

Development of a baculovirus-based fluorescence resonance energy transfer assay for measuring protein–protein interaction

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A new baculovirus-based fluorescence resonance energy transfer (Bv-FRET) assay for measuring multimerization of cell surface molecules in living cells is described. It has been demonstrated that gonadotropin-releasing hormone receptor (GnRH-R) was capable of forming oligomeric complexes in the plasma membrane under normal physiological conditions. The mouse gonadotropin-releasing hormone receptor GnRH-R was used to evaluate the efficiency and potential applications of this assay. Two chimeric constructs of GnRH-R were made, one with green fluorescent protein as a donor fluorophore and the other with enhanced yellow fluorescent protein as an acceptor fluorophore. These chimeric constructs were coexpressed in an insect cell line (BTI Tn5 B1-4) using recombinant baculoviruses. Energy transfer occurred from the excited donor to the acceptor when they were in close proximity. The association of GnRH-R was demonstrated through FRET and the fluorescence observed

using a Leica TSC-SPII confocal microscope. FRET was enhanced by the addition of a GnRH agonist but not by an antagonist. The Bv-FRET assay constitutes a highly efficient, reliable and convenient method for measuring protein–protein interaction as the baculovirus expression system is superior to other transfection-based methods. Additionally, the same insect cell line can be used routinely for expressing any recombinant proteins of interest, allowing various combinations of molecules to be tested in a rapid fashion for protein–protein interactions. The assay is a valuable tool not only for the screening of new molecules that interact with known bait molecules, but also for confirming interactions between other known molecules.

Keywords: FRET; baculovirus; membrane protein–protein interaction; dimerization; GnRH receptor.

The dimerization of cell surface molecules represents one of the most important phenomena in signal transduction because it opens a new level of understanding of the basic function and interactions of these molecules. Many molecules that were thought to function as monomers are in fact capable of forming dimeric or oligomeric complexes, and many membrane proteins such as receptor tyrosine kinases [1,2], membrane lymphotoxin- $\alpha\beta$ ligands [3–6], receptors for growth hormone [7–10], and many G protein-coupled receptors associate as functional oligomeric complexes [11–14]. Consequently, there is an increasing demand for a reliable and convenient assay for measuring protein–protein interactions in living cells.

In the past few years, a number of different fluorescence resonance energy transfer (FRET)-based assays have been developed [15–20]. FRET is a useful method for investigating the associations of molecules. It is based on the transfer of energy from one fluorophore (the donor) to another fluorophore (the acceptor) that usually emits fluorescence of a different colour. As FRET efficiency depends on the distance between the donor and acceptor (usually less than 100 Å apart) [21–25], it provides a useful assessment for protein–protein interaction, especially the dimerization of cell surface molecules.

So far, most FRET assays performed *in vivo* have been performed primarily in transfected cells. A major disadvantage of the transfection-based FRET assays lies in the difficulty of controlling the level of individual recombinant protein expression in transfected cell cultures. The expression of recombinant proteins in transient-transfected cells is influenced by many factors, including transfection efficiency of a given cell type, the quality of the DNA, the quantity of DNA taken up by the cells, the cytotoxicity of the transfection reagents, and the condition of the cells. For example, low transfection efficiency results in having insufficient cells that coexpress both donor and acceptor fluorophores. In addition, a low level of protein expression may result in insufficient amounts of donor and acceptor fluorophores located in close vicinity, reducing the probability of their interaction. Furthermore, FRET efficiency also depends on the ratio of coexpression between donor and acceptor fluorophores (Table 1). To exclude artefacts

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Abbreviations: FRET, fluorescence resonance energy transfer;

Bv-FRET, baculovirus-based FRET; GnRH, gonadotropin-releasing

hormone; GnRH-R, GnRH receptor; GFP, green fluorescent protein;

EYFP, enhanced yellow fluorescent protein; Tn5 cells, BTI Tn5 B1-4

cells; MOI, multiplicity of infection; LT α , lymphotoxin α ; LT β ,

lymphotoxin β ; IL-2R α , interleukin-2 receptor α -subunit.

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Table 1. The probability of formation of various complexes with reference to the ratios between molecules A and B. The Hardy–Weinberg law was used as the mathematical model for calculating the frequency of heterodimeric and homodimeric complexes formation.

A : B ratios	Probability (%)		
	AB	AA	BB
1 : 1	50.0	25.0	25.0
2 : 1	44.4	44.4	11.2
3 : 1	37.5	56.2	6.3
4 : 1	32.0	64.0	4.0
5 : 1	27.8	69.4	2.8

due to aberrant donor to acceptor expression ratios and to increase the FRET signal-to-noise ratio, an optimal level of expression for both donor and acceptor is a basic requirement for FRET. Although some of the drawbacks associated with expressing recombinant proteins through transient-transfected cells could be minimized by using stable-transformed cells with the capability of coexpressing multiple recombinant proteins at a desirable level and ratio, these procedures are time consuming as well as labor intensive. Therefore, FRET assays using stable-transformed cells are unlikely to be a popular choice for many researchers.

As the accuracy and sensitivity of FRET assays rely on the ability to optimize protein expression in cell culture, it is necessary to perform them using a reliable protein expression system. The baculovirus system has proven to be one of the most powerful and reliable eukaryotic protein expression systems that can be used to express functionally active recombinant proteins [26–32]. Many of the post-translational modification pathways, such as phosphorylation, glycosylation, myristoylation and palmitoylation present in mammalian systems are also utilized in insect cell lines, allowing the production of recombinant protein that is functionally similar to the native mammalian protein [33,34]. Most importantly, the baculovirus system allows one to achieve a fine control on the level of recombinant protein expression, manipulating it by adjusting the multiplicity of infection (MOI).

By combining the merits of both FRET and the baculovirus system, a new baculovirus-based FRET (Bv-FRET) assay was developed for detecting protein–protein interaction. This system offers all the advantages of FRET assays but overcomes the shortcomings of the transfection-based methods. The Bv-FRET assay has two major advantages. Firstly, it allows protein–protein interactions to be observed in living cells with confocal microscopy. Secondly, it allows direct control of the level of individual recombinant protein expression and coexpression of both donor and acceptor fluorophores in a desirable ratio.

Lundin *et al.* reported a FRET-based assay for measuring protein expression on the cell surface using a baculovirus expression system. Their study used europium as a donor attaching to the biotinylated cell surface of the Sf9 cells. The human interleukin-2 receptor α -subunit (IL-2R α) was also expressed on the cell surface by infecting the cells with recombinant baculoviruses. FRET was used as an assessment for protein expression on the cell surface

through the Cy5-labeled antibody against IL-2R α as an acceptor fluorophore. Although their assay was not designed to study protein dimerization, it demonstrated the potential application of baculovirus in the FRET-based assays [15].

GnRH-R is a member of the G protein-coupled receptors superfamily, which represents the largest grouping of cell surface receptors, mediating a wide variety of extracellular stimuli, such as light, Ca²⁺, odors, pheromones, peptides and proteins [35]. All G protein-coupled receptors have a common central core, which is composed of seven transmembrane domains connected by three extracellular loops and three intracellular loops [36]. Recent studies showed that GnRH-R was capable of forming multimeric complexes in the cell surface under normal physiological conditions [37,38]. It has also been shown that functionally active GnRH-R can be expressed in insect cells using recombinant baculovirus [29,30]. In the present study, the mouse GnRH-R was used to evaluate the efficiency of the new Bv-FRET assay.

Materials and methods

Construction of expression plasmids

A mouse GnRH-R/green fluorescent protein (GFP) baculovirus expression plasmid was constructed by inserting GnRH-R cDNA in multiple cloning sites upstream of the GFP of a PVL1393 BioGreen vector (Pharmingen, San Diego, CA, USA). The mouse GnRH-R insert was synthesized by PCR using *Pfu* DNA polymerase (Promega, Madison, WI, USA) and mouse GnRH-R cDNA (generous gift of M. Perrin, Salk Institute, San Diego, CA, USA) as a template. A *Bgl*II restriction site (bold) was introduced into the forward primer (5'-CCTGTCAGATCTCCGCCATGGCTAACAAATGCATCTCT-3'), and a *Bam*HI site (bold) was introduced into the reverse primer (5'-TCTCCCGGATCCAAAGAGAAATACCCATA-TA-3') to facilitate vector–insert ligation. Amplification conditions were 4 min at 92 °C, followed by 35 cycles of 1 min at 92 °C, 30 s at 55 °C, and 2 min and 30 s at 72 °C. A final extension was carried out at 72 °C for 10 min. PCR products were purified by QIAquick PCR purification columns (Qiagen, Hilden, Germany), and a double digestion with *Bgl*II and *Bam*HI restriction enzymes was carried out. The PVL1393 BioGreen vector was linearized by *Bam*HI digestion, and the prepared GnRH-R insert was ligated into the prepared vector. The ligation mixture was transformed into XL1-Blue cells (Stratagene, San Diego, CA, USA) according to the manufacturer's protocol.

A mouse GnRH-R/enhanced yellow fluorescent protein (EYFP) expression plasmid was made by removal and replacement of GFP from the GnRH-R–GFP expression plasmid with EYFP. GFP was removed by *Bam*HI and *Eco*RI digestions. The EYFP insert was synthesized by PCR using *Pfu* DNA polymerase and pEYFP-N1 vector (Clontech Laboratories, Palo Alto, CA, USA) as a template. PCR was carried out as described above using the forward primer (5'-AATTCTGCAGTCGACGGTAC-3') and the reverse primer (5'-GATTATGAATTCGAGTCGCGGCCGCTTTACTT-3'). An *Eco*RI site (bold) was introduced into the reverse primer. The PCR product

was purified, and a double digestion with *Bam*HI and *Eco*RI carried out. The GnRH-R–PVL1393 vector arm was prepared by removal of GFP from the *Bam*HI and *Eco*RI sites, and the prepared EYFP insert was ligated into the vector arm. The ligation mixture was transformed into XL1-Blue cells according to the manufacturer's protocol.

PVL1393 BioGreen expression plasmid was used for the expression of cytosolic GFP. Cytosolic EYFP expression plasmid was constructed by the removal and replacement of GFP from the PVL1393 BioGreen vector with EYFP. GFP was removed from the PVL1393 BioGreen vector by *Bam*HI and *Eco*RI digestions. The EYFP insert was prepared as described above, then ligated in the prepared vector. The ligation mixture was transformed into XL1-Blue cells according to the manufacturer's protocol.

Transfection and amplification of recombinant baculoviruses

BTI Tn5 B1-4 cells (generous gift of S. Ford, Australian National University, Canberra, Australia) were used for expression of fusion proteins. BTI Tn5 B1-4 cells (Tn5 cells) are a cell line derived from the *Trichoplusia ni* egg cells and are commonly used for the expression of proteins using recombinant baculoviruses. Tn5 cells (0.7×10^6) were seeded in a T25 tissue culture flask containing 5 mL of Ex-Cell 405 medium (JRH Biosciences, Lenexa, KS, USA). The sample was placed at room temperature and the cells were allowed to attach firmly to flask (approximately 15 min). Transfection was performed using Lipofectin reagent (Life Technologies, Gaithersburg, MD, USA). The expression plasmids were cotransfected with the BaculoGold baculovirus DNA (Pharmingen), according to the manufacturer's instructions. The transfected cells were incubated at 27 °C for 4 days. Afterwards, culture medium was collected and used to infect freshly prepared cells for viral amplification. An end-point titration was carried out to isolate a single clone. The recombinant baculovirus was amplified to obtain a high titer stock solution by infecting freshly seeded Tn5 cells at $\text{MOI} = 0.5 \text{ U}\cdot\text{cell}^{-1}$. The infected cells were incubated at 27 °C for 4 days before the medium was harvested. End-point dilution was used to determine the viral titer.

GnRH-R–GFP and GnRH-R–EYFP expression

To examine the expression and subcellular localization of the GnRH-R–GFP and GnRH-R–EYFP fusion proteins, protein expression using recombinant baculovirus was carried out by infecting freshly seeded Tn5 cells in a Laboratory-Tek II chambered coverglass (Nalge Nunc International, Naperville, IL, USA). The cells were infected with GnRH-R–GFP or GnRH-R–EYFP recombinant baculovirus at 3 $\text{MOI}\cdot\text{cell}^{-1}$ and incubated at 27 °C for 2 days. Cells expressing the recombinant proteins were detected with a Leica TCS-SPII confocal system (Leica, Heidelberg, Germany) fitted to a DMIRBE microscope (Leica) using a 63×1.2 numerical aperture water immersion objective. The pinhole was set at 1 Airy disc unit, and an appropriate dichroic beam-splitting mirror was used. GnRH-R–GFP expressing cells were visualized by illumination using a Coherent Enterprise 651 Ar-UV laser (Coher-

ent, Santa Clara, CA, USA) with the laser line set at 364 nm, and the fluorescence was detected at an emission window of 480–602 nm. For the detection of GnRH-R–EYFP fluorescence, the cells were illuminated by an Ar-visible laser (JDS Uniphase, San Jose, CA, USA) with the laser line set at 514 nm, and the fluorescence was detected at an emission window of 520–602 nm. The cells were illuminated with minimum level of laser power, and images were recorded at a frame-average of eight. To minimize photobleaching and cell movement during imaging, the recording was completed in approximately 5 s.

Spectral characterization of GFP and EYFP

For GFP, GnRH-R–GFP expressing cells were illuminated with an Ar-UV laser and the laser line set at 364 nm. Spectral scanning was carried with the interval of scanning set at 2.24 nm. For EYFP, GnRH-R–EYFP expressing cells were illuminated with an Ar-visible laser and the laser line set at 488 nm. Spectral scanning was carried out as above.

FRET assay

Cell culture and expression of the GnRH-R–GFP, GnRH-R–EYFP, cytosolic GFP and EYFP were performed as described above. The principle of the FRET assay is illustrated in Fig. 1. GnRH agonist (pGlu-His-Trp-Ser-Tyr-D-Ala-N-methyl-Leu-Arg-Pro-Gly-NH₂; Sigma, St. Louis, MO, USA) was added to the culture at a final concentration of 100 nM [37]. Five minutes after the addition of the GnRH agonist, the prepared cells were visualized by illumination with an Ar-UV laser and the laser line set at 364 nm. The GnRH-R–GFP expressing cells were observed in the green channel with the detection window at 484–512 nm. FRET, GFP fluorescence bleed-through and EYFP emission resulting from the Ar-UV excitation were detected at the FRET channel with the detection window at 530–570 nm. The net FRET image was obtained after subtracting the GFP fluorescence bleed-through and the emission of EYFP from Ar-UV laser excitation.

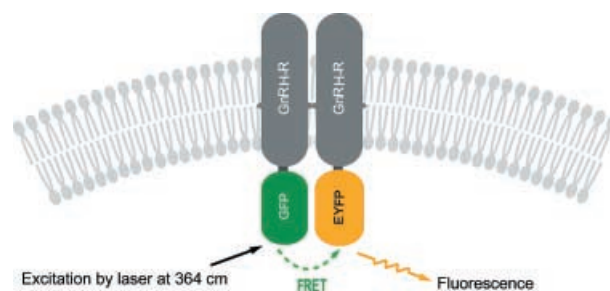


Fig. 1. Schematic illustrations of baculovirus-based fluorescence resonance energy transfer (Bv-FRET) assay. GFP is fused at the C-terminal end of the mouse GnRH-R as a donor fluorophore, and EYFP is fused at the C-terminal end as an acceptor fluorophore and these fusion proteins are coexpressed in a cell line (Tn5 cells). GFP is excited by an Ar-UV laser at 364 nm, and energy transfer occurs from GFP to EYFP that emits yellow fluorescence. The fluorescence is detected at the FRET channel with the detection window at 530–570 nm using a Leica TCS-SPII confocal microscope.

The amount of GFP fluorescence bleed-through subtracted was derived from the fluorescence obtained from cells only expressing GFP. To ensure the GFP fluorescence bleed-through from the GFP and EYFP coexpressing cells was fully subtracted, cells expressing GFP only should have similar, preferably equal, levels of GFP expression compared to that in the GFP and EYFP coexpressing cells. Similarly, the value of the EYFP background fluorescence (resulting from the Ar-UV laser excitation) subtracted was based on the fluorescence obtained from cells only expressing EYFP. To ensure this background fluorescence from the GFP and EYFP coexpressing cell was fully subtracted, the cells only expressing EYFP should have similar, preferably equal, levels of EYFP expression compared to that in the GFP and EYFP coexpressing cells. The subtraction (below) was carried out using the Leica TCS-SPII data analysis software (version 2002).

$$\begin{aligned} \text{FRET} = & \text{Total emission collection at 530–570nm} \\ & \text{window} - \text{GFP fluorescence bleed-through} \\ & - \text{EYFP emission resulting from the} \\ & \text{Ar-UV laser excitation.} \end{aligned}$$

In addition, GnRH-R–EYFP expressing cells were visualized by illumination using an Ar-visible laser with the laser line set at 514 nm. Although this excitation wavelength was suboptimal for EYFP, it did not cause coexcitation of GFP. EYFP expressing cells were detected in the yellow channel with the detection window at 520–602 nm. Images were recorded at a frame-average of eight. Each experiment was repeated a minimum of three times.

For the time series experiment, the yellow to green fluorescence ratio as an indicator of FRET was measured in the presence or absence of GnRH analogues. Cell culture, protein expression, excitation setting and emission channels were the same as described above. A GnRH agonist (pGlu-His-Trp-Ser-Tyr-D-Ala-N-methyl-Leu-Arg-Pro-Gly-NH₂; Sigma) or antagonist (pGlu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH₂; Sigma) was added to the cells at a final concentration of 100 nM. Five minutes after the addition of GnRH agonist or antagonist, images from the green and FRET channels were recorded at a frame average of eight every 2 min for up to 20 min. A minimum level of laser power and duration of recording time were set for imaging to minimize photobleaching and cell movement during recording, respectively. The average intensity of the yellow and green fluorescence in the membrane region was measured at each time point, and values were normalized to unity with reference to the set value at time zero. The yellow to green ratio was calculated and the values were plotted against time. Each assay was repeated at least three times.

Results

Receptor expression and subcellular localization

Recent studies have shown that GnRH-R is capable of forming stable oligomeric complexes in the plasma membrane [37,38]. In this study, mouse GnRH-R was used to examine the efficiency of our Bv-FRET assay. Two chimeric

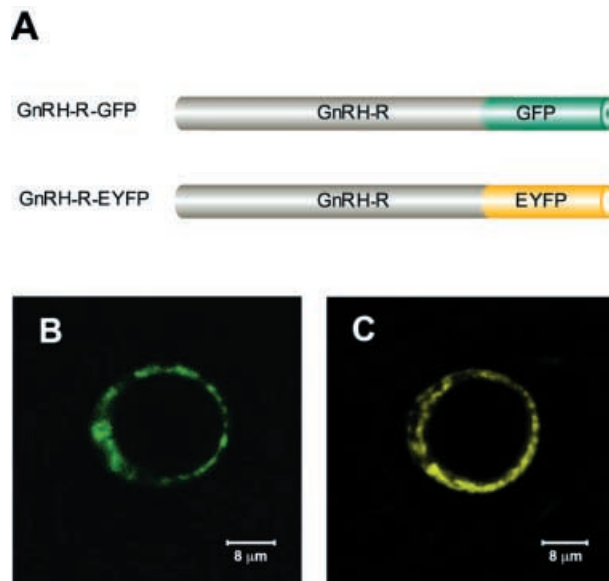


Fig. 2. Expression and subcellular localization of GnRH-R-GFP and GnRH-R-EYFP fusion proteins. (A) Schematic diagrams of the recombinant protein cassettes in the PVL1393 expression vector. (B) Expression and subcellular localization of GnRH-R-GFP. GnRH-R-GFP expressing cells were visualized using an Ar-UV laser with the laser line set at 364 nm, and the fluorescence was detected at an emission window of 480–602 nm. Fluorescence is coloured in green. (C) Expression and subcellular localization of GnRH-R-EYFP in a Tn5 cell. GnRH-R-EYFP expressing cells were illuminated by an Ar-visible laser with the laser line set at 514 nm, and the fluorescence was detected at an emission window of 520–602 nm. Fluorescence is coloured in yellow. These images were recorded with a Leica TCS-SPII confocal microscope in a frame-average of eight.

constructs were made. One of them was fused at the C-terminal end of the mouse GnRH-R with GFP and the other one with EYFP. These fusion proteins were expressed in Tn5 cells using a baculovirus expression system (Fig. 2A). The subcellular localization of the recombinant receptors was examined with confocal microscopy. In cells expressing each of these receptor constructs, GFP and EYFP were localized on the plasma membrane (Fig. 2B,C), showing that the addition of GFP or EYFP to the C-terminal end of GnRH-R did not affect its membrane localization.

Assay optimization

The assay was optimized in order to determine the optimal infection intensity for individual recombinant protein expression and conditions of measuring FRET. Protein expression was optimized by a series titration on the intensity of viral infection from 0.1 to 10 MOI per cell. The results (data not shown) indicated that 2–5 MOI of each recombinant virus per cell gave a sufficient level of protein expression for the FRET assay while it still allowed some cells expressing only GFP or EYFP to be found in the cell culture. The presence of these single fluorophore-expressing cells were essential for the FRET assay because the fluorescence of these cells served as references for the subtraction of background fluorescence (the GFP

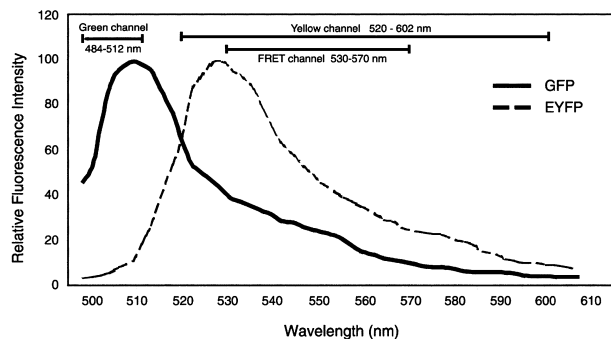


Fig. 3. Spectral characterization of GnRH-R-GFP and GnRH-R-EYFP fusion proteins. Emission spectra of both GnRH-R-GFP and GnRH-R-EYFP were determined by the spectral scanning program of Leica TSC-SPII confocal system. The peak of each emission curve was normalized to a set value of 100 units. Bar lines show the range that the detection windows were set for the FRET assay.

fluorescence bleed-through and the EYFP emission resulting from the Ar-UV laser excitation) of the GFP and EYFP coexpressing cells. The assay was also optimized for the measurement of FRET. Excitation was carried out using an Ar-UV laser with the laser line set at 364 nm to minimize coexcitation of EYFP, and a minimum energy level was used for imaging to minimize photobleaching. Optimum emission windows for both GFP and EYFP were determined with the spectral scanning program of a Leica TSC-SPII confocal microscope. To minimize the bleed-through of GFP fluorescence, FRET was measured at the emission window of 530–570 nm (Fig. 3).

Dimerization of GnRH receptor

Tn5 cells were infected with both GnRH-R-GFP and GnRH-R-EYFP recombinant baculoviruses. GnRH agonist was added to the cells at a final concentration of 100 nM [37]. Five minutes after the addition of GnRH agonist, the cells were illuminated using an Ar-UV laser with the laser line set at 364 nm, and the GnRH-R-GFP expressing cells were observed in the green channel with the detection window at 484–512 nm. This channel is primarily for detecting GFP expressing cells (Fig. 4A). For detection of GnRH-R-EYFP expressing cells, EYFP was illuminated with an Ar-visible laser with the laser line set at 514 nm, a wavelength that did not cause coexcitation of GFP. Fluorescence was detected in the yellow channel with the detection window set at 520–602 nm (Fig. 4B).

Total fluorescence (FRET + GFP fluorescence bleed-through + EYFP fluorescence resulting from the Ar-UV laser excitation) under the Ar-UV laser excitation with the laser line at 364 nm was measured at the FRET channel with the detection window at 530–570 nm (Fig. 4C). The fluorescence signal caused by FRET was obtained after subtracting the GFP fluorescence bleed-through and the emission of EYFP resulting from the Ar-UV laser excitation at 364 nm (Fig. 4D). The remaining fluorescence was due to FRET. The signal of FRET was observed under a confocal microscope. Only in the GnRH-R-GFP and GnRH-R-EYFP coexpressing cell did the results show the dimerization of receptor in the plasma membrane.

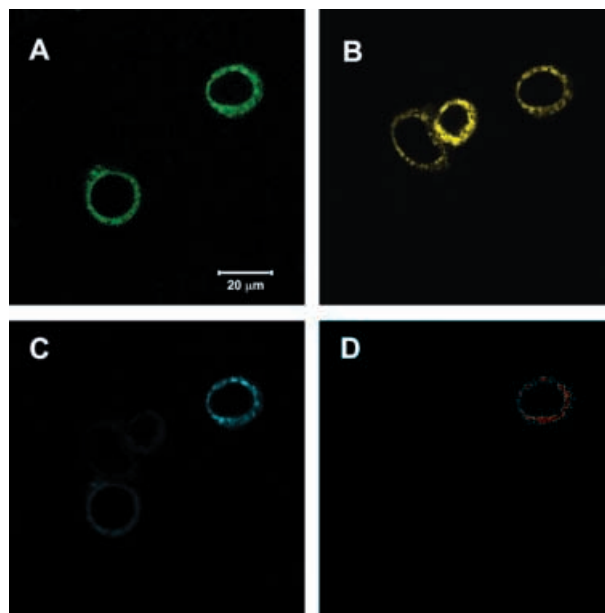


Fig. 4. Assessment of the association of GnRH-R using Bv-FRET assay. (A) GnRH-R-GFP expressing cells. Tn5 cells were infected by both GnRH-R-GFP and GnRH-R-EYFP recombinant baculoviruses. GnRH-R-GFP expressing cells were visualized by illumination using an Ar-UV laser with the laser line set at 364 nm, and the cells were observed in the green channel with the detection window at 484–512 nm. Fluorescence is coloured in green. (B) GnRH-R-EYFP expressing cells. Cells were visualized by illumination using an Ar-visible laser with the laser line set at 514 nm. EYFP fluorescence was detected in the yellow channel with the detection window at 520–602 nm. Fluorescence is coloured in yellow. (C) Fluorescence observed in the FRET channel. FRET, GFP fluorescence bleed-through and EYFP emission resulting from the Ar-UV excitation were detected in the FRET channel with the emission window at 530–570 nm. Fluorescence is coloured in cyan. (D) Signal of FRET. Net fluorescence resulting from FRET was obtained after subtracting the GFP fluorescence bleed-through and the emission of EYFP resulting from the Ar-UV laser excitation as observed in the FRET channel. Fluorescence resulting from FRET is printed in shades of gray. Calibration bar in A (20 μm) also refers to B–D.

Negative controls

To rule out the possibility that FRET observed in Fig. 4 might be due to protein–protein interaction between GFP and EYFP, GFP and GnRH-R, or EYFP and GnRH-R, two negative control experiments were designed. The principles of these negative controls are illustrated in Fig. 5. Tn5 cells were expressed with cytosolic GFP as well as membrane-bound GnRH-R-EYFP, and FRET was measured. Figure 6A shows the cytosolic GFP expressing cells, and Fig. 6B shows the GnRH-R-EYFP expressing cells. In cells coexpressing both GFP and GnRH-R-EYFP, FRET was not seen after subtracting the GFP fluorescence bleed-through and the emission of EYFP from Ar-UV laser excitation as described in the FRET assay section (Fig. 6C,D). These results indicate that GFP did not interact with EYFP or GnRH-R.

An additional control experiment was carried out using membrane-bound GnRH-R-GFP and cytosolic

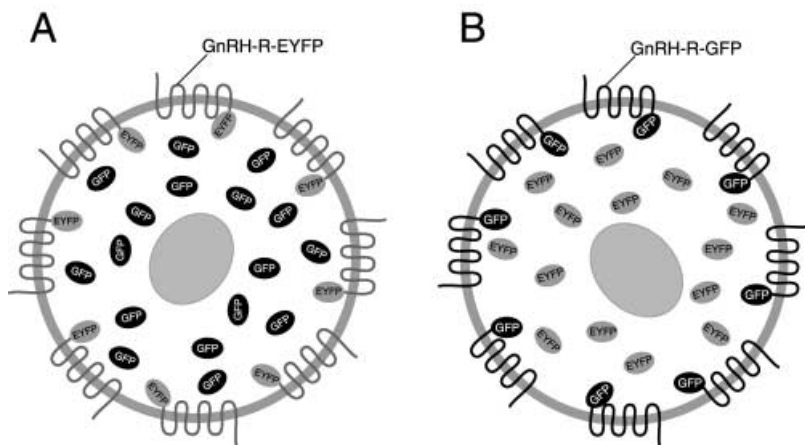


Fig. 5. Schematic illustrations of the negative controls. (A) Coexpression of GnRH-R-EYFP and cytosolic GFP in a Tn5 cell. (B) Coexpression of GnRH-R-GFP and cytosolic EYFP in a Tn5 cell.

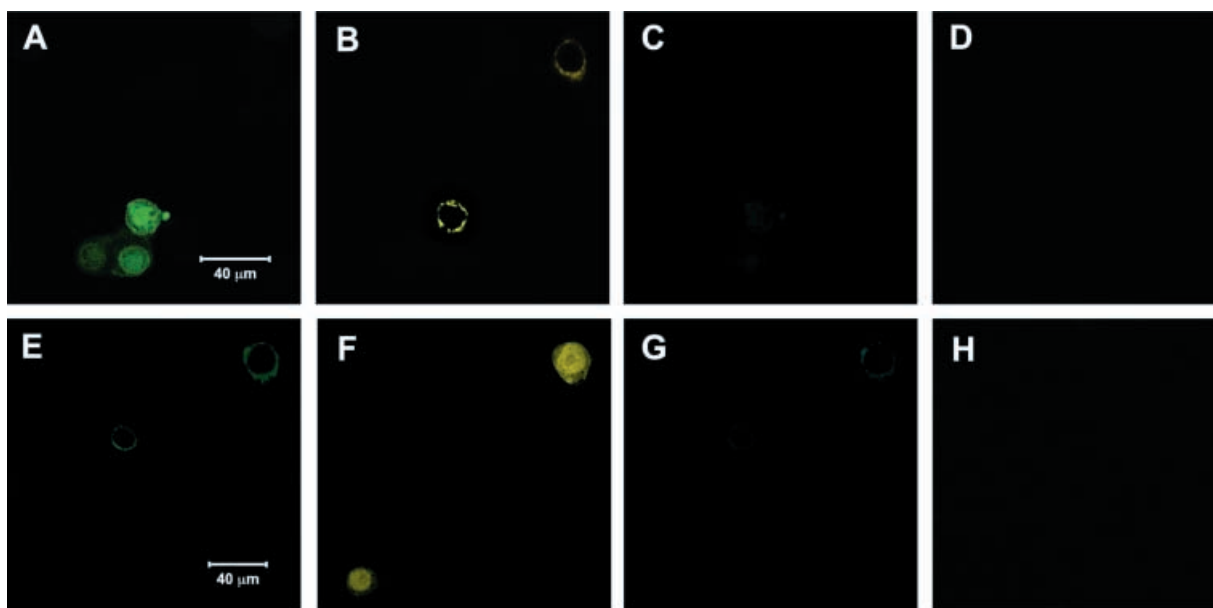


Fig. 6. The results of FRET assays on the negative controls. To examine any potential interaction between GFP, EYFP and GnRH-R, FRET assay was carried out in the GFP and GnRH-R-EYFP coexpressing cell culture. (A) Cytosolic GFP expressing cells. GFP expressing cells were detected by illumination using an Ar-UV laser with the laser line set at 364 nm, and the cells were observed in the green channel with the detection window at 484–512 nm. Fluorescence is coloured in green. Note the presence of GFP fluorescence in the cytoplasm. (B) GnRH-R-EYFP expressing cells. Cells were visualized by illumination using an Ar-visible laser with the laser line set at 514 nm. EYFP fluorescence was detected in the yellow channel with the detection window at 520–602 nm. Fluorescence is coloured in yellow. Note the presence of EYFP fluorescence only in the membrane region. (C) Fluorescence recorded in the FRET channel. FRET (if any), GFP fluorescence bleed-through and EYFP emission resulting from the Ar-UV excitation were detected in this channel with the emission window at 530–570 nm. Fluorescence is coloured in cyan. (D) Remaining fluorescence after subtracting the GFP fluorescence bleed-through and EYFP emission resulting from the Ar-UV excitation. Fluorescence was not seen after the subtraction. To examine any potential interaction between EYFP and GnRH-R, FRET assay was carried out in the GnRH-R-GFP and EYFP coexpressing cell culture. (E) GnRH-R-GFP expressing cells. Fluorescence is coloured in green. Note green fluorescence in the membrane region. (F) Cytosolic EYFP expressing cells. Fluorescence is coloured in yellow. Note yellow fluorescence in cytoplasm. (G) Fluorescence recorded in the FRET channel. Fluorescence is coloured in cyan. (H) Remaining fluorescence after subtracting the GFP bleed-through and EYFP emission resulting from the Ar-UV excitation. Fluorescence was not seen after the subtraction. Calibration bar in A (40 μm) also refers to B–D; bar in E (40 μm) also refers to F–H.

EYFP to exclude the possibility of any potential interaction between EYFP and GnRH-R. Cells expressing GnRH-R-GFP and EYFP are shown in Fig. 6E,F, respectively. In cells coexpressing both GnRH-R-GFP and EYFP, FRET did not occur (Fig. 6G,H). Taken

together, these results indicate that protein–protein interaction did not occur between GFP and EYFP, GFP and GnRH-R, or EYFP and GnRH-R. FRET observed in Fig. 4D was a result of specific interaction between GnRH-R molecules.

Fig. 7. Effect of GnRH analogues on the association of GnRH-R molecules. Tn5 cells coexpressing both GnRH-R-GFP and GnRH-R-EYFP were prepared. A GnRH agonist or antagonist was added to the cells 5 min before the measurement of FRET was taken. Images from the green channel (with detection window from 484 to 512 nm) and FRET channel (with detection window from 530 to 570 nm) were recorded at intervals of 2 min for up to 20 min after the addition of GnRH agonist or antagonist in a final concentration of 100 nM. The average intensity of the yellow and green fluorescence in the membrane region was measured at each time point, and values were normalized to unity with reference to the set value at time 0. The yellow to green ratios were calculated and the values were plotted against time. (A) Control experiment. A time series was taken in the absence of GnRH analogues. (B) Cells with GnRH agonist. (C) Cells with GnRH antagonist.

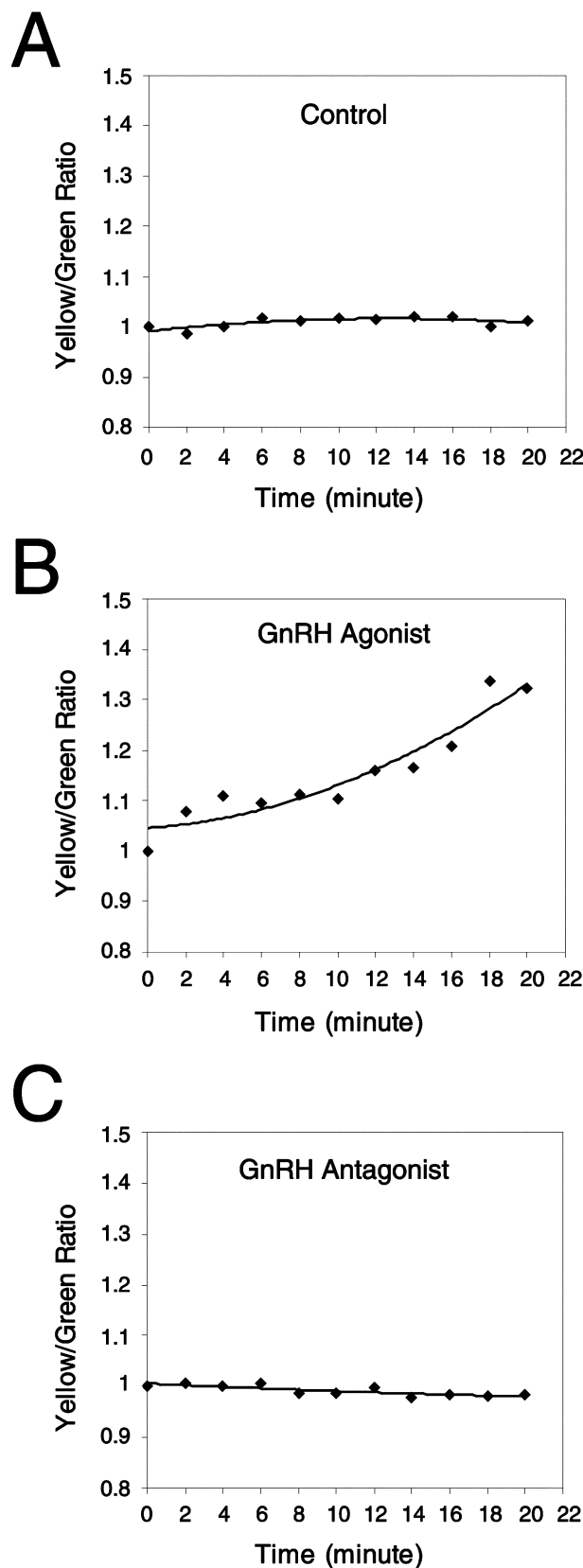
Effect of GnRH analogues on the association of GnRH receptor

Recent data shows that GnRH agonists play a positive role in rat GnRH-R multimerization [37,38]. The effect of a GnRH agonist and antagonist on mouse GnRH-R dimerization has been examined. Tn5 cells expressing both GnRH-R-GFP and GnRH-R-EYFP were prepared. A GnRH agonist or antagonist was added to the cells 5 min before measuring FRET. Images from the green and FRET channels were recorded at intervals of 2 min up to 20 min after the addition of the GnRH agonist or antagonist to the final concentration of 100 nM [37]. The average intensity of the yellow and green fluorescence in the membrane region was measured at each time point, and the yellow to green ratio was calculated. In the presence of 100 nM GnRH agonist, there was an increase in the yellow to green ratio (Fig. 7B) compared to the control (Fig. 7A). However, the addition of an antagonist did not result in an increase of the yellow to green ratio (Fig. 7C). These results showed that a GnRH agonist enhanced the association of GnRH-R.

Discussion

The efficiency and potential applications of a new Bv-FRET assay have been demonstrated by examining protein-protein interaction between mouse GnRH-R molecules on cell surfaces. Insect cells coexpressing GnRH-R-GFP and GnRH-R-EYFP were prepared by infecting Tn5 cells with recombinant baculoviruses. Additionally, cells coexpressing GnRH-R-GFP and cytosolic EYFP, and cells coexpressing GnRH-R-EYFP and cytosolic GFP were used as negative controls. The association of GnRH-Rs in the plasma membrane was demonstrated through FRET, and the FRET signals were visualized with a confocal microscope (Fig. 4). In contrast, when GFP or EYFP were not membrane-anchored with GnRH-R, FRET did not occur, as shown in the negative controls (Fig. 6). These observations indicated that FRET took place through the specific interaction between GnRH-R molecules.

The effect of a GnRH agonist and antagonist on GnRH-R association has also been examined. The data showed that FRET was enhanced by the addition of a GnRH agonist but not by an antagonist (Fig. 7), suggesting that the GnRH agonist facilitates receptor association. Although



the molecular basis of this action has not yet been precisely defined, it has been suggested that GnRH agonists provoke microaggregation of the receptor [37].

The Bv-FRET assay has a number of advantages over the transfection-based FRET assays. Firstly, the Bv-FRET system constitutes a reliable method. It allows a researcher to have direct control on the level of recombinant protein expression. This not only enhances the possibility of having a sufficient number of cells that coexpress both the donor and acceptor fluorophores, but also to produce them in a desirable ratio. Thus, it enhances the signal-to-noise ratio and greatly increases the sensitivity of FRET assays.

The Bv-FRET system allows the achievement of a high signal-to-noise ratio through protein expression. Since the efficiency and sensitivity of FRET assays depend on certain ratios between donor and acceptor fluorophores, the signal to noise ratio decreases if donor to acceptor is in an undesirable ratio. For the molecules that form homodimers or oligomers, the ideal ratio between the two fluorophores is 1 : 1 (Table 1). Based on the Hardy–Weinberg law [39,40], approximately 50% of the complexes would contain both donor and acceptor fluorophores, which are essential for FRET. However, when the ratio of the two fluorophores becomes greater or less than 1, the chances of forming complexes that contain both of the fluorophores will decrease. At a ratio of 5 : 1, only around 28% of the complexes may contain both of the fluorophores, and approximately 72% of the complexes may contain only one of the fluorophores. It is important to note that these single fluorophore-forming complexes are not capable of participating in FRET. Instead, they increase the noise and reduce the sensitivity of FRET assays. Therefore, the excess amount of these complexes must be minimized.

Although baculovirus-infected cells eventually start to die on the fourth or fifth day after infection, and there is a possibility that some recombinant proteins might induce apoptosis of cells [41], the baculovirus has proven its ability to reconstitute functionally active cell surface multimeric complexes in insect cells at an early stage of infection. A well-known example is the reassembly of membrane lymphotoxin- $\alpha\beta$ ligands (LT α 1 β 2 and LT α 2 β 1) [32]. A LT α 1 β 2 complex is composed of one LT α and two LT β s, and a LT α 2 β 1 complex contains two LT α s and one LT β . By simply adjusting the relative ratio of infection between the two recombinant baculoviruses (LT α and LT β), each of these molecules was correctly reconstituted. A FRET assay incorporating a baculovirus protein expression system is a sophisticated method as it enables coexpression of both donor and acceptor fluorophores in a desirable ratio with a high signal-to-noise ratio.

Secondly, the Bv-FRET assay constitutes a highly efficient and convenient method for measuring protein–protein interaction. The same insect cell line can be routinely used to express any recombinant proteins of interest, allowing various combinations of molecules to be tested in a rapid fashion for protein–protein interactions. Once recombinant viral stocks are obtained, FRET measurement can be performed 2 days after infection of the cells. Another benefit of using recombinant baculoviruses is that they are very stable when stored properly. Existing viral stocks can be used for the screening of new molecules or confirming the interaction between other known molecules. Furthermore, the study on the effect of GnRH agonist and antagonist on GnRH-R association shows that the Bv-FRET assay has the potential to be further developed and used for

investigating the molecular mechanism involved in protein–protein interaction and for screening novel molecules that might enhance or block the protein–protein interactions of molecules of interest.

In summary, the Bv-FRET assay represents a powerful method for studying protein–protein interactions. This assay can reveal the interaction between GnRH-R molecules and the effect of GnRH analogues on this association. It is a convenient, reliable, accurate and sensitive method of visualization of FRET and assessment of protein–protein interactions. It can potentially allow for studying the associations of molecules of any proteins of interest.

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