Functional complementation of high efficiency resonance energy transfer. A new tool for the study of protein binding interactions in living cells.

Paola Molinari, Ida Casella and Tommaso Costa

Department of Pharmacology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy

Correspondence:
T.Costa,
Dipartimento del Farmaco
Istituto Superiore di Sanità
Viale Regina Elena 299
00161 – Roma, Italy
Tel. 0039-0649902386
Fax 0039-0649902014
Email: tomcosta@iss.it

***Short title*:** Complementation-induced BRET using *Renilla* photo proteins.

Keywords: Resonance energy transfer, G protein-coupled receptors, β-arrestin, Renilla Luciferase, Renilla GFP, bioluminescence, Functional complementation

SYNOPSIS

Green bioluminescence in *Renilla* species is generated by a ~ 100% efficient resonance energy transfer (RET) process that is caused by the direct association of a blue-emitting luciferase (Rluc) and a green fluorescent protein (RGFP). Despite the high efficiency, such system was never evaluated as a potential reporter of protein-protein interactions. To address the question, we compared and analyzed in mammalian cells the bioluminescence of Rluc and RGFP co-expressed as free native proteins, or as fused single-chain polypeptides and tethered partners of self-assembling coiled-coils. Here we show that: (a) No spontaneous interactions generating detectable BRET signals occur between the free native proteins. (b) High efficiency BRET similar to that observed in *Renilla* occurs both in fusion proteins and self-interacting chimeras, but only if the amino terminal of RGFP is free. (c) The high efficiency BRET interaction is associated to a dramatic increase in light output when the luminescent reaction is triggered by low-quantum yield coelenterazine analogs.

Here we propose a new functional complementation assay based on the detection of the high efficiency BRET signal that is generated when the reporters Rluc and RGFP are brought into close proximity by a pair of interacting proteins to which they are linked. To demonstrate its performance we implemented the assay to measure the interaction between G protein-coupled receptors (GPCRs) and β–arrestins. We show that complementation-induced BRET allows detecting the GPCR–β–arrestin interaction in a simple luminometric assay with high signal to noise ratio, good dynamic range, and rapid response.

INTRODUCTION

Resonance energy transfer (RET) [1] between donor-acceptor pairs of chromophores linked to different proteins or protein domains has been extensively used to investigate nanoscale molecular interactions in protein biochemistry [2, 3]. The progress in genetically encoded fluorescent protein, such as GFP and its spectral variants [4], has made fluorescence-induced RET (FRET) a very attractive tool also in cell biology. Although the intrinsic structural limitations of GFP-like probes make rigorous measurements of distances impracticable, genetically encoded reporters allow to investigate – at least in a qualitative fashion – the network of macromolecular interactions occurring in the living cell [5-7].

Bioluminescence-induced RET (BRET) is a recently introduced variation that exploits energy tranfer occurring between a luciferase-bound donor and a compatible fluorescent protein acceptor [8-11]. BRET has two advantages over conventional FRET. First, it does not require incident light, which can be damaging to living cells under several conditions. Second, it may lead to assays with better signal-to-noise ratio, because endogenous luminescence is far lower than auto fluorescence in mammalian cells. In practice, however, the benefits of the lower background are offset by the small efficiency of the RET process, which is usually observed in such assays, even when donors and acceptors are fused in tandem within a single polypeptide chain [12].

Two types of BRET assays have been described so far. Both utilize the reaction of *Renilla Reniformis* luciferase (Rluc) with coelenterazine, as light-emitter, and mutants of *Aequorea victoria* GFP, as acceptors of energy tranfer. One type uses the yellow-shifted mutant YFP (λ max = 530 nm) [13], which provides a better spectral separation of the RET emission peak from that of native coelenterazine (λ max = 475 nm). In the second type, the acceptor is a GFP mutant with a blue-shifted absorption spectrum [14]. If used in conjunction with coelenterazine 400A (a blue-shifted and weakly luminescent didehydroxylated analogue), this assay can achieve a similar improvement of spectral resolution, but at the expenses of dramatic reductions in quantum yield. In both cases, however, only a small fraction of photons are emitted via RET, even under conditions where covalent bonding ensure irreversible proximity between Rluc and the fluorescent acceptor [13, 14].

Unlike in artificially created probes, BRET can reach close to 100% efficiency [15] in some natural macromolecular systems [16-18]. For example, in the marine anthozoan *Renilla*, green luminescence is generated by a high efficiency RET process, which effectively converts most of the blue photoemission of excited coelenterazine [19] into green light [20]. This efficiency is the result of a specific protein-protein interaction between Rluc and a *Renilla* GFP (RGFP) [21]. Presumably the binding of the two proteins can optimize the spatial relationship between the chromophores, thus maximizing the transfer of energy via resonance. An additional important feature of this system, although still requiring clarification, is the apparent enhancement of quantum yield of the RET signal. Depending on the coelenterazine analogue used, the photon emission per mole of substrate is enhanced 5-100 folds in the presence of the GFP acceptor [22].

On one hand, such features suggest that Rluc and RGFP would constitute an ideal reporter system for the investigation of protein-protein interactions. On the other, however, the tendency to spontaneous association

might pose an impossible limitation, as it could make interacting proteins that normally do not. The importance of such limitation is crucially dependent on the extent to which the "background" affinity of the reporter system differs from that of the "reported" proteins under study. Earlier measurements [21, 23] described a significant affinity between Rluc and RGFP, with an apparent K_d located in the micro molar range. However, such determinations required conditions of virtually no ionic strength in the reaction, since the RET signal was readily disrupted at relatively low salt concentrations [21, 23]. This suggests that the true affinity between Rluc and RGFP under physiological ionic strength might be far lower than the value estimated in those determinations.

In this study we have investigated if the "natural" BRET between the two *Renilla* proteins can be used as a reporter system. To evaluate the interaction between Rluc and RGFP under conditions that are as close as possible to the reaction occurring between genetically encoded tags, we engineered a series of specifically designed chimeric proteins, and recorded the spectral properties of the luminescence both in cell extracts and intact cells.

Here we show that no detectable spontaneous interaction occurs between Rluc and RGFP when they are co-expressed as individual proteins in cells. In contrast, both the highly efficient RET process and the characteristic enhancement in photon yield is readily observed when the two proteins are covalently linked into a single polypeptide chain, or are driven into proximity by a complementary pair of Leu-zipper peptides tethered to their sequences. We also show that the *Renilla* proteins can be used as an excellent reporter system for the study of the interaction between G protein-coupled receptors and arrestin in living cells.

EXPERIMENTAL

Materials - Cell cultures media, G418, hygromicin and fetal calf serum were from Invitrogen. All ligands and DEAE-dextran sulfate (500 kDa average mass) were from Sigma. C-Myc monoclonal antibody was from Santa Cruz Biotechnology. Coelenterazine was from Prolume, LDT. Coelenterazine 400A (also known as Deep Blue®) was purchased from Biotium, Inc.

cDNA constructs - The RGFP cDNA was obtained from the plasmid pRrGFP (Prolume, LDT). All constructs were synthesized by PCR using standard procedures. To generate the fusion between the *Renilla* proteins the N-terminal methionine or the stop codon of RGFP were replaced, respectively, with a 21-mer (GDLGELSRILEQKLISEEDLL) or a 19-mer (EEQKLISEEDLGIPPARAT) linker peptide containing a c-Myc epitope (underlined). The obtained cDNAs were sub cloned into pRluc-C1 or pRluc-N1 (PerkinElmer, Life science) to have Rluc-RGFP and RGFP-Rluc fusion proteins, respectively.

The fusion between EYFP (Clontech) and Rluc was obtained by linking the two cDNAs through a sequence encoding a 10-mer linker (GDLGELSRIL). Tagged versiosn of native *Renilla* proteins were obtained by extending the N-terminal of Rluc and C-terminal of RGFP with a c-Myc epitope.

The CZ and NZ leucine zipper peptides tethered to c-Myc epitopes (bold and underlined, respectively,in the following amino acid sequences) were generated by annealing two synthetic oligonucleotides partially complementary, carrying appropriate restriction sites at their ends. Subsequently mutually primer synthesis was carried out.

The obtained fragments, encoding the CZ helix (*M*EQLISEEDL**EQLEKKLQALEKKLAQLEWKNQALEKKLAQ**GGSGIPPARAT) and the NZ helix (EEQKLISEEDGIQGGSGSG**ALKELQANKKLAQLKWELQALKKELAQ)**, were tethered to the ends of *Renilla* proteins, which had been previously sub cloned into pcDNA3 vector (Invitrogen) without Met initiator or stop codon.

The sequence encoding the thrombin cleavage site and the hirudin domain (Leu^{38}-Asp^{58}) of the human Proteinase-activated receptor 1 (PAR1) was PCR-amplified with only one substitution of glycine for aspartate 39. This sequence was extended at the N or C-terminus with a c-Myc epitope and the amplified fragments were inserted into the chimeric constructs RGFP-Rluc or Rluc-RGFP, to generate the respective cleavable fusion proteins.

To construct the GPCR Rluc-tagged fusion proteins we replaced the stop codon with a sequence coding for a 9-mer linker peptide (PGSPPARAT) in the human β_2AR and rat δ-opioid receptor, and a 15-mer peptide (KLAVPRARDPPARAT) in the rat vasopressin 2 receptor. The tagged receptors were then sub cloned into the retroviral expression vector pQIXN (Clontech).

Rat β-arrestin 1 and 2, with their Met initiator codon removed, were linked to Asp^2 of RGFP through a peptide (EQKLISEEDLRT) and sub cloned into pQIXH (Clontech).

Cell culture, transfection and extracts preparation: COS-7, Hek-293, and GP2-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate, in a humified atmosphere of 5% CO_2 at 37°C.

Transient transfection of COS-7 cells was performed by the DEAE dextran/chloroquine procedure [24]

HEK-293 cell lines permanently co-expressing chimeric GPCR-Rluc and RGFP-βarrestins, were generated using the pantropic retroviral expression system by Clontech. Briefly, recombinant retroviruses expressing receptor-Rluc or RGFP-βarrestins fusion proteins, were prepared by transfection of GP2-293 packaging cell with different retroviral vectors using FuGene, according to the manufacturer's instruction. Cells were allowed to increase the viral titer for 48-72 hours before collecting the virus-containing supernatants. HEK-293 cells were infected with the RGFP-βarrestins 1 or 2 retroviruses in presence of 8 μg/ml polybrene for 48 hours, and selected under hygromicin (100 μg/ml active drug). These polyclonal cells expressing RGFP-βarrestin1 or 2 were later super-infected with the different receptor-Rluc retroviruses, as described above, and selected under G418 (600 μg/ml active drug) and hygromicin. Receptor and arrestin expressions were confirmed by appropriate radioligand biding assay and western blot analysis, respectively, as described previously [25].

To prepare cytosol extracts for luminescence and fluorescence determinations, detached COS-7 cells, transiently transfected with vectors expressing the different fusion proteins, were homogenized in 50 mM Tris-HCl pH 7.4 with a Dounce homogenizer and centrifuged 10 min at 500 x g, to pellet the nuclei. The resulting supernatants were centrifuged at 55,000 x g for 1 hour at 4°C, to separate the cytosol from the particulate fraction, and stored frozen at – 80°C. For measurements in living cell suspensions, cell monolayers were detached in Ca^{2+}/Mg^{2+}-free PBS containing 1mM EDTA, centrifuged at 500 x g, and resuspended in PBS supplemented with 0.1% D-glucose prior to the addition to the microcuvette. Protein determinations were done according to Peterson [26]

Measurements of luminescence and fluorescence: Bioluminescence and fluorescence measurements were made in a Fluorolog-3 spectrofluorometer (Horiba, Jobin Yvon Inc.), equipped with two identical emission monochromators and photon-counting detectors arranged in a T-format configuration. Emission spectra were corrected with company provided correction files. Luminescence was recorded with the excitation lamp turned off. The Rluc substrates, coelenterazine (CLZ) and coelenterazine 400A (CLZ400A) were prepared as methanolic solutions (1 mM) and stored at – 80°C in light-shielded vials. Aliquots were dissolved in sample buffer before use, and added to cell extracts at the final concentration of 5 – 10 μM.

Bioluminescence emission spectra were typically collected starting 2 sec after the addition of substrate, using a combination of scanning steps and integration times such that a full spectrum could be completed in 10-25 sec. When necessary, time-corrected spectra (i.e. corrected for the decay of Rluc emission during the acquisition time) were obtained by simultaneously recording light from both monochromators, one of which scanned the desired range of wavelengths, while the other was maintained at a fixed wavelength (either 475 or 510 nm) to record the rate of decay of light intensity, which was then used to apply the corrections to the spectrum.

To measure the ratios of light intensity at the emission maxima of donor and acceptor (from which BRET ratios were computed) the light emitted from the sample was read simultaneously from both monochromators, one set at 475 nm and the other at 510 nm, using a slits of 5 nm. Readings were started 5-8

sec prior to substrate addition, to record dark current values, and were continued thereafter for 60-120 sec using time increments and integration of 0.2 sec. Small differences in light response between the two photomultipliers were corrected using factors computed by the simultaneous recording of the water Raman emission bands generated at similar wavelength maxima. All data points corresponding to the following 30 sec starting 0.4 sec after the peak value of light emission were used and averaged for calculation of BRET ratios.

Thrombin hydrolysis: Cytosol extracts (≈ 30 µg of total proteins) from COS-7 cells transiently transfected with plasmids encoding the cleavable fusion proteins were incubated in 50 µl of a buffer containing 1mM Tris-HCl, pH 7.4, 100 mM NaCl, in the presence and absence of thrombin (10 U/ml) for 30 min at 25°C. The reactions were arrested by adding hirudin (15 U/ml) to each sample. For luminescence measurements, the samples were diluted in 50 µl of 1mM Tris-HCl, pH 7.4. For western blot analysis, sample aliquots (15 µg of proteins) treated or not treated with thrombin were diluted in Laemmli sample buffer and separated by SDS-polyacrylamide gel electrophoresis (10%). Immunoblotting was performed using monoclonal anti-cMyc antibody (0.2 µg/ml), and the reactive bands were visualized by phosphatase staining with Promega reagents.

Fluorescence index of constructs: Since all our constructs were studied in cytosol extracts, we devised to measure an index related to the intrinsic fluorescence of the GFP domain, in order to compare different chimeric proteins. For each cytosol prepared from cells transfected with a given construct, serial dilutions were used to build two titration curves relating, respectively, total fluorescence and cMyc-immunoreactivity, to the total protein concentration of the extract. Total fluorescence was measured in the spectrofluorometer adding each diluted aliquot into 1mM Tris/HCl pH 7.4, with excitation and emission monochromators set at 475 nm and 510 nm, respectively. For the assessment of immunoreactivity, the same aliquots were diluted into Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis (10%), and electrotransfered onto polyvinylidene difluoride membranes (Millipore Immobilon). Immuno blotted bands were revealed using mouse anti-cMyc antibody followed by fluorescein conjugate anti-mouse (Amersham Biosciences) as secondary antibody, and were quantified by fluorescence scanning in a Typhoon 9200 (GE, Healthcare).

From the slopes of such curves (obtained by linear regression) we calculated the fluorescence intensity (FI, in cps) and the immunoreactivity (IR, in relative fluorescence units, RFU) per µg unit of extract. Their ratio FI/IR (cps/RFU) is a number related to the intrinsic fluorescence of the expressed protein. It can be used to compare chimeric proteins expressed in different cell extracts, under the assumption that the antibody affinity for the c-Myc epitope does not diverge significantly across the different constructs.

BRET assay of chimeric receptors and β–arrestins: : Luminescence emission spectra of cells expressing pairs of luminescent GPCR and fluorescent β–arrestins were measured as described above for intact cells determinations. Cell suspensions (1-3 x 10^5 cells) were incubated with or without the different ligands for 5 minutes in a 200 µl cuvette before adding coelenterazine (5 µM). For BRET determinations in attached monolayers, cells were plated into either 96-well or 24-well sterile white plastic plates (Packard View-plate)

at a density of 1 x 10^5 and 5 x 10^5 cells per well, respectively. To measure BRET ratios, the medium was replaced with PBS containing CLZ (5μM) and after 10 min the plates were inserted in a plate luminometer (Viktor light, Perkin Elmer) equipped with automatic injectors and two bandpass emission filters (blue: 470(20) nm and green: 510(20) nm, 3^{RD} Millenium, Omega Optical, Brattleboro, VT, USA). Following injection of agonist ± antagonist, or PBS, the wells were incubated for further 5 min and sequentially read through the green and blue filters (5 sec integration). For recording the enhancement of light emission with the low quantum yield substrate CLZ400A, the medium was replaced with PBS containing CLZ400A (10 μM) with or without the receptor agonist, and the total luminescence of the wells was immediately read. For kinetics studies, (as shown in fig.9), cells expressing β_2AR/βarrestin2 in 24-well plates were incubated for 5 minutes with CLZ400A. Next, the plates were inserted in the luminometer and the emission of total luminescence was recorded at 1 sec intervals following a first injection (at 90 sec) of either PBS or isoproterenol (100 nM final conc.) and a second injection (at 210 sec) of PBS or alprenolol (10 μM).

Data analysis and calculations: To accurately fit the non symmetrical luminescence spectra of CLZ, CLZ400A and RGFP we used a skewed Gaussian distribution function [27, 28]:

$$Y = Y_0 \exp\left[-\ln 2\left(\frac{\ln(z)}{b}\right)^2\right], \text{ with : } z = 1+\left[\frac{2b(\lambda-\lambda_0)}{\Delta\lambda}\right] \text{ and } Y=0, \text{ for } z \le 0.$$

Here, Y_0 is the maximal emission of the peak and λ_0 is the wavelength at the peak maximum, while b and $\Delta\lambda$ are, respectively, an asymmetry factor and a parameter of peak spread, from which the peak width at half-maximal intensity can be computed as: $W_{0.5} = \Delta\lambda\ (\sinh(b)/b)$.

A single Gaussian distribution was sufficient to describe the spectrum of Rluc alone, whereas a linear combination of three distributions was needed to accurately describe the spectra generated by the Rluc-RGFP interaction. Total light emission was computed either by numerical integration of the experimental data using the trapezoidal rule, or by summing the areas under the best fitting distribution functions calculated [28] as:

$$Area = Y_0 \frac{\Delta\lambda}{2}\left(\frac{\pi}{\ln 2}\right)^{1/2} \exp\left(\frac{b^2}{4\ln 2}\right)$$

BRET ratios were computed from the light intensities in counts per second (cps) recorded at the two wavelengths maxima (λ_{510} and λ_{475}) corrected for spectral overlap. That is:

BRET ratio = $\dfrac{cps_{510}(1-m)}{cps_{475}(1-n)}$, where m is the ratio $\lambda_{510}/\lambda_{475}$ in the emission spectrum of Rluc/coelenterazine, and n is the ratio $\lambda_{475}/\lambda_{510}$ in the fluorescence emission spectrum of RGFP.

RESULTS

Absence of BRET signals in wild-type *Renilla* proteins Efficient RET between Rluc and RGFP was previously described *in vitro* using purified proteins [15]. To investigate whether energy transfer can occur in the extracts of cells expressing the genetically encoded proteins, we expressed either individually or jointly wild-type RGFP and Rluc in COS-7 cells.

Bioluminescence spectra triggered by the addition of 5 µM coelenterazine were recorded in extracts containing each individual protein, either co-expressed in the same cell, or prepared as mixtures obtained by reconstituting individual extracts in various proportions. Although both RGFP and Rluc were well expressed, as indicated by the luminescence and fluorescence signals of the cell extracts, no detectable BRET emission could be detected. The bioluminescence spectrum of the RGFP/Rluc mix was always superimposible to that recorded for Rluc in the absence of RGFP (Figure 1A). Identical results were obtained either under very low (1 mM Tris-HCl) or normal (50 mM Tris-HCl, 100 mM NaCl) ionic strength reaction conditions.

Thus, RGFP and Rluc are not capable to form a BRET emitting association complex under such conditions.

High and low efficiency BRET in fusion proteins Next, we generated two dimeric-fusion proteins in which the luciferase donor was alternatively linked to the N- or the C-terminal end of the fluorescent acceptor, through spacer residues (see Experimental Procedures, under cDNA constructs). Bioluminescence spectra were recorded from Cos-7 cells extracts expressing the fusion proteins.

In both cases emission spectra were bimodal, exhibiting, in addition to the 475 nm peak of Rluc, a second green-shifted component (λ_{max} = 510), corresponding to the peak emission of RGFP (Figure 1B, C). This indicates that energy transfer from the coelenterazine excited state to the fluorescent chromophore readily occurs in such chimeras. However there was an impressive difference in transfer efficiency between the constructs. In the protein with RGFP in amino terminal position, the BRET peak was predominant, as light was almost entirely emitted through the green pathway (Figure 1C). In contrast, when RGFP was downstream to Rluc, the broad emission spectrum of coelenterazine was the main component of the emitted light and the BRET signal appeared as a small shoulder on the low energy side of the spectrum (Figure 1B).

Thus, high efficiency BRET as previously observed in vivo [20] and in reconstitution experiments [21, 23], only takes place in the chimera where RGFP is in amino terminal position.

Essential role of the N-terminus of RGFP The difference in BRET efficiency between the two proteins suggests that the modifications due to the fusion (which in the construct with reduced BRET involve the GFP N-terminal and the Rluc C-terminal) might either impair the intrinsic fluorescence of GFP or interfere with the protein-protein interaction generating efficient energy transfer. Alternatively, the C-terminus modification in Rluc might adversely affect catalytic efficiency. To sort out such possibilities, we modified the chimera with the best RET response (i.e. RGFP-Rluc) by extending either the N-terminus of the fluorescent protein domain, or the C-terminus of the luciferase, with 10 histidine residues.

The fluorescence index (in cps/RFU, see Experimental procedures) of the N-terminus extended construct (4.4 ± 0.6) was 60% compared to the C-terminus modified chimera (7.4 ± 1.1), and 40% compared to c-Myc

tagged wild-type RGFP (10.7 ± 1.9). This suggests that the two modifications have only minor effects on the intrinsic fluorescence of the fluorophor. We also measured excitation fluorescence spectra of modified constructs and wild type GFP. They were superimposible, suggesting that there are no changes in the absorption properties of the chromophore.

In contrast, the luminescence spectra shown by the construct with the N-terminus extended GFP sequence displayed a marked reduction of the RET peak, similar to that observed for Rluc-RGFP, whereas the C-terminal extension of Rluc had no significant effect (Figure 2). Thus, the data clearly indicate that a free N-terminus in RGFP is an essential requirement for the occurrence of the highly efficient energy transfer process.

Proteins tethered to self-assembling leucine zippers We next evaluated if the two proteins could generate nonradiative energy transfer when brought into molecular proximity by the interaction of two tethered complementary partners. We used a pair of antiparallel "leucine zipper" sequences, which are a useful self-assembling tool for the detection of very weak protein-protein interactions [29, 30].

Four different fusion constructs were designed, such that the antiparallel peptide helices CZ and NZ were alternatively encoded on the carboxyl and amino terminal of the two proteins. This resulted in two complementary pairs of interacting proteins capable to associate with opposite orientations, only one of which involves GFP with a free α-amino group (see scheme in Figure 3).

As observed for the fusion constructs described above, the bioluminescence spectra studied in reconstituted cell extracts show that only when the GFP N-terminus is free, high efficiency BRET was observed (Figure 3A, B). This again underscores the fundamental role that the amino terminus of the fluorescent proteins plays for the occurrence of high efficiency BRET.

To verify whether the interaction driven by the Leu zipper tags is also detectable in living cells, different ratios of cDNA mixes encoding GFP-NZ and CZ-Rluc fusion proteins were transfected in COS-7 cells and the luminescent spectra were compared with those of cells transfected using equivalent proportions of plasmids encoding the corresponding native proteins. BRET signals were clearly present in cells co-transfected with the Leu zippers-linked proteins, and the relative height of the 510 nm peak steadily rose as the fraction of acceptor coding cDNA increased (Figure 4A). In contrast, no BRET was observed in cells expressing the native proteins, despite the level of wild-type expression was roughly 10-fold greater than that of zipper chimeras (Figure 4B) – as judged from the intensity of luminescence and fluorescence (not shown). There is a clear correlation between the relative size of the RET peak (gauged as wavelength intensity ratios at 510 / 475 nm) and the transfection ratio of the cDNAs coding acceptor and donor. This is consistent with the notion that RET results from the Leu-zipper driven self-association of Rluc and RGFP inside the cells (Figure 4C).

Cleavable constructs and reversibility of the high-efficiency BRET interaction. The data presented above clearly demonstrate that Rluc and RGFP cannot form a spontaneous complex in solution, but they do so when forced into close proximity via linked binding partners that have sufficient affinity. However, one important question is whether the complex, once formed, can dissociate when the interaction between the linked partners is interrupted.

To answer the question we made two cleavable fusion proteins RGFP-Thr-Rluc and Rluc-Thr-RGFP. In both, we introduced a thrombin cleavage site within the linker region of the fused proteins, and to ensure efficient thrombin cleavage we also inserted a flanking sequence corresponding to the "hirudin-like" domain (Phe^{41}-Asp^{58}) of the protease-activated receptor-1 [31] .

Western blot analysis of cytosolic extracts containing the expressed proteins (shown for RGFP-Thr-Rluc in Figure 5A), prior and following thrombin treatment, indicated that the enzyme can accomplish the complete cleavage of the chimera under mild reaction conditions. The luminescence spectra of the cleavable proteins were identical to those of the corresponding non cleavable constructs. In both cases, thrombin treatment converted the spectrum into the single broadband peak ($\lambda_{max,}$ 475 nm) typical of Rluc emission in the absence of RGFP. Furthermore, the hydrolysis was specific, as no effect of thrombin was observed in the constructs lacking the cleavage site, nor cleavage of the constructs was detected in the presence of equimolar amounts of hirudin (data not shown).

One advantage of the cleavable constructs is the possibility to compare light output prior and after thrombin treatment, without altering reactants concentrations. That allows to quantify the difference in RET efficiency between associated and dissociated proteins. Using this strategy we measured the BRET ratios (e.g. the ratio of light emissions at peak wavelengths, 510/475, corrected for spectral cross-talk) in the RGFP-Thr-Rluc (17.1 ± 0.1) and Rluc-Thr-RGFP (1.72 ± 0.03) constructs prior and following thrombin hydrolysis (Figure 5B). RET efficiency is thus 10-fold greater when the interaction between the proteins entails a free amino terminal in RGFP. In both chimeras RET was abolished by thrombin cleavage (Figure 5B). Therefore, the data reassert the lack spontaneous RET-producing interaction between free Rluc and RGFP in solution, but also indicate that even when the interaction is assisted by a covalent link, can be readily reversed upon link removal.

Quantum yield enhancement of luminescence An additional important feature emerged when we studied the luminescence spectra of the substrate coelenterazine 400A (CLZ400A) in the cleavable construct. The emission spectrum of this analog (λ_{max} = 396 nm) has marginal overlap with the absorption spectrum of RGFP. That makes it a poor donor of energy transfer in this system. Yet in the GFP-Thr-Luc construct the spectrum of CLZ400A displayed a prominent narrow bandwidth peak at 510 nm, in addition to the typical 395 nm peak of the CLZ400A excited state, indicating energy transfer to the RGFP chromophore. Thrombin hydrolysis virtually abolished the green component, but also dramatically reduced light output through the blue pathway (Figure 6).

Spectra integration in control and thrombin treated samples indicated that the enhancement in overall light output from free luciferase to RGFP-bound enzyme was roughly 44-fold. This gain of apparent quantum

yield of the CLZ400A luminescence emitted from the GFP-Luc complex is of the same order of magnitude of that previously reported using purified proteins [22]. Unlike those studies, however, we find that both the blue (395 nm) and the green (510 nm) emissions are enhanced as a result of the interaction of Rluc with RGFP.

The *Renilla* system as reporter of GPCR–β–arrestin interactions. We speculated that this gain in luminescent emission can constitute a novel and alternative readout for BRET signal detection in protein-protein interaction studies. To verify this idea we tested the performance of the *Renilla* proteins to act as reporters of the interaction between G protein coupled receptors (GPCR) and arrestins [32].

A number of vectors encoding complementary sets of chimeric proteins were prepared. To ensure the presence a free α-amino group in its sequence, the acceptor RGFP was fused to the amino terminal ends of both β-arrestin 1 and arrestin 2. The Rluc donor was instead fused on the C-terminus of three GPCRs: the β_2-adrenergic receptor (β_2AR), the δ-opioid receptor (DOP) and the vasopressin 2 receptor (V_2R). Using retroviral vectors, six HEK293 cell lines were prepared, each co-expressing one luminescent donor receptor with one fluorescent acceptor βarrestin.

As shown in Figure 7, the luminescence spectra of CLZ400A recorded in intact cells co-expressing either β_2AR-Rluc (Figure 7A) or DOP-Rluc (Figure 7B) with RGFP-βarrestin2 were sharply changed by the addition of the corresponding receptor agonists (isoproterenol and [D-Ala^2, D-Leu^5]enkephalin, respectively). In both cases agonist-induced receptor association to β-arrestin was revealed by a major increase of the 510 nm peak and an overall enhancement of total light emission over basal conditions. These changes were largely prevented in the presence of receptor antagonists (Figure 7A, B). We found no detectable agonist effects on luminescence in cells co-expressing DOP-Rluc or β_2AR-Rluc with RGFP-βarrestin 1, which is in agreement with data indicating that β_2AR and DOP do not interact tightly with this protein. Conversely, Arg-vasopressin (AVP) induced strong luminescence enhancements when either RGFP-βarrestin 2 or RGFP- βarrestin 1 were present in cells expressing V_2R-Rluc, a receptor known to interact with both βarrestin types [32] (data not shown). A small, but detectable 510 nm peak was present in the absence of agonists (see Figure 7A, B). This basal emission was absent in cells where β_2AR and DOP are co-expressed with RGFP- βarrestin 1 (not shown). Thus, it is likely that the signal reflects some extent of constitutive interaction between unbound receptor and βarrestin, rather than background noise due to spontaneous binding of the reporters.

The light enhancement occurring when BRET is triggered via CLZ400A is large enough to allow for the detection of the interaction as simple increase of total luminescence, without need of spectral resolution of the emitted light. This was tested in Figure 8, where cells co-expressing V_2 receptors and βarrestins 1 or 2 were plated in multiwell flasks, and the AVP-induced interaction of the V2 receptor with the two arrestins was compared using two alternative methods of detection. In one, we made conventional ratiometric determinations of the change in BRET ratios, using cells incubated with CLZ. AVP produced a ~ 3 fold-enhancement of BRET ratio in both V_2R-βArr1 and V_2R-βArr2 cells, and both prevented in the presence of

the antagonist [d(CH_2)$_3$[1],D-Ile[2],Ile[4],Arg[8]]-vasopressin (fig. 8A). In the second method, the enhacement of the total luminescence was recorded at consecutive time intervals following the addition of CLZ400A plus or minus the agonist AVP (Fig. 8B). The ratios of light intensities in the presence and absence of agonist increased with time up to ten fold, indicating that RET induced by the GPCR-βarrestin association can also be revealed as an increase of total luminescence.

We further tested if this enhancement of quantum yield resulting from the binding of the two Renilla tags can also detect both the association and the dissociation of the arrestin-receptor complex in the cell. As shown in fig.9, the addition of the agonist isoproterenol to cells coexpressing β_2AR-Rluc and RGFP-βarrestin2 in the presence of CLZ400A, induced a rapid increase of total luminescence,which was reversed upon the further injection of the antagonist alprenolol. This data confirm and extend the results obtained with cleavable constructs, as they demonstrate that the reversibility of the RGFP-Rluc complex is maintained also when the Renilla reporters are linked to proteins that interact in living cells.

DISCUSSION

The luciferase and the fluorescent protein of *Renilla* coelenterates represent the first natural bioluminescent system where radiationless energy transfer was investigated [16, 20]. Although quantitative studies demonstrated that the process - modeled according to a Föster-type mechanism - can reach a surprising 100% efficiency in these proteins [15], no work was ever done to assess if such molecules can make a useful gene reporter system for the detection of protein-protein interactions in cells.

In this paper we have reinvestigated the interaction between Rluc and RGFP with this question in mind. Unlike previous studies, which were based on the analysis of the purified proteins in diluted, low-salt solutions, here we adopted a protein engineering approach to probe their interaction. Using Rluc and RGFP sequences embedded into chimeric vectors to force their intramolecular interaction, or proteins tethered to self-assembling α-helices to drive their intermolecular association, we tested which configuration was required for optimal energy transfer from the Rluc bioluminescent reaction center to the GFP chromophore.

Taking advantage of thrombin cleavable constructs, we also measured differences in RET efficiency, and quantified the net gain of light output that occurs when the oxidation of the low-quantum-yield substrate CLZ400A is compared in the presence and absence of the acceptor RGFP.

We finally used the well-known interaction between membrane-bound GPCRs and βarrestins [32-34] as a benchmark tool to test in living cells the performance of the *Renilla* proteins as reporter system. Three clear results emerge from this study.

First, there is no detectable background interaction between Rluc and RGFP when these molecules are expressed as free native proteins in cytosolic extracts or living cells. The inability to undergo spontaneous association is further confirmed by additional evidence. One is the total disruption of RET observed upon disconnection of the tethered proteins from the cleavable constructs. Another is the absence of ground reporter signals in the βarrestin -receptor assay. In fact, the small levels of RET observed in the absence of

agonist in cells expressing adrenergic or opioid receptors reflect a basal interaction between those GPCRs and βarrestin 2, as it was not displayed when the same receptors were co-expressed with arrestin 1, a protein to which they bind far less strongly [32].

Thus, we note that Rluc and RGFP do not generate more background noise than other pairs of reporters previously used for BRET assays, at least under the experimental conditions that are normally employed in such type of assays. This finding, although positive with regard to the use as reporter system, is puzzling when confronted with the dissociation constant of ≈ 1μM estimated for the binding between Rluc and RGFP [21, 23]. As we did not attempt to measure the intracellular concentrations of the expressed proteins, we must assume them to be equal or below nanomolar, to account for the lack of any detectable spontaneous association. Alternatively, we may suspect that the binding affinity was overestimated in previous experiment.

This lack of spontaneous interaction may seem a paradox, if one considers that in living *Renilla* cells the two proteins readily interact, and the resulting RET is so efficient as to cause complete conversion of the emitted light spectrum. However, it was demonstrated in anthozoan cells that Luc and GFP are sequestered into sub micron-sized organelles called lumisomes [35]. It is thus likely that high local concentration due compartmentalization, and perhaps membrane docking mechanisms within the lumisome, are obligatory factors that promote the interaction between Luc and GFP, despite the low intrinsic affinity between the proteins.

Molecular weight estimates obtained by gel chromatography also suggested that RGFP may exist in dimeric form [21, 23]. It was proposed that the minimal required complex for high efficiency BRET emission might be the heterotrimer between one Rluc and two RGFP molecules [23]. Our data obtained in RGFP-Rluc fusion proteins would suggest that a 1:1 complex is sufficient for an efficient interaction, although we cannot exclude a spontaneous association of the chimeric constructs through their GFP domains. We found that BRET ratios of fusion proteins did not change even when they were diluted up to 20-fold (not shown), which makes it difficult to believe that high efficiency BRET depends on their self-association. Obviously, more detailed investigations are necessary to verify conclusively if dimeric GFP is an essential intermediate of the interaction

Regardless of such unsolved questions on reaction strength and stoichiometry, our data clearly indicate that the tendency of *Renilla* proteins to form a complex is not an obstacle for the use of this system as BRET reporter of protein-protein interactions in transfected mammalian cells.

A second novel result in this study is that the high efficiency RET process generating the green luminescence of *Renilla* requires a free α-amino group in the molecule of RGFP. This finding explains previous observations showing that the reaction of purified RGFP with amino group modifying agents disrupted its RET acceptor activity [21]. An effect of the free N-terminus in enhancing BRET efficiency was also reported for the Aequorea victoria mutant GFP2, by comparing fusion constructs in which this protein was linked to either the amino or the carboxy terminus of Rluc [36].

The N-terminal modification of RGFP does not appear to produce important changes in the fluorescence

of the fluorophor, or to prevent the transfer of energy between the optical centers of the two proteins. It does, however, cause a dramatic diminution of RET efficiency. The decrease is 10-fold, if deduced from the difference in BRET ratios, and affects either the intramolecular RET in fusion proteins or the intermolecular RET induced via the docking of antiparallel α-helices.

Our data suggest that the α-NH_2 group in the N-terminus of GFP is crucial for the formation of the dimeric complex that allows RET to achieve maximum efficiency. This complex might optimize the physics of energy transfer to an extent that would never be possible by simply pulling into maximal proximity the molecular shells of the two proteins. This means that there are two different ways in which the *Renilla* proteins can be used as a reporter system.

One is when the RGFP amino terminal is blocked within the encoded fusion protein. The system in this case behaves and performs just like any other BRET reporter assay described so far [10, 12]. There might be the advantage of a slightly improved BRET efficiency, due to the better spectral overlap between the emission of Rluc and the absorption of RGFP. However that comes with the disadvantage of a poorer separation between the maxima of the BRET peak and the emission of coelenterazine.

The second is when GFP is encoded with a free amino terminal. The system, in this case, has the potential to deliver BRET emissions that display the same high efficiency as in the intact *Renilla*. Such unprecedented efficiency should allow engineering assays with far superior sensitivity and accuracy than those described so far.

There is, however, an important caveat. Unlike conventional BRET assays, where the signal depends on the extent to which the reporters are brought into the range of distances in which energy transfer can occur, in the *Renilla* system BRET results from the complex that is formed when the reporters reach a threshold distance. Once that complex is made, it is not clear if the intermolecular distance and orientation of the optical centers can still be influenced by the molecular motion of the carrier proteins. For this reason, we consider this type of BRET assay much more alike those based on functional complementation of split enzymes [37, 38] or split fluorescent protein fragments [30, 39, 40]. Further work is necessary to investigate if this "complementation-induced" BRET system can be useful for engineering intramolecular BRET biosensors, (i.e. proteins carrying BRET labels tethered at distal ends of the molecule, so that the binding of signaling intermediates, like Ca^{2+} or cAMP, can induce conformational changes that are reported as changes of RET efficiency [41-45]). One clear application, however, is the utilization of this *Renilla* system for engineering single-chain optical sensors of proteolysis [46]. As shown here, thrombin hydrolysis of the cleavable construct engineered with an amino terminal GFP caused a drop of BRET ratio from 17.1 (± 1.5) to 0.11 (± 0.07). This means a > 150-fold dynamic range in the response to thrombin hydrolysis. Conceivably, by inserting between the Rluc and the RGFP labels peptides containing specific cleavage sites for proteases of pharmacological interest, (such as caspases or viral hydrolases), highly sensitive and specific probes can be prepared that may be particularly suited use in high-throughput assay systems.

The third point emerging from this study is that the quantum yield enhancement that occurs in the emission of the didehydroxy analog CLZ400A can be effectively exploited as readout of the interaction

between Rluc and RGFP. As deduced from the difference between intact and cleaved fusion proteins, we measured more than 40-fold enhancement of light output when the luminescence emitted from CLZ400A/Rluc takes place in the presence of RGFP. This property allows measuring "complementation-induced" BRET in a simple luminometric assay, which does not require multiple filter readings. When adapted to the study of the interactions GPCR/arrestin in living cells, the system reports agonist-induced binding of receptor to arrestin as several-fold enhancements of total light emission, a remarkable improvement of signal to noise ration for the detection of a protein-protein interaction in an intact cellular system. Moreover, both the association and dissociation of the receptor-arrestin complex can be detected.

The mechanism underlying the phenomenon of quantum yield enhancement is still unclear. To explain the emission of low-quantum yield analogues, such as CLZ400A, two singlet excited states were proposed. Both a neutral (λ = 395 nm, bad RET donor) and a monoanionic (λ = 475 nm, good RET donor) species would be produced during oxidation of CLZ400A. Since the mode of binding of this substrate to the enzyme pocket would cause fast quenching of the monoanionic state through solvent and/or protein interactions, there is no detectable emission at 475 nm. In the presence of the acceptor GFP, the energy transfer process would compete with the quenching, and unveil the monoanion as 510 nm RET emission [22].

This theory was based on the observation that the addition of GFP to CLZ400A/Rluc induced the emergence of the 510 nm peak but did not change the 395 nm emission [22]. In contrast, we found here that the interaction with GFP not only generates the green emission band, but also results in a dramatic enhancement of the bioluminescent emission at 395 nm, suggesting that GFP binding may change the overall catalytic properties of the enzymatic reaction between CLZ400A and Rluc, rather than merely "rescue" one nonradiating intermediate of the reaction. A recent and interesting study shows that a single site mutation in the molecule of Rluc can produce enhancements of quantum yield that are identical to those induced by GFP binding to Rluc, and suggests that the mutated residue might be involved in the interaction of Rluc with the fluorescent protein. [47]. If the effect of GFP can be reproduced by a mutation in the Rluc sequence, it is likely that the mechanism of luminescence enhancement involves a conformational change that the binding of the fluorescent protein induces in the molecule of Rluc, and cannot be solely explained by the dynamics of luminescence excited states.

In summary our data demonstrate that the *Renilla* system provides several opportunities for engineering optical reporter assays that are useful to measure macromolecular interactions in living cells. Besides its use as a conventional BRET assay, when both GFP and Luc are fused in frame with the C-terminal ends of the proteins under study, our results highlight a novel strategy for exploiting its properties. That relies on the natural capability of the *Renilla* proteins to form a high efficiency RET emitting complex when the proteins to which they are attached reach a permissive intermolecular distance.

This strategy, that we call "complementation-induced BRET", requires that the energy acceptor must be positioned on the N-terminus of the protein of interest. Moreover, depending on the substrate that is used, complementation-induced BRET can be detected in two different ways: as ratiometric determination of RET efficiency, when using native CLZ, or as enhancement of total luminescence, when exploiting the large

increase of quantum yield observed for CLZ400A.

An obvious limitation of complementation-induced BRET, as of any other assay based on functional complementation, is that it cannot provide a quantitative measure of the intermolecular distances between the interacting proteins. Quantification, at any rate, is a hard task even in conventional FRET and BRET assays, particularly when they are based on genetically encoded labels [3], and the quantitative interpretation of the data is still highly controversial matter [48, 49]. Even if only capable of qualitative information, this new strategy should allow building assays with unrivaled characteristics in terms of signal to noise ratio, accuracy and sensitivity.

REFERENCES

1 Förster, T. (1948) Zwischenmolekulare Energiewanderung Und Fluoreszenz. Ann. Physik. **2**, 55-75

2 Stryer, L. and Haugland, R. P. (1967) Energy transfer: a spectroscopic ruler. Proceedings of the National Academy of Sciences of the United States of America. **58**, 719-726

3 Selvin, P. R. (2000) The renaissance of fluorescence resonance energy transfer. Nature structural biology. **7**, 730-734

4 Tsien, R. Y. (1998) The green fluorescent protein. Annu Rev Biochem. **67**, 509-544

5 Nguyen, A. W. and Daugherty, P. S. (2005) Evolutionary optimization of fluorescent proteins for intracellular FRET. Nature biotechnology. **23**, 355-360

6 Verkhusha, V. V. and Lukyanov, K. A. (2004) The molecular properties and applications of Anthozoa fluorescent proteins and chromoproteins. Nature biotechnology. **22**, 289-296

7 Giepmans, B. N., Adams, S. R., Ellisman, M. H. and Tsien, R. Y. (2006) The fluorescent toolbox for assessing protein location and function. Science. **312**, 217-224

8 Bouvier, M. (2001) Oligomerization of G-protein-coupled transmitter receptors. Nat Rev Neurosci. **2**, 274-286

9 Milligan, G. (2004) Applications of bioluminescence- and fluorescence resonance energy transfer to drug discovery at G protein-coupled receptors. Eur J Pharm Sci. **21**, 397-405

10 Pfleger, K. D. and Eidne, K. A. (2005) Monitoring the formation of dynamic G-protein-coupled receptor-protein complexes in living cells. Biochem J. **385**, 625-637

11 Prinz, A., Diskar, M. and Herberg, F. W. (2006) Application of bioluminescence resonance energy transfer (BRET) for biomolecular interaction studies. Chembiochem. **7**, 1007-1012

12 Pfleger, K. D. and Eidne, K. A. (2006) Illuminating insights into protein-protein interactions using bioluminescence resonance energy transfer (BRET). Nat Methods. **3**, 165-174

13 Xu, Y., Piston, D. W. and Johnson, C. H. (1999) A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proceedings of the National Academy of Sciences of the United States of America. **96**, 151-156

14 Hamdan, F. F., Audet, M., Garneau, P., Pelletier, J. and Bouvier, M. (2005) High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer 1-based beta-arrestin2 recruitment assay. J Biomol Screen. **10**, 463-475

15 Ward, W. W. and Cormier, M. J. (1976) In Vitro Energy Transfer in Renilla Bioluminescence. J Phys Chem. **80**, 2289-2291

16 Morin, J. G. and Hastings, J. W. (1971) Energy transfer in a bioluminescent system. J. Cell Physio. **77**, 313-318

17 Cormier, M. J., Hori, K. and Anderson, J. M. (1974) Bioluminescence in coelenterates. Biochim Biophys Acta. **346**, 137-164

18 Morise, H., Shimomura, O., Johnson, F. H. and Winant, J. (1974) Intermolecular energy transfer in the bioluminescent system of Aequorea. Biochemistry. **13**, 2656-2662

19 Hori, K. and Cormier, M. J. (1973) Structure and Chemical Synthesis of a Biologically Active Form of Renilla (Sea Pansy) Luciferin. Proceedings of the National Academy of Sciences of the United States of America. **70**, 120-123

20 Wampler, J. E., Hori, K., Lee, J. W. and Cormier, M. J. (1971) Structured bioluminescence. Two emitters during both the in vitro and the in vivo bioluminescence of the sea pansy, Renilla. Biochemistry. **10**, 2903-2909

21 Ward, W. W. and Cormier, M. J. (1979) An energy transfer protein in coelenterate bioluminescence. Characterization of the Renilla green-fluorescent protein. J Biol Chem. **254**, 781-788

22 Hart, R. C., Matthews, J. C., Hori, K. and Cormier, M. J. (1979) Renilla reniformis bioluminescence: luciferase-catalyzed production of nonradiating excited states from luciferin analogues and elucidation of the excited state species involved in energy transfer to Renilla green fluorescent protein. Biochemistry. **18**, 2204-2210

23 Ward, W. W. and Cormier, M. J. (1978) Energy transfer via protein-protein interaction in Renilla bioluminescence. Photochemistry and Photobiology. **27**, 389-396

24 Cotecchia, S., Exum, S., Caron, M. G. and Lefkowitz, R. J. (1990) Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. Proc Natl Acad Sci U S A. **87**, 2896-2900

25 Molinari, P., Ambrosio, C., Riitano, D., Sbraccia, M., Gro, M. C. and Costa, T. (2003) Promiscuous coupling at receptor-Galpha fusion proteins. The receptor of one covalent complex interacts with the alpha-subunit of another. J Biol Chem. **278**, 15778-15788

26 Peterson, G. (1983) Determination of Total Protein. Methods in Enzymology. **91**, 95-119

27 Fraser, R. D. B. and Suzuki, E. (1969) Resolution of Overapping Bands: Functions for Simulating Band Shapes. Analytical chemistry. **41**, 171-173

28 Rusch, P. F. and Lelieur, J. P. (1973) Analytical Moments of Skewed Gaussian Distribution Functions. Analytical chemistry. **45**, 1541-1543

29 Ghosh, I., Hamilton, A. D. and Regan, L. (2000) Antiparallel Leucine Zipper-Directed Protein Reassembly: Application to the Green Fluorescent Protein. J. Am. Chem. Soc. **122**, 5658-5659

30 Magliery, T. J., Wilson, C. G., Pan, W., Mishler, D., Ghosh, I., Hamilton, A. D. and Regan, L. (2005) Detecting protein-protein interactions with a green fluorescent protein fragment reassembly trap: scope and mechanism. J Am Chem Soc. **127**, 146-157

31 Ishii, K., Gerszten, R., Zheng, Y. W., Welsh, J. B., Turck, C. W. and Coughlin, S. R. (1995) Determinants of thrombin receptor cleavage. Receptor domains involved, specificity, and role of the P3 aspartate. J Biol Chem. **270**, 16435-16440

32 Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G. and Barak, L. S. (2000) Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem. **275**, 17201-17210

33 Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M. and Bouvier, M. (2000) Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci U S A. **97**, 3684-3689

34 Vrecl, M., Jorgensen, R., Pogacnik, A. and Heding, A. (2004) Development of a BRET2 screening assay using beta-arrestin 2 mutants. J Biomol Screen. **9**, 322-333

35 Anderson, J. M. and Cormier, M. J. (1973) Lumisomes, the cellular site of bioluminescence in coelenterates. J Biol Chem. **248**, 2937-2943

36 Wang, Y., Wang, G., O'Kane, D.J. and Szalay, A.A. (2001) A study of protein-protein interaction in living cells using luminescence resonance energy transfer (LRET) from Renilla luciferase to aequorea GFP. Mol. Gen. Genet. **264**, 578-587

37 Galarneau, A., Primeau, M., Trudeau, L. E. and Michnick, S. W. (2002) Beta-lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein protein interactions. Nature biotechnology. **20**, 619-622

38 Paulmurugan, R. and Gambhir, S. S. (2005) Firefly luciferase enzyme fragment complementation for imaging in cells and living animals. Analytical chemistry. **77**, 1295-1302

39 Cabantous, S., Terwilliger, T. C. and Waldo, G. S. (2005) Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. Nature biotechnology. **23**, 102-107

40 Nyfeler, B., Michnick, S. W. and Hauri, H. P. (2005) Capturing protein interactions in the secretory pathway of living cells. Proc Natl Acad Sci U S A. **102**, 6350-6355

41 Allen, G. J., Kwak, J. M., Chu, S. P., Llopis, J., Tsien, R. Y., Harper, J. F. and Schroeder, J. I. (1999) Cameleon calcium indicator reports cytoplasmic calcium dynamics in Arabidopsis guard cells. Plant J. **19**, 735-747

42 Zaccolo, M., De Giorgi, F., Cho, C. Y., Feng, L., Knapp, T., Negulescu, P. A., Taylor, S. S., Tsien, R. Y. and Pozzan, T. (2000) A genetically encoded, fluorescent indicator for cyclic AMP in living cells. Nat Cell Biol. **2**, 25-29

43 Ponsioen, B., Zhao, J., Riedl, J., Zwartkruis, F., van der Krogt, G., Zaccolo, M., Moolenaar, W. H., Bos, J. L. and Jalink, K. (2004) Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. EMBO Rep. **5**, 1176-1180

44 Nikolaev, V. O., Bunemann, M., Hein, L., Hannawacker, A. and Lohse, M. J. (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. J Biol Chem. **279**, 37215-37218

45 Jiang, L. I., Collins, J., Davis, R., Lin, K. M., DeCamp, D., Roach, T., Hsueh, R., Rebres, R. A., Ross, E. M., Taussig, R., Fraser, I. and Sternweis, P. C. (2007) Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. J Biol Chem. **282**, 10576-10584

46 Waud, J. P., Bermudez Fajardo, A., Sudhaharan, T., Trimby, A. R., Jeffery, J., Jones, A. and Campbell, A. K. (2001) Measurement of proteases using chemiluminescence-resonance-energy-transfer chimaeras between green fluorescent protein and aequorin. Biochem J. **357**, 687-697

47 Loening, A. M., Fenn, T. D., Wu, A. M. and Gambhir, S. S. (2006) Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output. Protein Eng Des Sel. **19**, 391-400

48 James, J. R., Oliveira, M. I., Carmo, A. M., Iaboni, A. and Davis, S. J. (2006) A rigorous experimental framework for detecting protein oligomerization using bioluminescence resonance energy transfer. Nat Methods. **3**, 1001-1006

49 Bouvier, M., Heveker, N., Jockers, R., Marullo, S. and Milligan, G. (2007) BRET analysis of GPCR oligomerization: newer does not mean better. Nat Methods. **4**, 3-4; author reply 4

FOOTNOTES

We gratefully appreciate the gift of cDNAs for β-arrestins from Dr. Antonio De Blasi (University of Rome "La Sapienza," Rome, Italy) and for V_2-receptor from Dr. Fabio Naro (University of Rome "La Sapienza,"). This work was supported in part by the FIRB “internationalization” program grant RBIN04CKYN_001.

The abbreviations used are: RET, resonance energy transfer; FRET and BRET, RET induced by fluorescence and bioluminesence, respectively; RGFP, *Renilla* GFP; Rluc, *Renilla* luciferase; CLZ, coelenterazine; CLZ400A, didehydroxy-coelenterazine; GPCR, G protein-coupled receptor.

FIGURE LEGENDS

Figure 1 BRET between Rluc and RGFP can only be detected in fusion proteins

Bioluminescence emission spectra generated with native coelenterazine recorded in extracts of COS7 cell transfected with various proteins (**A**) Comparison of spectra obtained in extracts of cells transfected with Rluc cDNA (*solid line*), or with a 1:3 mix of Rluc cDNA and RGFP cDNA (▲), and spectrum recorded from a 1:6 cytosol mixture of Rluc and RGFP extracts (□).

Bimodal spectral distribution of the bioluminescent reaction triggered with coelenterazine in cell extracts expressing the dimeric fusion proteins, (**B**) Rluc-RGFP, and (**C**) RGFP-Rluc. The structure of the transfected proteins is schematically represented on the right-hand side of the corresponding plots. All the spectra were normalized to the light intensity recorded at the wavelength of maximal emission.

Figure 2 Effect of the N-terminus modification of RGFP on BRET efficiency.

Comparison of the luminescence spectra emitted with native coelenterazine in cell extracts expressing the RGFP-Rluc fusion protein, modified by adding 10 histidine residues at either the N-terminus (His10X-RGFP-Rluc, *dashed line*) or the C-terminus (RGFP-Rluc-His10X, *solid line*). To illustrate the change in the relative proportion of the BRET peak compared to that of coelenterazine, both spectra were normalized to the light emission recorded at 475 nm (i.e. the λ_{max} of coelenterazine/Rluc emission in the absence of GFP).

Figure 3 BRET resulting from reassembling the interaction between Rluc and RGFP via leucine zippers.

Rluc and RGFP tethered to antiparallel coiled coils in either N terminal or C terminal position were expressed in COS-7 cells. Pairs of cell extracts, each containing either one luciferase donor or one fluorescent acceptor, attached to complementary helices, were mixed as schematically shown on the right-hand side of each plot, at a donor: acceptor ratio (vol/vol) of 1:3. Luminescence spectra of the reassembled proteins were recorded after addition of native coelenterazine and were plotted as ratios of the maximal emission of the spectrum (λ_{max}). Note that in one reassembled pair the amino terminal group of RGFP is free (*upper panel*), while in the other it is blocked by the peptide helix (*lower panel*).

Figure 4 Comparison of luminescence spectra emitted by *Renilla* proteins intracellularly reassembled as leucin zipper chimeras or native proteins.

Mixtures of plasmids coding either for wild type Rluc and RGFP, or for the same leucine zippers tethered proteins, RGFP-NZ and CZ-Rluc, were cotransfected in Cos-7 cells. A constant DNA concentration was maintained, by transfecting variable ratios (as indicated) of donor-coding cDNAs (CZ-Rluc in (**A**) and wild-type Rluc in (**B**)) and acceptor coding cDNAs (RGFP-NZ in (**A**) and wild-type RGFP in (**B**)). Bioluminescence spectra were recorded by adding native coelenterazine (5μM) to aliquots of cell suspensions containing 1 x 10^5 cells. Note that no BRET signal (**B**) can be detected in the spectra obtained

with native proteins. (**C**) The ratios of λ_{max} (510/475 nm) computed in the spectra recorded for leucine zippers (■) and native proteins (○) were replotted as a function of the ratios of transfected cDNAs.

Figure 5 Quantification of the RET efficiency using RGFP-Thr-Rluc and Rluc-Thr-RGFP cleavable fusion proteins.

(A) Western blot analysis of RGFP-Thr-Rluc fusion protein after SDS-PAGE separation. Cell extracts were incubated in presence or absence of thrombin plus or minus hirudin as described in the Experimental section. The samples were visualized with c-Myc monoclonal anti-body. (**B**) BRET ratios were recorded and computed as described in the Experimental section from cell extracts expressing the cleavable fusion proteins RGFP-Thr-Rluc and Rluc-Thr-RGFP before (control) and following thrombin treatment. Data represent the mean ± s.e.m. of 3 independent experiments.

Figure 6 Quantum yield enhancement in CLZ400A emission.

Time-corrected luminescence emission spectra (8 nm slits) of CTZ400A (5 μM) were recorded from cell extracts expressing RGFP-Thr-Rluc, treated (□) or not (■) with thrombin as described in the Experimental section. The spectra were fitted using a skewed Gaussian distribution function (see Experimental section) and the best-fitting distributions are shown together with the data points (*solid lines*). The *inset* is a rescaled plot of the thrombin treated data (□) shown in the main plot. The data points represent means of six independent experiments. Total light output (in cps x nm x 10^6) was computed either from the sum of the areas of the best fitting distributions (0.65 , thrombin, and 28.6, no thrombin), or from numerical integration of the experimental data points (0.68 , thrombin and 28.9, no thrombin). If we consider only the photons emitted through the GFP chromophore (areas, 0.035, thrombin, vs. 21, no thrombin) the enhancement of light output due to Rluc/RGFP interaction was 592 fold.

Figure 7 The *Renilla* BRET system as reporter of GPCRs- β–arrestin interactions.

Time-corrected luminescence spectra of CTZ400A (10 μM) were recorded from suspensions of Hek-293 cell lines (1 x 10^5 cells) permanently co-expressing either β_2AR-Rluc (**A**) or DOP-Rluc (**B**) with RGFP-arrestin2 in the absence of the respective receptor ligands (*BASAL*) and after exposure to isoproterenol (*ISO*) or [D-Ala^2, D-Leu^5]enkephalin (*DADL*). The agonist-induced BRET signals were inhibited in presence of receptor antagonists CGP-12177A (*CGP*) or naltrindole (*NLT*). All ligands were used at 1 μM. The data are representative single experiments that were repeated at least three times with similar results.

Figure 8 Ratiometric and luminometric detection of V_2 receptor - β–arrestin interaction

A. Hek-293 cell lines co-expressing vasopressin 2 receptors linked to Rluc (V_2R-Rluc) with either arrestin 1 or arrestin 2, tethered to RGFP (RGFP-ARRx) were seeded in 24-well at a density of 5 x 10^5 cells/well. After 24 hr the cells were preincubated with CLZ and the BRET ratios in the absence or presence of agonist (AVP, 1μM) ± antagonist (ANT, 10μM) were measured as described in the Experimental section

B. The same cell lines were plated in 96-well white opaque plates at a density of 1 x 10^5 cells/well. Total luminescence was recorded 24 hr later in a Victor plate reader using time increments of 0.5 sec immediately following the addition of a solution containing either CTZ400A (10 μM) or CTZ400A plus Arg-vasopressin (1 μM). Data points are means of values obtained in three different wells and are plotted as ratios of counts recorded in the presence or absence of agonist.

Figure 9 Kinetics of arrestin-receptor interaction monitored by changes of luminescence .

Cells co-expressing β_2AR-Rluc and RGFP-βarr2 (plated in 24-well flasks) were incubated 5 min in the presence of CLZ400A, and inserted in the luminometer. Total light was continuously recorded at 1 sec intervals prior and after two sequential additions of PBS or ligands controlled by automatic injectors (as indicated by the arrow-heads on top of the plot). Each tracing plotted in the graph represents the average of light output (cps) recorded in 4 different experiments. The substances added with the two injectors in each trace were: (**Bas**), PBS in both; (**Ago**): Isoproterenol (100nM) in **1** and PBS in **2**; (**Ago + Ant**): Isoproterenol (100nM) in **1** and Alprenolol (10μM) in **2**.

Biochemical Journal Immediate Publication. Published on 17 Sep 2007 as manuscript BJ20070803

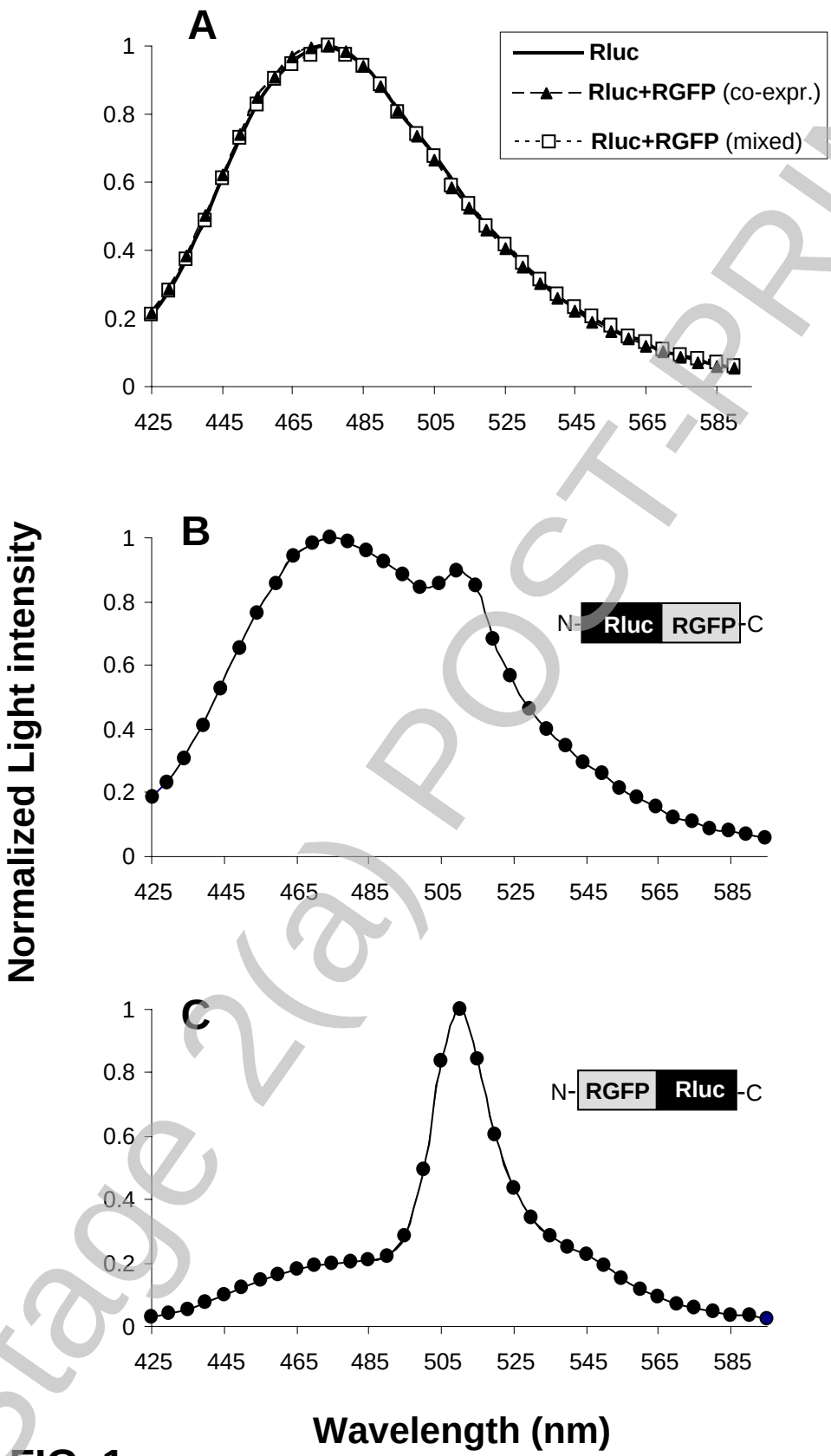

FIG. 1

Biochemical Journal Immediate Publication. Published on 17 Sep 2007 as manuscript BJ20070803

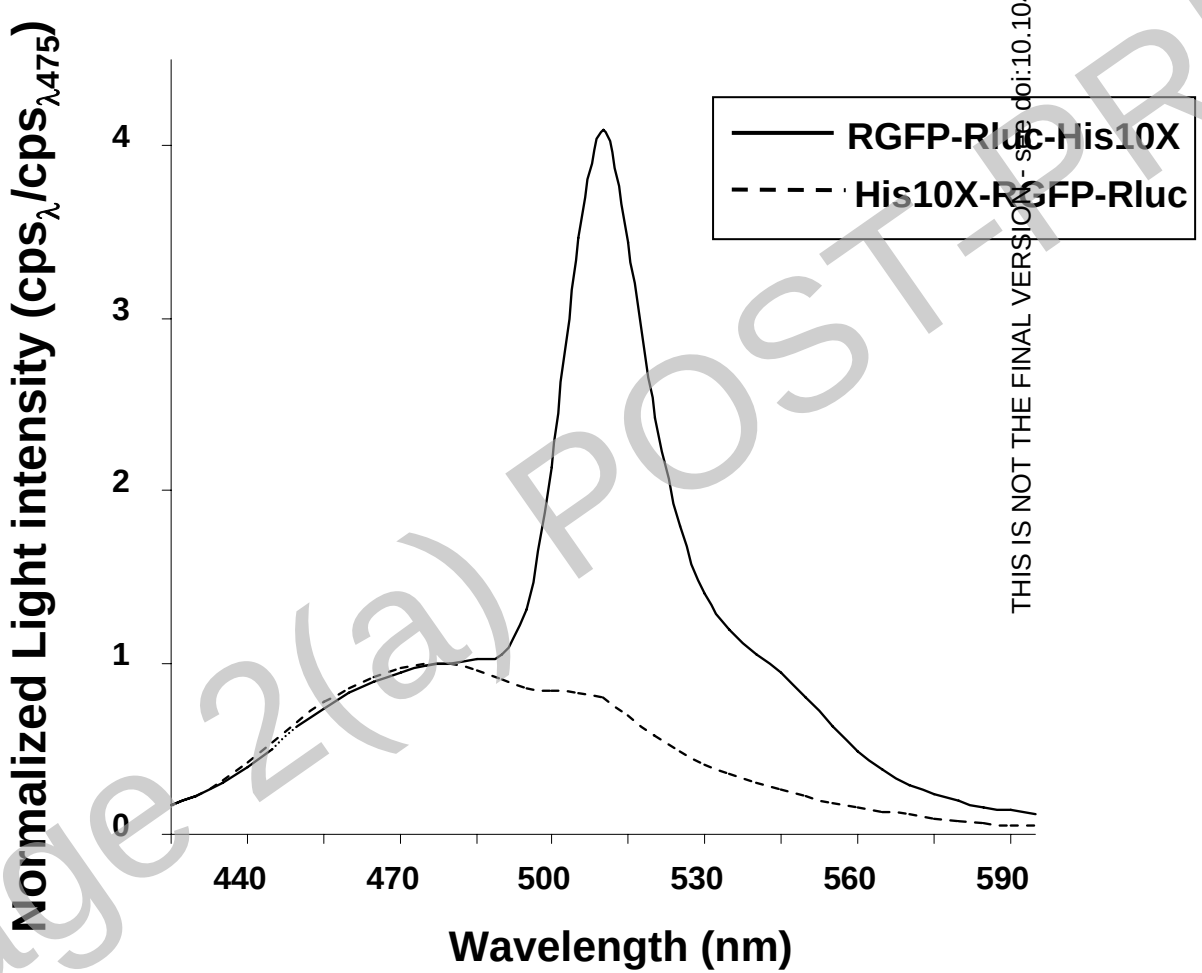

FIG. 2

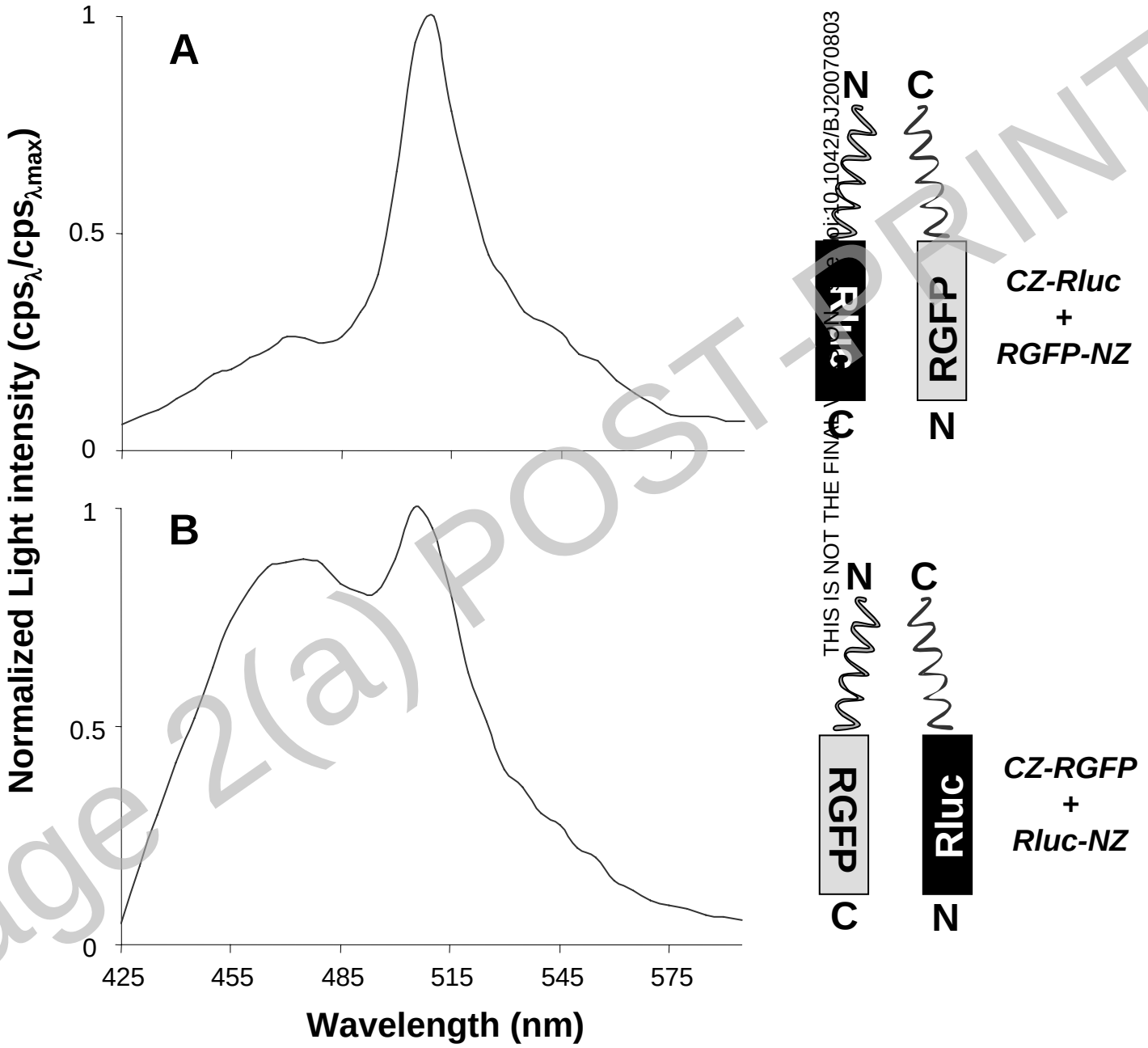

FIG. 3

Biochemical Journal Immediate Publication. Published on 17 Sep 2007 as manuscript BJ20070803

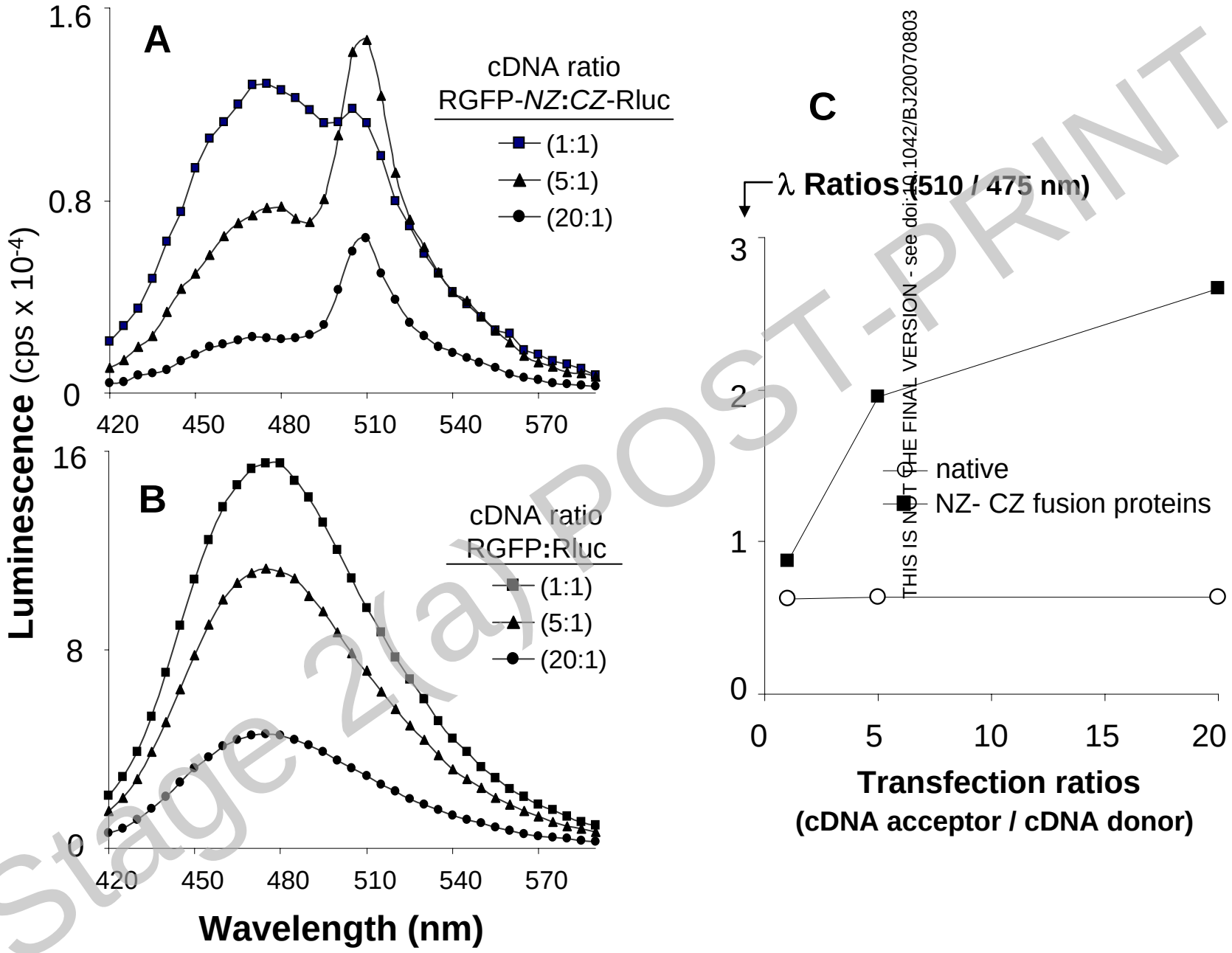

FIG. 4

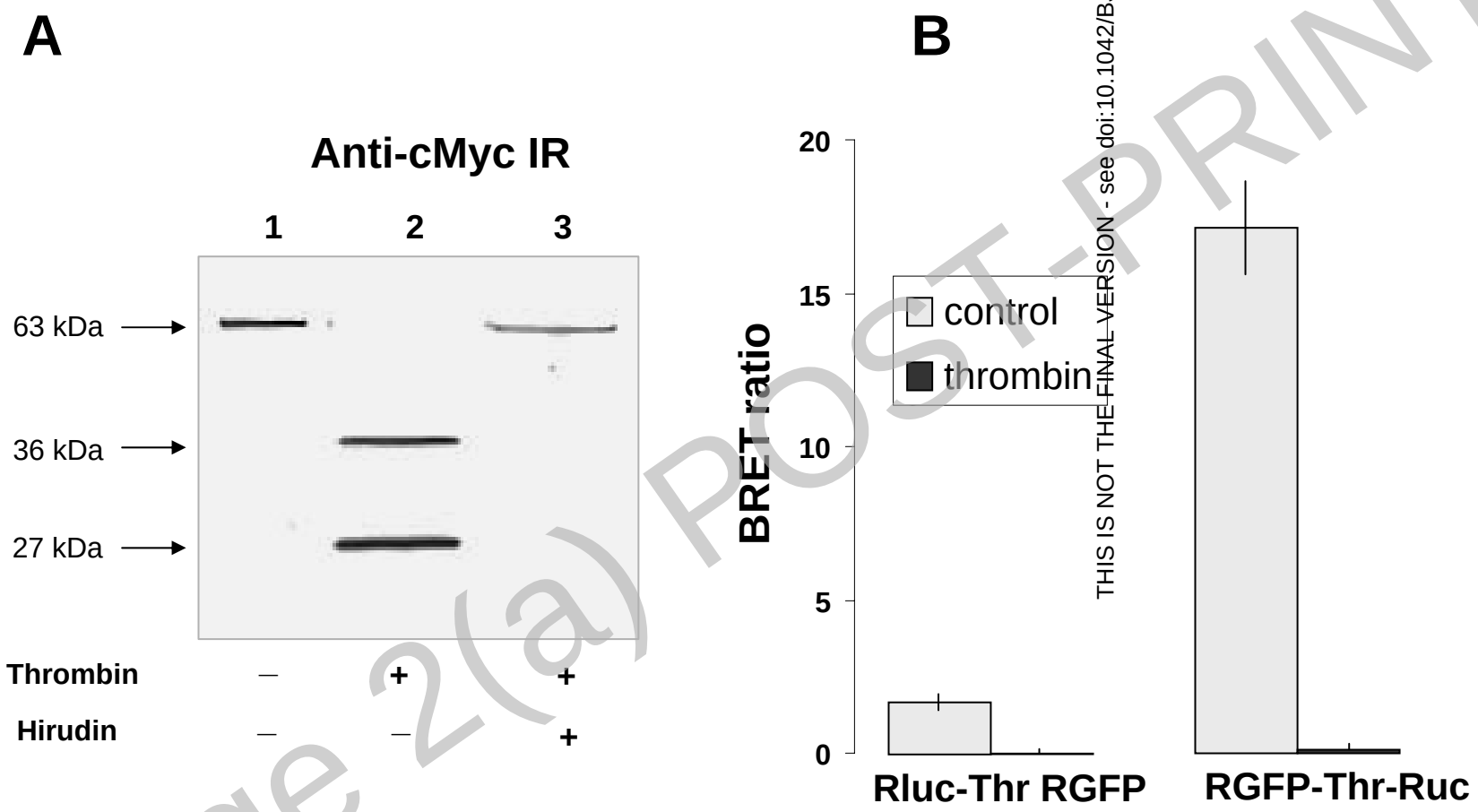

FIG. 5

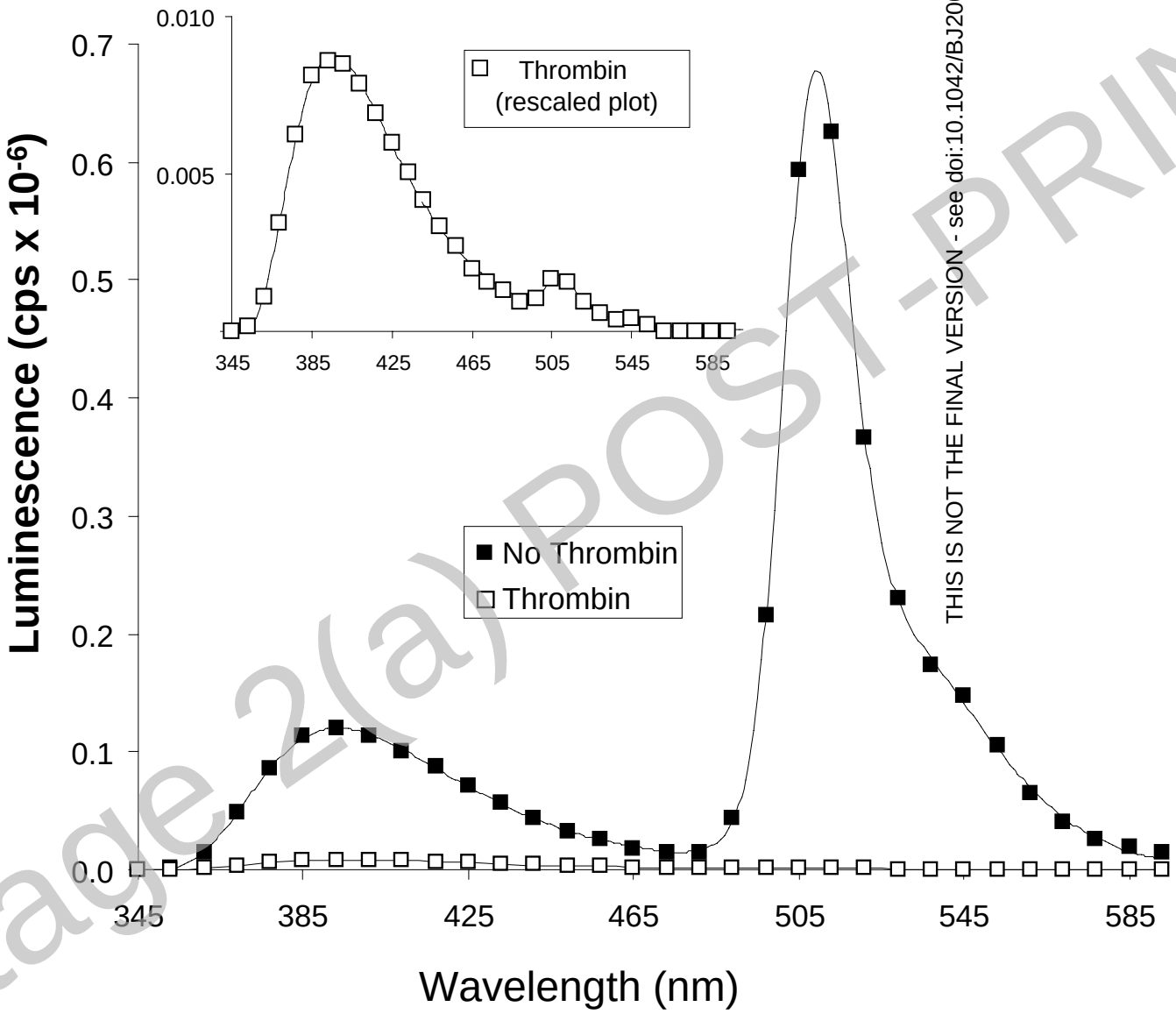

FIG. 6

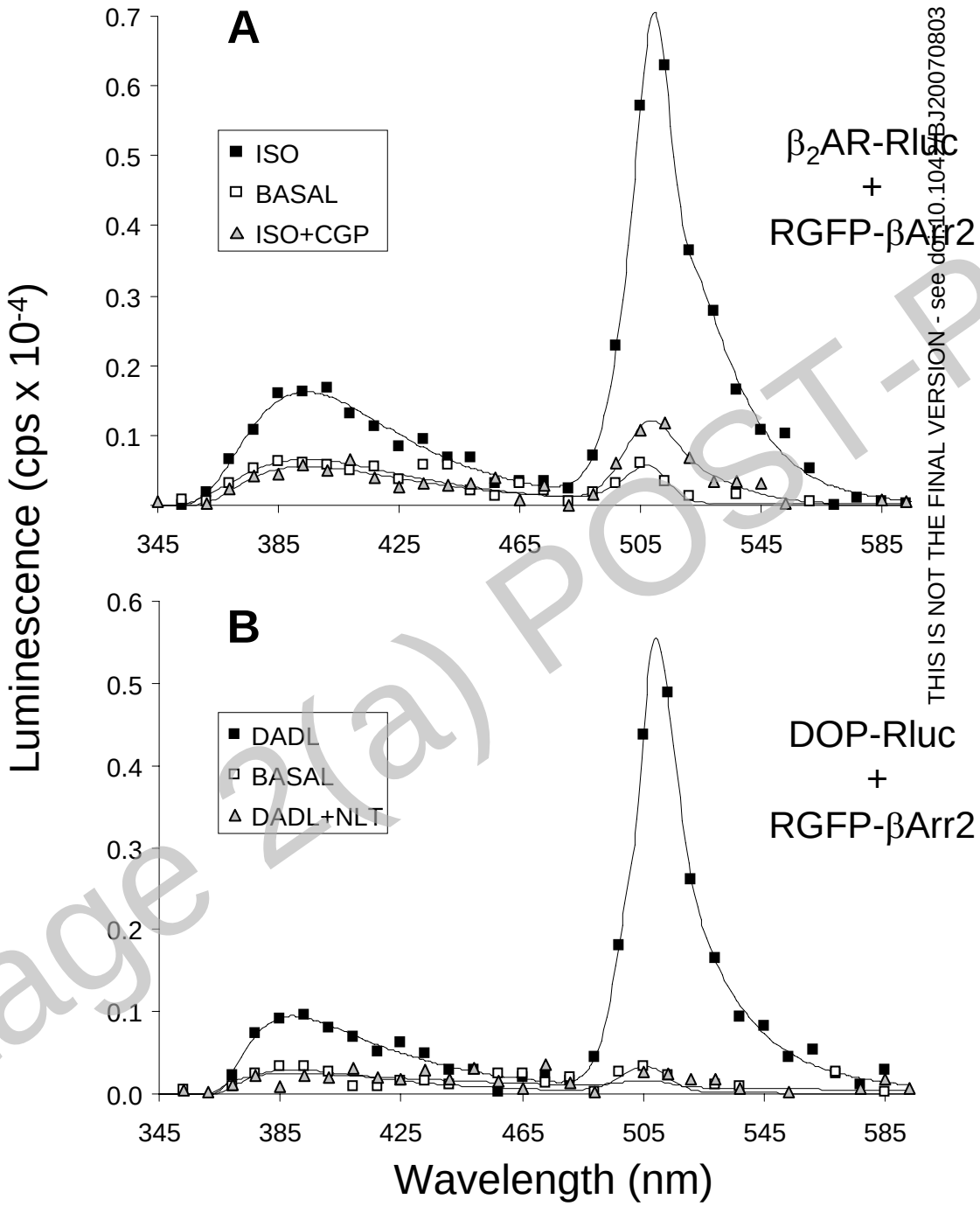

FIG. 7

Biochemical Journal Immediate Publication. Published on 17 Sep 2007 as manuscript BJ20070803

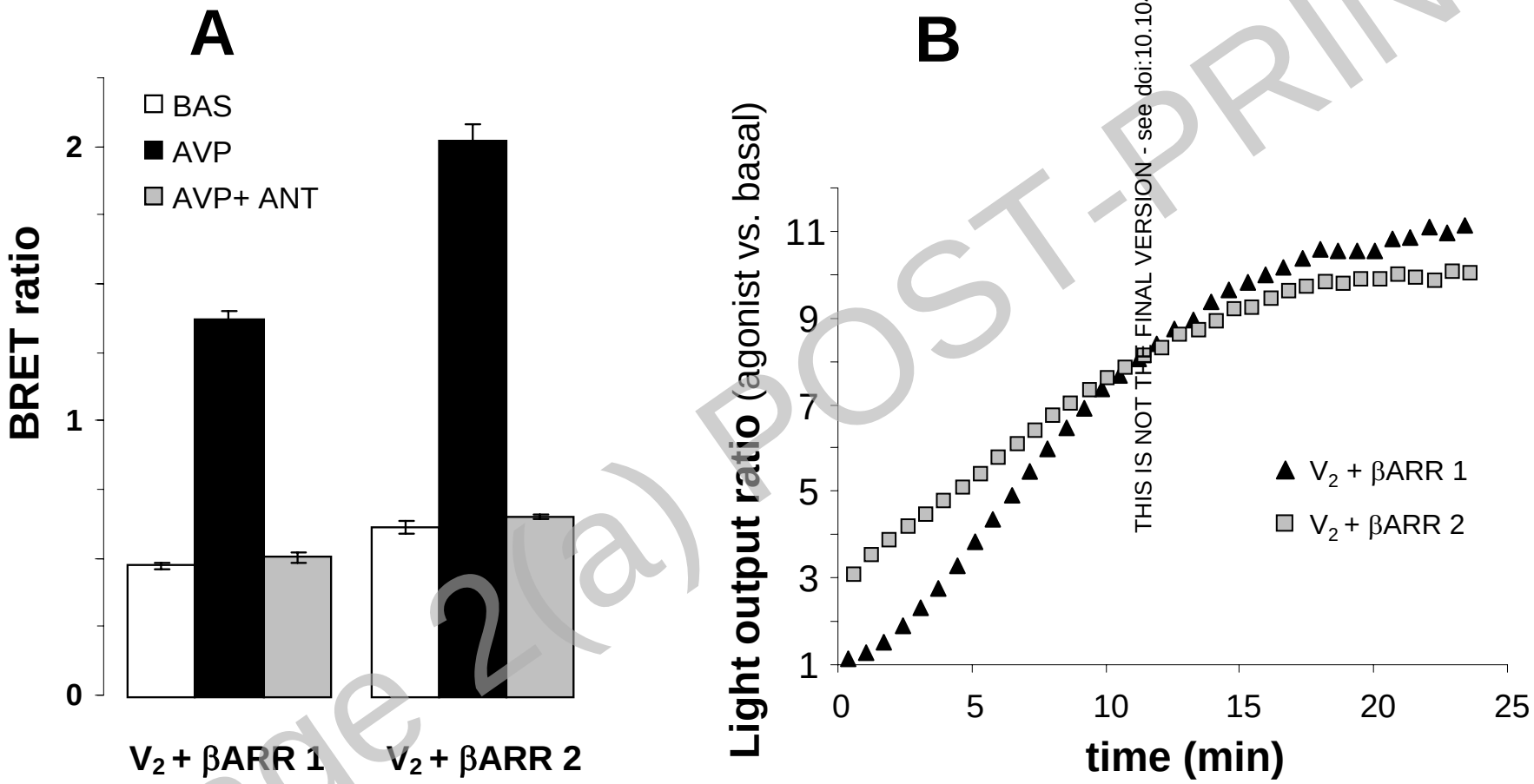

FIG. 8

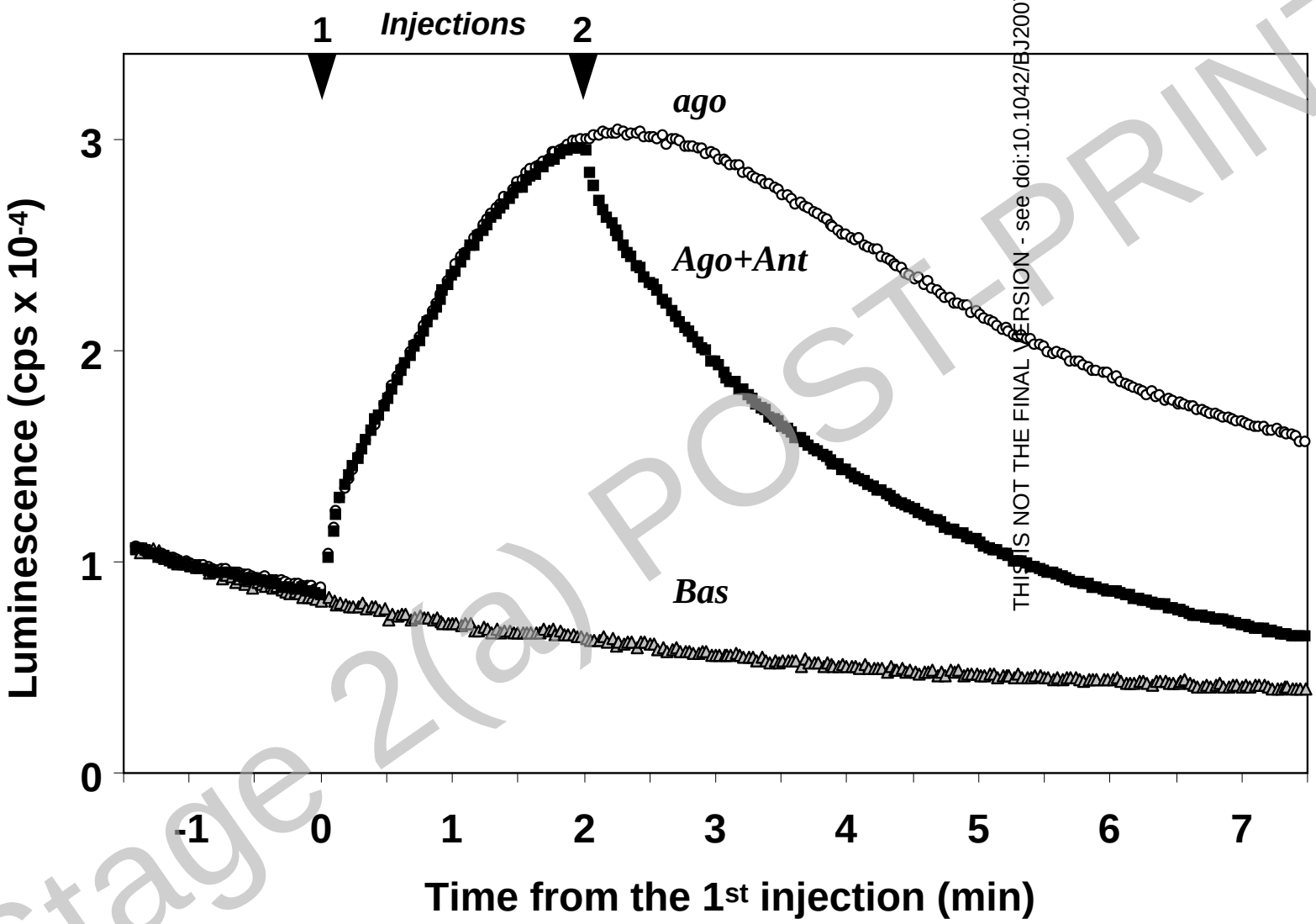

FIG. 9