and mitochondria

Finally, we briefly describe another case of protein-protein interaction at the sub-mitochondrial compartment structure. The influenza A viral protein PB1-F2 is a unique non-structural polypeptide known to localize at mitochondria [19]. Thorough biochemical analysis indicated that PB1-F2 translocates into the inner membrane space (IMS) of mitochondria via TOM machinery and associated tightly with the IM [20]. When the structural characteristics of the mitochondrially targeted PB1-F2 were investigated by a combination of BRET and BiFC assay in live cells, it was revealed that the viral protein was homotypically associated with itself and oligomerized into a highly ordered structure (Fig. 2D) [20]. Several studies using different experimental approaches have shown that the PB1-F2 construct also forms oligomeric/aggregated states [21–23], further implying the significance of the protein concentration at IMS of mitochondria. Indeed, the subcellular localization of PB1-F2 impairs the antiviral innate immunity of host cells [20, 24].

Future perspective

The BRET system combined with BiFC would be ideal to increase the number of molecules in an interaction to more than three (or more). A novel technique, so-called sequential BRET-FRET (SRET) [25], would be an attractive strategy by which to understand heteromerization complexes in a physiological environment. Recently, BRET has been used not only to study specific interactions of molecules described above, but also widely for other purpose, such as high-throughput screening to search for novel molecules and compounds [26] or monitoring cellular metabolism [27].

Finally, in this review we have demonstrated several examples of the use of the BRET assay to examine intermolecular interactions that have been advanced mainly in mitochondrial proteins. The application of other organelle-bounded molecules or cytoplasmic proteins is also expected.

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Conflict of interest

The author declare no competing financial interests.

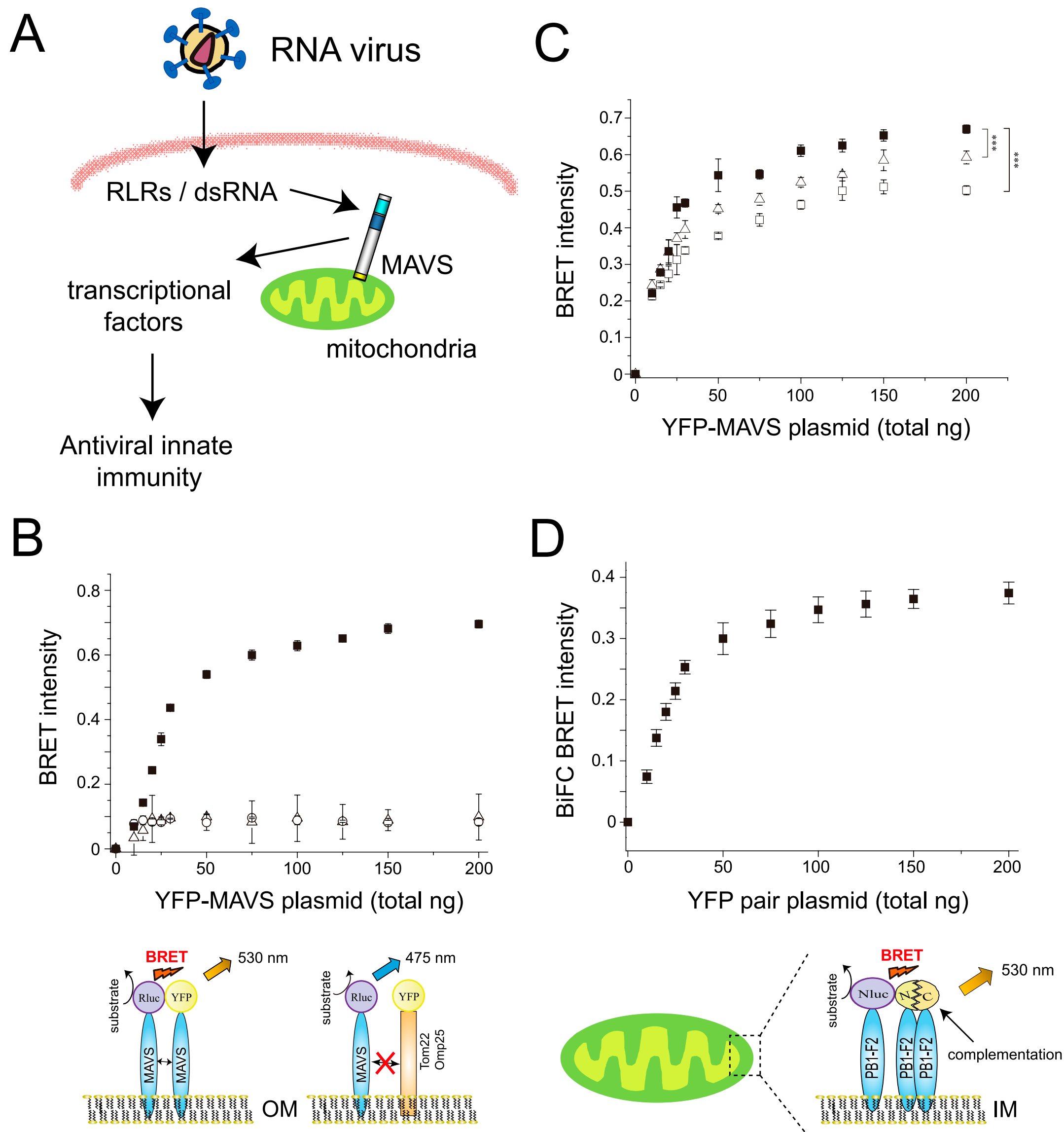

Figure 2 (A) A brief schematic of innate immunity against RNA virus in mammals. (B) BRET saturation curves of Rluc-MAVS/YFP-MAVS (closed squares), Rluc-MAVS/YFP-Tom22 (open triangles), and Rluc-MAVS/YFP-Omp25 (open circles). Tom22 and Omp25 were used as negative controls. Bottom diagram represents MAVS-MAVS homotypic oligomerization on the surface of OM. This figure is reproduced from a previous paper [16]. (C) Mfn2 affects MAVS oligomerization in a dose-dependent manner. BRET saturation curves of Rluc-MAVS/YFP-MAVS (closed squares) interaction were monitored in the presence of either with 50 (open triangles) or 100 ng (open squares) of Mfn2 expression plasmids. This figure is reproduced from a previous paper [16]. (D) BiFC BRET saturation curve was successfully monitored when cells were co-transfected with Nanoluc-PB1-F2 and split YFP-PB1-F2 plasmids. Bottom diagram represents a structural characteristic of the mitochondrially targeted PB1-F2 around IM. This figure is reproduced from a previous paper [20].

Author contribution

T.K. contributed to review the field of BRET study and wrote the manuscript.

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