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Improved donor/acceptor BRET couples for monitoring β -arrestin recruitment to G protein-coupled receptors

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Keywords:	BRET, RGFP, YPet, GPCR, β arrestin



Research Article ((4359 words))**Improved donor/acceptor BRET couples for monitoring β -arrestin
recruitment to G protein-coupled receptors****((Supporting information online))**

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Keywords: BRET, RGFP, YPet, GPCR, β arrestin

Abbreviations: BRET, Bioluminescence Resonance Energy Transfer; mBu,
milli-BRET unit; Rluc, *Renilla* luciferase; YFP, Yellow Fluorescent Protein;

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4 RGFP, *Renilla* green fluorescent protein; GPCR, G protein-coupled receptors;

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6 β ARR, β arrestin; V2R, vasopressin 2 receptor ; MT₁, melatonin 1 receptor; AVP,

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For Peer Review

Abstract

We report highly sensitive Bioluminescence Resonance Energy Transfer (BRET) assays with optimized donor/acceptor couples. We combined the energy donors *Renilla* luciferase (Rluc) and the Rluc8 variant with the energy acceptors yellow fluorescent protein (YFP), the YPet variant and the *Renilla* green fluorescent protein (RGFP). Different donor/acceptor couples were tested in well-established assays measuring ligand-induced β -arrestin (β ARR) intramolecular rearrangements and recruitment to G protein-coupled receptors (GPCRs). We show increased sensitivity with Rluc8/YPet and Rluc8/RGFP couples and measured previously undetectable BRET signals. These tools improve existing β ARR assays and offer new options for the development of future BRET assays.

1. Introduction

BRET is a natural phenomenon occurring in several marine organisms. In 1999, Xu *et al.* first applied BRET to the detection of protein-protein interactions in intact cells [1]. This initial version, nowadays called BRET¹, relies on the Rluc energy donor and its substrate, coelenterazine h, and the YFP energy acceptor. The overlap between the donor and acceptor energy spectra is substantial, resulting in a relatively high background signal that needs to be carefully controlled [2]. In contrast, the BRET² version uses Rluc in the presence of the blue-shifted substrate coelenterazine 400A and the GFP10 variant that shows a blue-shifted absorption spectrum [3, 4]. Despite improved spectral resolution in the BRET² version, BRET¹ is still the most widely used method due to its higher sensitivity (>100X higher luminescence signals) [5] allowing BRET measurements at physiological protein expression levels [6].

The primary goal of this study was to further improve the BRET¹ version by testing new donor/acceptor couples. We compared the original energy donor Rluc with the recently described Rluc8 variant, which differs by eight mutations from Rluc. Rluc8 was selected for its higher stability and quantum yield [7] and was successfully applied to BRET imaging in a BRET² setting [8]. Furthermore, we compared the original YFP with the YPet variant and the recently cloned RGFP. YPet was optimized for enhanced fluorescence resonance energy transfer (FRET) in a tandem CFP-YFP fusion protein [9], an experimental setting closely resembling the Rluc/YFP BRET couple. For RGFP, an improved energy transfer

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4 efficiency was reported in BRET² assays but only if the N-terminus of RGFP was
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6 free, considerably restricting the use of RGFP in BRET assays [10].
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9 GPCRs play a central role in the signal transduction of an enormous array of
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11 biological stimuli primarily through their coupling to heterotrimeric G proteins.
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13 GPCR activation initiates their feedback desensitization, which is mostly
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15 mediated by GPCR kinases (GRKs) and β ARR proteins. β ARRs can also act as
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17 scaffolds, linking GPCRs to mitogen-activated protein kinase signaling pathways
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19 [11]. Because β ARRs bind only the activated, phosphorylated form of the
20
21 receptor, BRET assays using β ARRs as biosensors for GPCR activation were
22
23 widely developed in the last decade [12, 13].
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26 In order to test the new BRET couples described above, we chose two well-
27
28 established BRET assays used to monitor agonist-promoted interactions upon
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30 GPCR activation: the ligand-induced β ARR intramolecular rearrangement and the
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32 ligand-induced β ARR recruitment to GPCRs.
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35 **2. Materials and Methods**

36 *2.1. Compounds*

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38 Arginine vasopressin (AVP) was purchased from Calbiochem/Merck (Nottingham,
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40 UK), melatonin from VWR (Fontenay sous Bois, France). The d(CH₂)₅[D-
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42 Tyr(Et)₂, Ile₄, Eda₉]AVP compound was provided by Dr. M. Manning (Toledo,
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44 Ohio, USA).
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49 *2.2. Construction of fusion proteins*

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4 The coding sequences for humanized Rluc, YFP, and *Renilla mullerie* GFP
5 (RGFP) were obtained by PCR amplification from previously described pcDNA3-
6 OBR-Rluc, pcDNA3-OBR-YFP [14] and pCMV-h-RM RGFP vectors (Prolume,
7 Arizona USA). The Rluc8 and YPet variants were generated by multi-site
8 mutagenesis (Promega, Charbonnières-les-Bains, France) from the Rluc and YFP
9 sequences. Tandem Acceptor-Rluc-Flag and Acceptor-Rluc8-Flag fusions were
10 generated by PCR by inserting the AENLYFQGA linker sequence between
11 acceptors and donors and adding a Flag epitope (DYKDDDDK) before the Stop
12 codon. Tandem Flag-Rluc-RGFP and Flag-Rluc8-RGFP fusion proteins contain
13 the same linker between donors and RGFP and the Flag epitope was inserted after
14 the Start codon of the donors.

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27 The human V2R coding region was cloned in frame with the Rluc coding region
28 to obtain the V2R-Rluc construct. The V2R-Rluc8 construct was obtained by
29 replacing Rluc by Rluc8. Both constructs contain a PVAT linker sequence.

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33 Rluc and YFP of the previously described Rluc- β ARR2-YFP vector [13] were
34 replaced by Rluc8, YPet and RGFP, respectively. The linker between donors and
35 β ARR2 is composed of the following amino acids
36 GDLRRALENSHASAGYQACGTGS and the linker between β ARR2 and the
37 acceptors is CLEDPRVPVAT.

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Accepter- β ARR2 fusion proteins were obtained by fusing the coding region of
YFP, YPet or RGFP in frame with that of rat β ARR2 without linker.

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4 MT₁-Rluc8 construct was obtained by replacing Rluc by Rluc8 from a previously
5 described MT₁-Rluc vector [15]. MT₁-Rluc and MT₁-Rluc8 contained the same
6 linker sequence (VWDPPVAT) between human MT₁ and the donor.
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10 11 12 2.3. Cell culture and transfections

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14 HEK 293T cells were grown in complete medium (Dulbecco's modified Eagle's
15 medium supplemented with 10% (v/v) fetal bovine serum, 4.5g/L glucose,
16 100U/mL penicillin, 0.1mg/mL streptomycin and 1mM glutamine) (Invitrogen,
17 CA). Transient transfections were performed using JetPEI (Polyplus Transfection,
18 France), or Fugene6 HD (Roche, Basel, Switzerland), according to manufacturer's
19 instructions.
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22 For Donor/Acceptor fusion protein BRET assays and corresponding separate co-
23 expressed soluble proteins, HEK 293T cells, which do not express endogenous V2
24 and MT₁ receptors, were transfected with 2 to 10µg of the different plasmids.
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27 For the double-brilliance βARR BRET assay, wild-type HEK 293T cell
28 transiently expressing myc-V2R or HEK 293 cells stably expressing Flag-MT₁
29 [16] were seeded in 6-well plates and transiently transfected with 500ng of Rluc-
30 βARR-YFP, Rluc-βARR-YPet, Rluc-βARR-RGFP, Rluc8-βARR-YFP, Rluc8-
31 βARR-YPet or Rluc8-βARR-RGFP expression vectors. 24h post-transfection,
32 cells were transferred into 96-well plates and left another 24h before BRET
33 measurements.
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37 For the βARR recruitment BRET assay, HEK293T cells were seeded in 10 cm
38 dishes and transiently transfected with 1500ng and 750ng of myc-V2R-Rluc and
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4 myc-V2R-Rluc8, respectively. 4h post-transfection cells were transferred into 12-
5 well plates, and transfected 24h later with increasing amounts of YFP-βARR,
6 YPet-βARR and RGFP-βARR expression plasmids. Cells were then transferred
7 into 96-well plates and left another 24h before BRET measurements.
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12 Concentration-response curves were performed in presence of saturating amounts
13 of YFP-βARR, YPet-βARR and RGFP-βARR expression plasmids.
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17 Measurements were performed 15 min after agonist addition.
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20 21 *2.4. BRET experiments*

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25 For Donor-Acceptor fusion proteins and the corresponding separate soluble forms,
26 cytoplasmic extracts were prepared 48h post-transfection as described previously
27 [10], diluted and submitted to BRET measurements in the presence of 5μM
28 coelenterazine h (Interchim, Montluçon, France).

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32 For the double-brilliance βARR2 and βARR2 recruitment assays, cells were
33 washed once with PBS 48h post-transfection, and coelenterazine h substrate was
34 added at a final concentration of 5μM and left for 10min at RT. Cells were then
35 stimulated by a saturating concentration of ligands for 15 min (double-brilliance
36 βARR2) or 5 min (βARR2 recruitment). Arginine vasopressin (AVP) and
37 melatonin were used as agonist for V2R and MT₁ experiments, respectively.

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46 BRET readings were performed using a lumino/fluorometer (Mithras; Berthold
47 technologies, France) allowing sequential integration of luminescence with three
48 filter settings (Rluc filter, 485 ± 10 nm; YFP (and YPet) filter, 530 ± 12.5 nm,
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4 RGFP filter, 515 ± 10 nm). Emission signals at 530 or 515 nm were divided by
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6 emission signals at 485 nm. The difference between this emission ratio obtained
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8 with donor and acceptor fused or co-expressed and that obtained with the donor
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10 protein expressed alone was defined as the BRET ratio. Results are expressed in
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12 milliBRET units (mBU) corresponding to the BRET ratio multiplied by 1000.
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14 Donor saturation curves and determination of maximal BRET values were
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16 described previously [17].
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21 2.5. Western Blot

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23 Proteins were separated by 10% SDS-PAGE and immunoblot analysis was carried
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25 out with the following antibodies: Mouse-monoclonal anti-Flag M2 (SIGMA,
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27 MO), mouse monoclonal anti-myc (Santa Cruz, CA), mouse monoclonal anti-
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29 Renilla Luciferase (Chemicon International, CA), rabbit polyclonal anti- β ARR
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31 (SIGMA, MO) and rabbit polyclonal anti- α -tubulin (Sigma, MO).
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33 Immunoreactivity was revealed using IRDye infrared secondary antibodies using
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35 the LI-COR Odyssey infrared imaging system (Courtaboeuf, France).
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39 3. Results

43 3.1 BRET efficiency of tandem fusion proteins

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48 We first generated a series of N-Acceptor-Donor-C fusion proteins composed of a
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50 N-terminal acceptor, a common linker sequence and a C-terminal donor.
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4 Expression of equivalent amounts of fusion proteins in HEK 293T cells revealed
5 the expected strong BRET for direct fusion proteins (**Fig. 1A**). Dilutions of these
6 samples up to 64 fold showed the expected stability of the BRET ratio (data not
7 shown). On the other hand, co-expression of the new donor/acceptor couples as
8 separate proteins resulted in negligible BRET signals (0-30 mBU) demonstrating
9 their suitability for the study of protein-protein interactions (**Fig. 1A**). BRET
10 values were highest for YPet fusion proteins followed by YFP and RGFP fusion
11 proteins and were always higher for Rluc8 fusion proteins as compared to the
12 corresponding Rluc fusion proteins. The use of RGFP in BRET has been reported
13 to be sensitive to its position in the fusion protein (N-terminal vs. C-terminal
14 fusion) [10]. We therefore tested N-Donor-RGFP-C fusion proteins with RGFP
15 located at the C-terminus. A robust BRET signal that was higher than that of N-
16 RGFP-Donor-C fusion proteins was observed (**Fig. 1B**). Taken together YPet
17 fusion proteins showed highest BRET signals, whereas BRET signals for RGFP
18 and YFP fusion proteins were comparable.

3.2. Improved BRET signals with Rluc8/YPet and Rluc8/RGFP couples when monitoring ligand-induced β ARR2 intramolecular conformational changes

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43 We next generated intramolecular BRET biosensors based on the recently
44 described double-brilliance β ARR assay [13]. This assay monitors the
45 conformational rearrangement of β ARR2 bringing its N-terminus and C-terminus
46 into closer proximity upon recruitment to agonist activated GPCRs. Equivalent
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4 amounts of the indicated N-Donor- β ARR2-Acceptor-C fusion proteins, as
5 indicated by Western Blot, were transiently expressed in myc-V2 vasopressin
6 receptor (myc-V2R)-expressing cells, known to recruit β ARR2. BRET values
7 were monitored for 15 min in the absence or presence of a saturating
8 concentration of the V2R agonist AVP (10 μ M). Maximal BRET signals were
9 generally observed as early as 4 min after agonist addition, indicating that the
10 double-brilliance fusion constructs are functional (Supplementary Fig. 1B).
11 Comparison of agonist-induced BRET after 15 min of stimulation showed
12 enhanced induced-BRET for N-Rluc8- β ARR2-YPet-C and N-Rluc8- β ARR2-
13 RGFP-C (2-3 fold, respectively) when compared with other constructs (Fig. 2A).

14
15 Agonist concentration-response curves were performed showing BRET EC₅₀
16 values ranging 75 +/- 45 nM and 79 +/- 29 nM for N-Rluc8- β ARR2-YPet-C and
17 N-Rluc8- β ARR2-RGFP-C constructs, respectively (Fig. 2B). Similar values were
18 obtained for the other double-brilliance fusion constructs (data not shown).

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19 We then expressed the different β ARR2 fusion proteins in HEK 293T cells stably
20 expressing Flag-MT₁ melatonin receptors a GPCR whose β ARRs recruitment is
21 difficult to monitor using the classical Rluc- β ARR2-YFP fusion protein (Fig. 2A).
22 Whereas agonist-induced BRET signals (10 μ M melatonin) were barely
23 detectable for all Rluc-containing fusions, BRET signals became clearly
24 detectable as early as 2 min after agonist addition for N-Rluc8- β ARR2-YPet-C
25 and N-Rluc8- β ARR2-RGFP-C constructs (Fig. 2A) (Supplementary Fig. 1C).
26 Comparable BRET EC₅₀ values of 6.3 +/- 3.7 nM and 8.2 +/- 4.9, were obtained
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4 for melatonin using N-Rluc8- β ARR2-YPet-C and N-Rluc8- β ARR2-RGFP-C
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6 constructs, respectively (**Fig. 2C**).
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9 Expression of equivalent amounts of the indicated N-Donor- β ARR2-Acceptor-C
10 fusion proteins in HEK 293T cells in the absence of V2R and MT₁ did not result
11 in agonist-induced BRET signals, demonstrating the specificity of the assay (data
12 not shown). Taken together, these results show that the Rluc8/YPet and
13 particularly the Rluc8/RGFP couple significantly amplify weak and previously
14 undetectable BRET signals of the intramolecular β ARR2 biosensor.
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20 21 22 23 *3.3. Improved BRET signals with Rluc8/YPet and Rluc8/RGFP couples when* 24 *monitoring ligand-induced β ARR2 recruitment to GPCR* 25 26

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28 We further analyzed the donor/acceptor couples in the β ARR2 recruitment assay
29 [12, 18]. Similar amounts of V2R-Rluc or V2R-Rluc8 C-terminal fusion proteins
30 were co-expressed with increasing amounts of N-Acceptor- β ARR2-C fusion
31 constructs and ligand-induced BRET was measured at maximal β ARR2
32 recruitment (5 min after agonist addition). BRET donor saturation curves were
33 generated in the absence or presence of AVP (10 μ M) as previously described [14,
34 19] to determine BRET_{max} values that correspond to the saturation of all donor
35 molecules by acceptor molecules (**Fig. 3**)(**Supplementary Fig. 2**). All couples
36 showed significant agonist-induced BRET signals with highest BRET_{max} values
37 for the V2R-Rluc8/ N-RGFP- β ARR2-C couple (**Table 1**). No significant ligand-
38 induced BRET signals were observed in the presence of the V2R antagonist
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4 Vd(CH₂)₅[D-Tyr(Et)₂, Ile₄, Eda₉]AVP (Table 1)(Supplementary Fig. 2). Basal
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6 BRET values were highest for the V2R-Rluc8/ N-RGFP-βARR2-C couple
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8 indicating the increased sensitivity of this couple to monitor basal βARR2
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10 recruitment. This recruitment is specific as expression of V2R-Rluc8 in the
11
12 presence of soluble RGFP gave negligible BRET values (Supplementary Fig.
13
14 **2A**). Basal βARR2 recruitment to V2R has been observed recently [10].

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17 Moreover, agonist concentration-response curves, in the presence of saturating
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19 amounts of acceptors, were performed showing BRET EC₅₀ ranging from 5 to 15
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21 nM AVP for the different N-Acceptor-βARR2-C constructs (data not shown).

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23 We then measured βARR2 recruitment to MT₁-Rluc and MT₁-Rluc8. Whereas the
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25 recruitment to MT₁-Rluc was undetectable, recruitment to MT₁-Rluc8 became
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27 detectable in combination with YPet and RGFP further demonstrating the
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29 superiority of these new BRET couples.
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31 32 33 **4. Discussion**

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37 The βARR recruitment assay has become increasingly popular to monitor GPCR
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39 activation. Since the development of the BRET technology in 1999 and the first
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41 βARR assay in 2000, several improvements have been reported to increase
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43 signaling amplitude and stability [20] [21]. Here, we focused on improving the
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45 most sensitive and widely used BRET¹ version by combining recently developed
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47 energy donors (Rluc, Rluc8) and acceptors (YFP, YPet, RGFP) in a systematic
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49 manner. By using two well-established BRET assays measuring βARR2
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4 intramolecular rearrangements and recruitment to GPCRs, we show that
5 Rluc8/YPet(RGFP) couples significantly increase the sensitivity of these assays,
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7 allowing the detection of previously undetectable BRET signals.
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10 We observed that Rluc8 and Rluc8 fusion proteins emit 2-4 times more light than
11 the corresponding Rluc proteins expressed at equivalent levels as previously
12 reported (**Supplementary Fig. 3**) [7, 8, 21]. The higher luminescence intensity of
13 Rluc8 implies a more efficient acceptor excitation and re-emission and the
14 possibility to further decrease protein expression levels to physiologically relevant
15 levels. In agreement with previous FRET experiments using YPet-CFP fusion
16 proteins, we show that YPet is a better BRET acceptor than YFP [9]. Whereas the
17 energy transfer efficiency in BRET assays is typically low, Molinari *et al.* recently
18 reported the striking observation that RGFP in combination with Rluc can
19 generate close to 100% energy transfer associated with a dramatic increase in
20 acceptor light output [10]. The underlying molecular basis of this phenomenon,
21 which is only observed when the N-terminus of RGFP is free, remains unclear. By
22 using N-terminal RGFP fusion proteins in two independent assays (RGFP-Rluc
23 tandem fusion protein and GPCR-Rluc/ RGFP- β ARR2 couple), we were unable to
24 confirm these observations. Indeed, no major difference in BRET signals
25 generated by YFP- and RGFP-containing fusion proteins were observed.
26 Accordingly, Rluc light output did not vary significantly with increased BRET
27 (not shown). The reason for this discrepancy remains currently unknown and
28 warrants further studies. We furthermore noted that BRET values obtained with
29 RGFP fusion proteins may be subject to some variation as illustrated by the
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4 scattering of BRET values obtained in the donor saturation experiment with
5 RGFP- β ARR2 and V2R-Rluc8 (Supplementary Fig. 2). These results indicate
6 that RGFP is a highly sensitive energy acceptor. RGFP can be therefore
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8 considered as an interesting BRET acceptor, which can result in superior BRET
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10 values, particularly when compared to YFP. The data show that the use of RGFP
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12 is not restricted to N-terminal fusion proteins, which considerably increases its
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14 attractiveness for the BRET assay. More importantly, RGFP shows improved
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16 sensitivity compared to YFP when combined with the Rluc8 variant.
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20 Overall, Rluc8/YPet and Rluc8/RGFP couples provide superior results compared
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22 to the classical Rluc/YFP couple. According to the data obtained in the β ARR2
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24 recruitment assay, these BRET couples primarily increase the overall sensitivity
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26 of the assay whereas the signal-to-noise ratio appears to be comparable for all
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28 combinations (3-5 fold increase between basal and AVP-induced BRET signals)
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30 (Table 1). Despite obvious conceptual advantages of the double brilliance β ARR2
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32 biosensor such as the use of unmodified wild-type GPCR and high assay stability
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34 due to the fixed donor/acceptor ratio, the assay is not broadly used in the literature
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36 [13, 22]. The low amplitude of ligand-induced BRET signals is one of these
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38 reasons. The increased sensitivity observed with the new donor/acceptor
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40 combinations clearly increases the attractiveness of the assay. A widespread
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42 application to monitor β ARR-dependent signaling events in the context of ligand-
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44 biased signaling can be anticipated.
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47 Several β ARR activation/recruitment assays have been developed over the years,
48 particularly for high-throughput screening purposes. Each of these assays has its
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4 own advantages and disadvantages. BRET assays measure ligand-induced signals.
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6 This is not the case for the fluorescence-based assay that monitors the cellular
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8 localization of β ARR-EGFP fusion proteins [23, 24]. If the changes in β ARR
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10 localization following agonist stimulation are small, data analysis may become
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12 difficult. The TANGO™ (Invitrogen corporation) β ARR assay that is coupled to a
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14 protease-mediated transcriptional reporter gene readout shows excellent assay
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16 sensitivity, due to the sensitive reporter gene read-out [25]. However, a high
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18 number of false positives can be anticipated in a high-throughput screening assays
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20 due to multiple intermediates between receptor activation and gene transcription
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22 initiation. BRET assays do not suffer from these potential drawbacks as receptor
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24 activation is closely coupled to the final read-out. This is also the case of the
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26 recently described enzyme fragment complementation assay for β ARR, now
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28 commercially available as PathHunter technology from DiscoverX [26].
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30 Conditions for successful enzyme complementation might be more demanding
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32 than conditions for successful energy transfer leading potentially to the detection
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34 of less compounds compared to BRET assays. Relatively low signals can be
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36 considered as one of the major disadvantages of β ARR-based BRET assays. The
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38 new donor-acceptor couples presented here improve this parameter, in particular
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40 in the double-brilliance assay.
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44 In conclusion, the new BRET¹ couples, Rluc8 and YPet/RGFP clearly widen the
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46 application of β ARR BRET assays as previously weakly detectable or even
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48 undetectable BRET signals are now reliably detected. These new BRET couples
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50 are likely to become of general interest for the detection of protein-protein
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4 interactions in intact cells at physiological expression levels. Enhanced ligand-
5 promoted BRET signals might also be beneficial for high-throughput screening
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7 procedures.
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10 11 12 **Acknowledgments**

13
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22 compound and the Myc-V2R expression plasmid.
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Legends to figures:

Figure 1: BRET measured with different donor/acceptor couples. (A) Donor/acceptor proteins were co-transfected (□) or transfected as N-Donor-Acceptor-C tandem proteins (■) and BRET was measured. (B) Comparison of BRET measured for N-Donor-RGFP-C and N-RGFP-Donor-C fusion proteins. n=5 independent experiments.

Figure 2: Monitoring of ligand-induced β ARR2 intramolecular rearrangement.

(A) Bar Charts showing the intramolecular rearrangement of the indicated β ARR2 double-brilliance constructs upon recruitment to agonist activated V2R (□) or MT₁ (■) in HEK 293T cells (15 min stimulation with agonist); n=4 independent experiments. (B) AVP concentration-response curves showing for N-Rluc8- β ARR2-YPet-C (○) and N-Rluc8- β ARR2-RGFP-C (□) (n=2). (C) Melatonin concentration-response curves for N-Rluc8- β ARR2-YPet-C (○) and N-Rluc8- β ARR2-RGFP-C (□) (n=2). Expression of β ARR2 was verified by western blotting using anti- β ARR antibodies. Further experimental details and controls are shown in **Supplementary Fig. 1**. A, acceptor; C, C-terminus; D, donor; N, N-terminus; α -tub, α -tubulin.

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Figure 3: BRET measured with the β ARR recruitment assay. Agonist-induced recruitment of the indicated β ARR2 fusion constructs to V2R-Rluc or V2R-Rluc8 (□) (10 μ M AVP, 5 min), and MT₁-Rluc or MT₁-Rluc8 (■) (10 μ M melatonin, 5 min). Expression of V2R-Rluc and V2R-Rluc8 was verified by western blot using

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4 anti-Rluc antibodies (that recognize Rluc and Rluc8 equally well, data not
5 shown). Ligand-induced BRET was defined as the difference between $BRET_{max}$
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7 values obtained from donor saturation curves +/- ligand. Saturation curves and
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9 further controls are shown in **Supplementary Fig. 2**; α -tub, α -tubulin. n=4
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11 independent experiments.
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Table 1: BRET β ARR2 recruitment assay.

	BRET max (mBU)			Fold stimulation
	Basal	AVP	Antagonist	AVP/Basal
V2R-Rluc/YFP-bARR2	9 \pm 1	35 \pm 3	12 \pm 1	3.9
V2R-Rluc/Ypet-bARR2	10 \pm 2	59 \pm 5	10 \pm 3	5.5
V2R-Rluc/RGFP-bARR2	9 \pm 1	37 \pm 3	13 \pm 1	4.1
V2R-Rluc8/YFP-bARR2	13 \pm 1	52 \pm 4	13 \pm 1	4.0
V2R-Rluc8/Ypet-bARR2	16 \pm 1	67 \pm 4	20 \pm 2	4.2
V2R-Rluc8/RGFP-bARR2	58 \pm 4	192 \pm 18	65 \pm 5	3.3
V2R-Rluc8/RGFP	6 \pm 1	4 \pm 1	3 \pm 1	0.7

BRET_{max} and fold stimulation values following agonist stimulation.

BRET_{max} values were determined as described in the “Material and Methods” section and ligand-induced BRET (shown in Fig. 2) was defined as the difference between BRET_{max} values in the absence and presence of ligand. BRET was measured in the absence and presence of the V2R agonist AVP and V2R antagonist Vd(CH₂)₅[D-Tyr(Et)₂, Ile₄, Eda₉]AVP. The fold induction values are expressed as the ratio of the agonist induced BRET_{max}/Basal BRET_{max}.

Figure 1

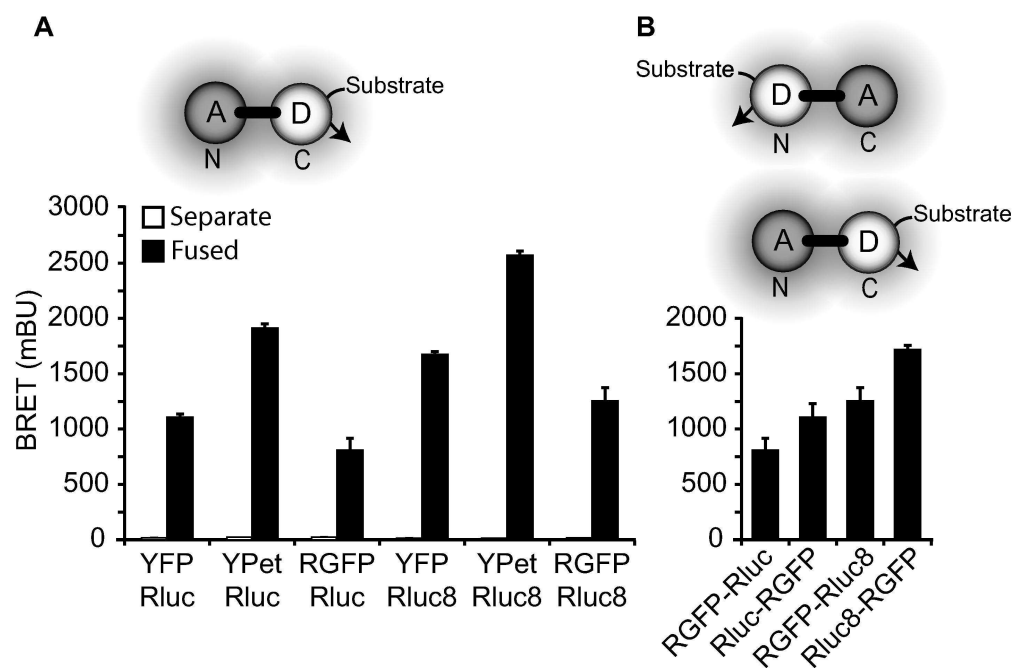


Figure 1: BRET measured with different donor/acceptor couples. (A) Donor/acceptor proteins were co-transfected (o) or transfected as N-Donor-Acceptor-C tandem proteins (<) and BRET was measured. (B) Comparison of BRET measured for N-Donor-RGFP-C and N-RGFP-Donor-C fusion proteins. n=5 independent experiments.

122x87mm (600 x 600 DPI)

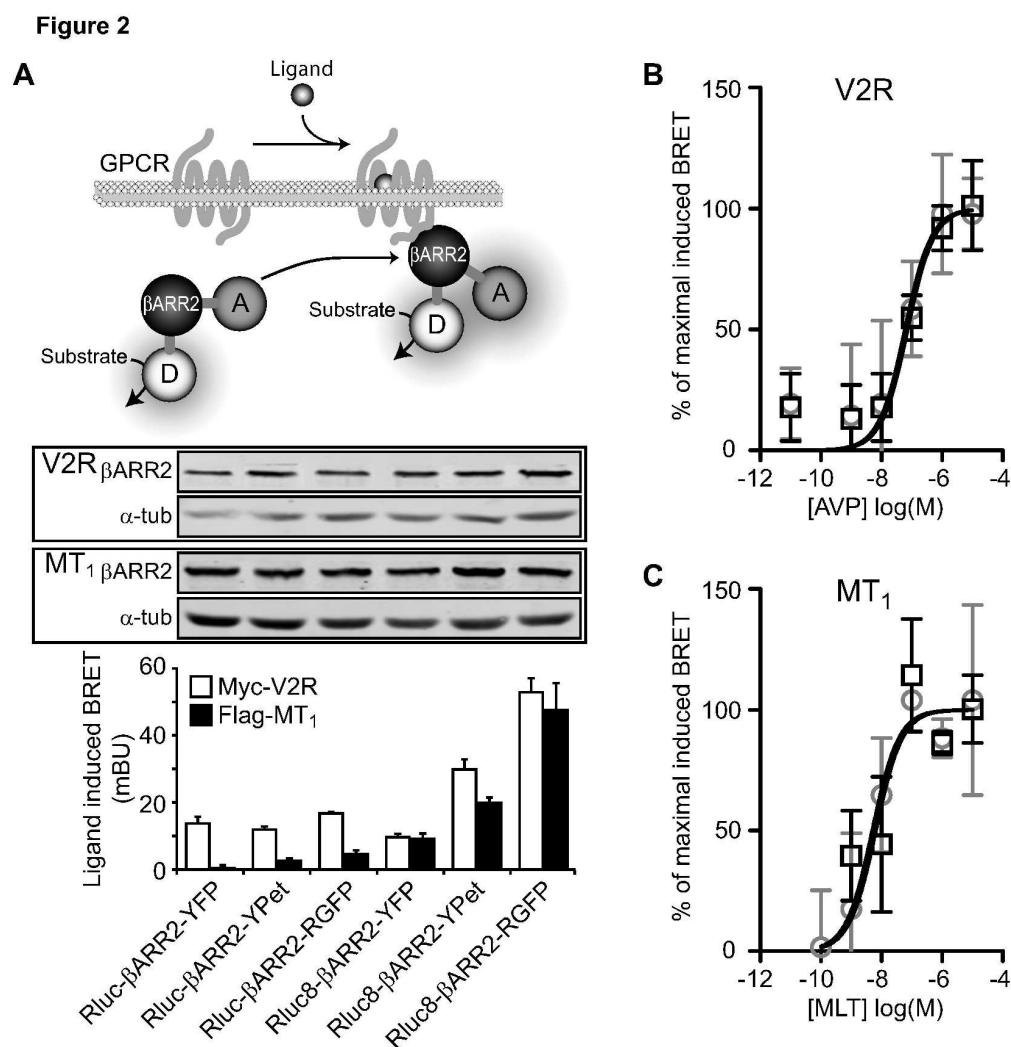


Figure 2: Monitoring of ligand-induced β ARR2 intramolecular rearrangement. (A) Bar Charts showing the intramolecular rearrangement of the indicated β ARR2 double-brilliance constructs upon recruitment to agonist activated V2R (\circ) or MT₁ (\triangleleft) in HEK 293T cells (15 min stimulation with agonist); $n=4$ independent experiments. (B) AVP dose-response curves showing for N-Rluc8- β ARR2-YPet-C (γ) and N-Rluc8- β ARR2-RGFP-C (\circ) ($n=2$). (C) Melatonin dose-response curves for N-Rluc8- β ARR2-YPet-C (γ) and N-Rluc8- β ARR2-RGFP-C (\circ) ($n=2$). Expression of β ARR2 was verified by western blotting using anti- β ARR antibodies. Further experimental details and controls are shown in Supplementary Fig. 1. A, acceptor; C, C-terminus; D, donor; N, N-terminus; α -tub, α -tubulin.

138x144mm (600 x 600 DPI)

Figure 3

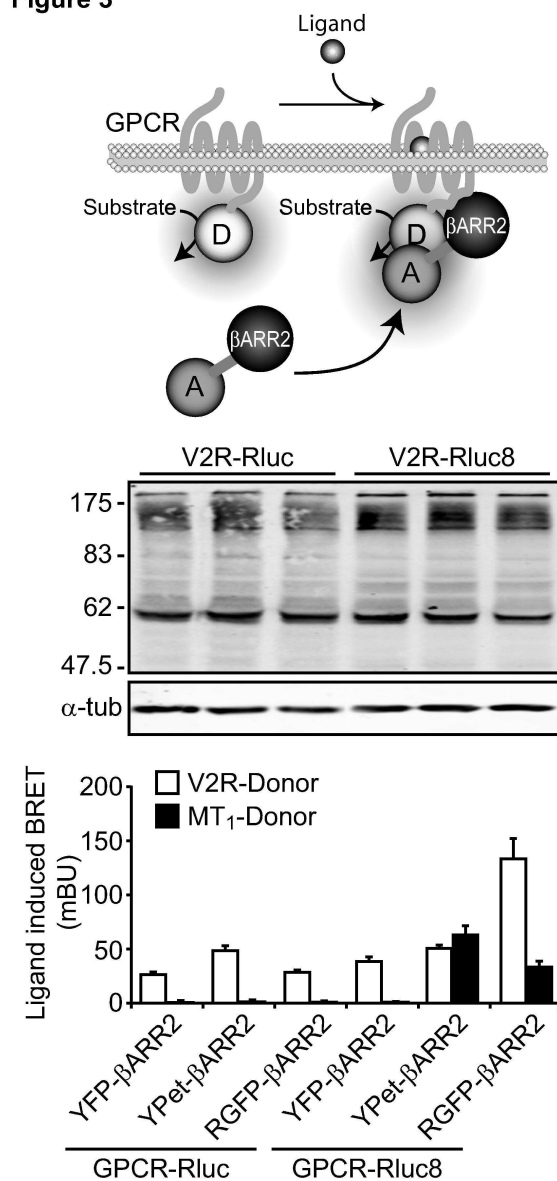


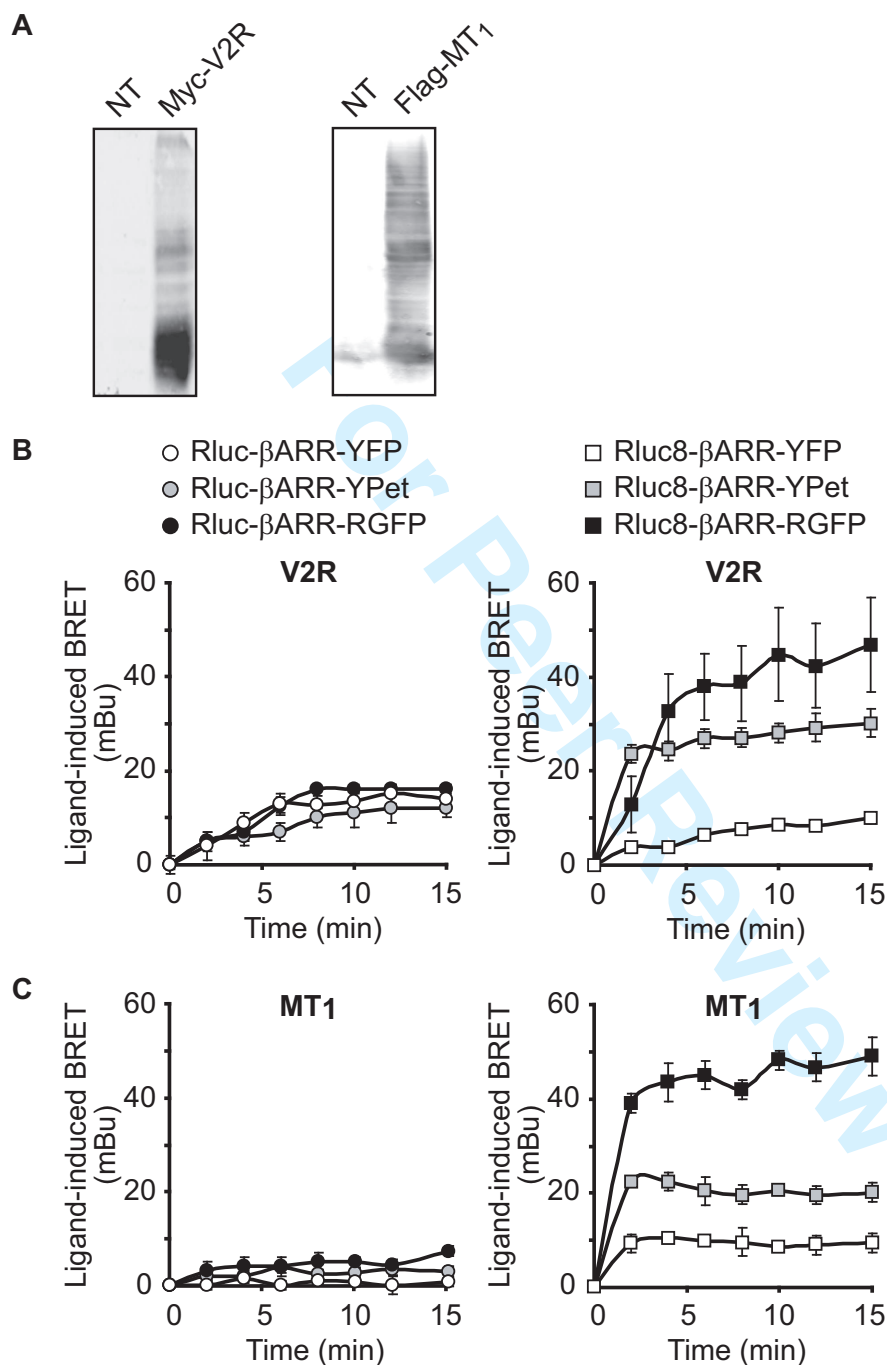
Figure 3: BRET measured with the β ARR recruitment assay. Agonist-induced recruitment of the indicated β ARR2 fusion constructs to V2R-Rluc or V2R-Rluc8 (\circ) (10 μ M AVP, 5 min), and MT1-Rluc or MT1-Rluc8 (\blacktriangleleft) (10 μ M melatonin, 5 min). Expression of V2R-Rluc and V2R-Rluc8 was verified by western blot using anti-Rluc antibodies (that recognize Rluc and Rluc8 equally well, data not shown). Ligand-induced BRET was defined as the difference between BRET_{max} values obtained from donor saturation curves +/- ligand. Saturation curves and further controls are shown in Supplementary Fig. 2; α -tub, α -tubulin. n=4 independent experiments. 71x155mm (600 x 600 DPI)

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7 **Supplementary Figures:**
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10 **Supplementary Fig. 1:** Supplementary data of donor/acceptor tandem fusion protein BRET assay and
11 double-brilliance β ARR2 biosensors shown in Fig. 1. (A) Expression of Myc-V2R and Flag-MT₁ in Fig.
12 1B was verified by western blot using anti-Myc and anti-Flag antibodies. (B) Kinetics of AVP- and
13 melatonin-promoted BRET changes of the indicated double-brilliance β ARR2 biosensors in HEK 293T
14 cells. AVP (V2R agonist) and melatonin (MT₁ agonist) were used at 10 μ M. NT, non-transfected.
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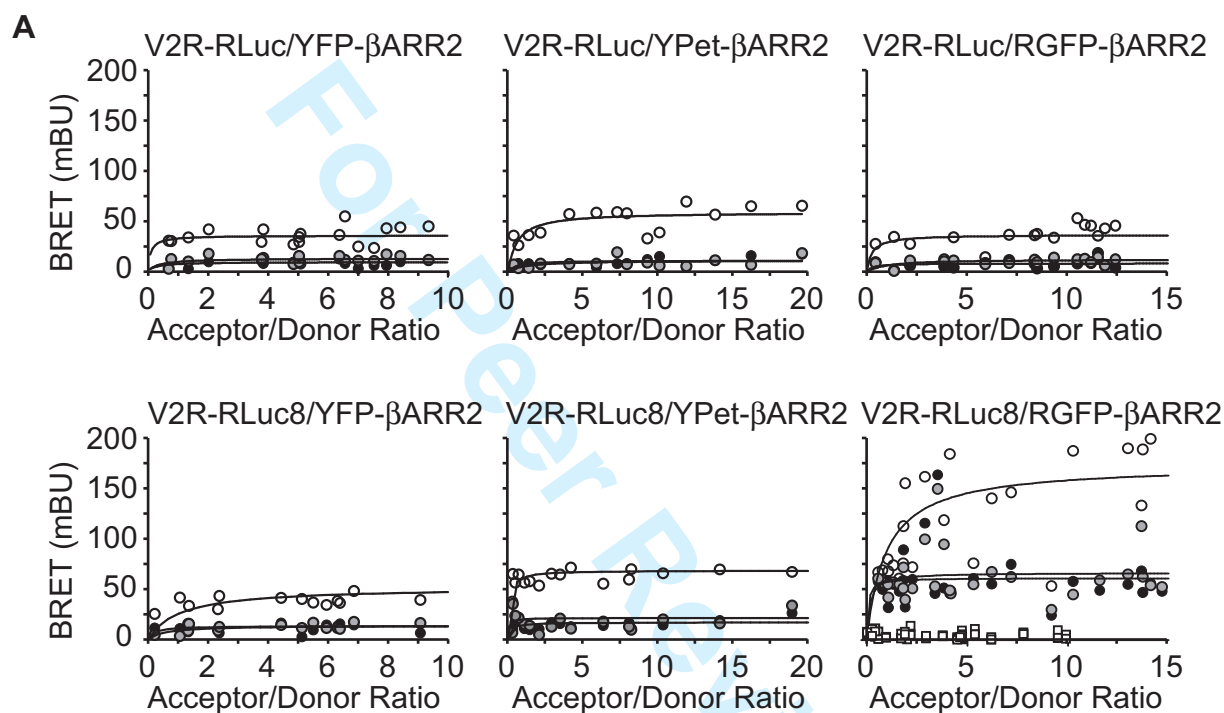
Supplementary figure 1



Supplementary Fig. 1: Supplementary data of donor/acceptor tandem fusion protein BRET assay and double-brilliance β ARR2 biosensors shown in Fig. 1. (A) Expression of Myc-V2R and Flag-MT1 in Fig. 1B was verified by western blot using anti-Myc and anti-Flag antibodies. (B) Kinetics of AVP- and melatonin-promoted BRET changes of the indicated double-brilliance β ARR2 biosensors in HEK 293T cells. AVP (V2R agonist) and melatonin (MT1 agonist) were used at 10 μ M. NT, non-transfected.

Supplementary Fig. 2: Supplementary data of the β ARR2 recruitment assay shown in Fig. 2. BRET donor saturation curves for V2R-RLuc and V2R-RLuc8 were generated in HEK 293T cells in the presence of different N-Acceptor- β ARR2-C fusion constructs. BRET was measured 5 min after ligand addition. BRET was measured in the absence and presence of the V2R agonist AVP and V2R antagonist Vd(CH₂)₅[D-Tyr(Et)₂, Ile₄, Eda₉]AVP. The absence of ligand-induced BRET in the presence of the antagonist demonstrates the specificity of the assay. Basal condition (●); 0.1 μ M Vd(CH₂)₅[D-Tyr(Et)₂, Ile₄, Eda₉]AVP (●); 0.1 μ M AVP (○); soluble RGFP (□).

Supplementary figure 2

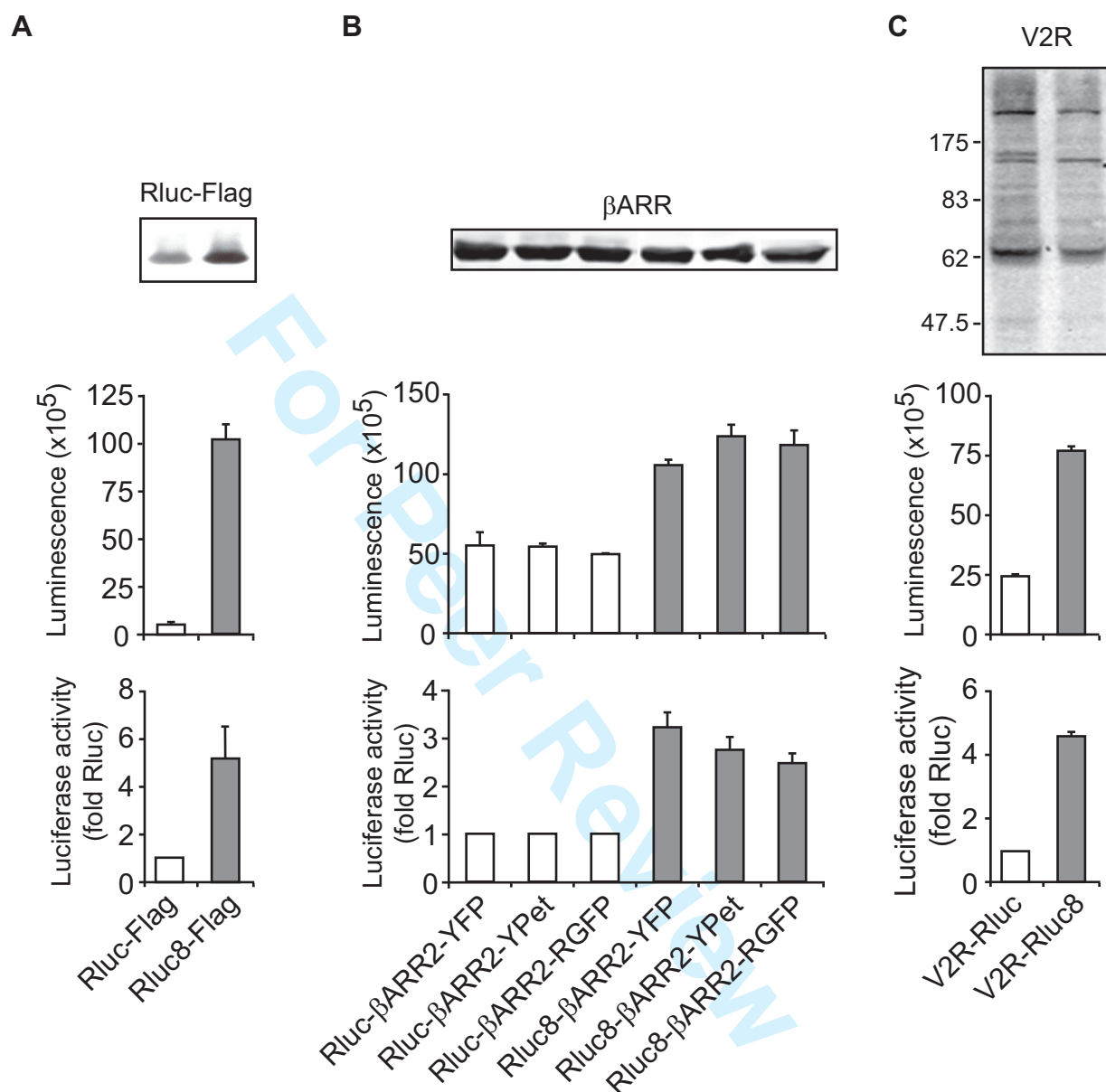


Supplementary Fig. 2: Supplementary data of the β ARR2 recruitment assay shown in Fig. 2. (A) BRET donor saturation curves for V2R-RLuc and V2R-RLuc8 were generated in HEK 293T cells in the presence of different N-Acceptor- β ARR2-C fusion constructs. BRET was measured 5 min after ligand addition. BRET was measured in the absence and presence of the V2R agonist AVP and V2R antagonist Vd(CH₂)₅[D-Tyr(Et)₂, Ile₄, Eda₉]AVP. The absence of ligand-induced BRET in the presence of the antagonist demonstrates the specificity of the assay. Basal condition (●); 0.1 μ M Vd(CH₂)₅[D-Tyr(Et)₂, Ile₄, Eda₉]AVP (●); 0.1 μ M AVP (○); soluble RGFP (□).

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7 **Supplementary Fig. 3:** Comparison of emitted light intensities of Rluc and Rluc8 fusion proteins. Rluc
8 (A) and the indicated β ARR2 double-brilliance (B) and V2R (C) constructs were expressed in HEK 293T
9 cells. Luciferase activity was determined in the presence of 5 μ M coelenterazine h (middle panels) and
10 another aliquot submitted to western blot analysis (upper panels) using anti-Flag (A), anti- β ARR (B) and
11 anti-Rluc (C) antibodies. Protein bands were quantified and luciferase activity of Rluc8 fusion proteins
12 expressed as % of the corresponding Rluc fusion protein (lower panels).
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Supplementary figure 3



Supplementary Fig. 3: Comparison of emitted light intensities of Rluc and Rluc8 fusion proteins. Rluc (A) and the indicated β ARR2 double-brilliance (B) and V2R (C) constructs were expressed in HEK 293T cells. Luciferase activity was determined in the presence of 5 μ M coelenterazine h (middle panels) and another aliquot submitted to western blot analysis (upper panels) using anti-Flag (A), anti- β ARR (B) and anti-Rluc (C) antibodies. Protein bands were quantified and luciferase activity of Rluc8 fusion proteins expressed as % of the corresponding Rluc fusion protein (lower panels).