Supporting Information for:

NanoBRET – a Novel BRET Platform for the Analysis of Protein-Protein Interactions

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Materials

Materials: The cDNA's for FKBP Frb (amino acids 2019-2112 of mTOR, , β-arrestin2 and Vasopressin V2 receptor, CBP andBromodomain containing protein 4 (BRD4) were obtained from the Kazusa clone collection (Kazusa DNA Research Institute)The vectors for TurboYFP (pTurboYFP-N) and TagRFP (pTagRFP-N) were purchased from Evrogen (US distributor Axxora). The cDNA's for Rluc8, Histone H3.3, Histone H4 and all BAZ2A truncations were generated by full gene synthesis (Gene Dynamics or Integrated DNA Technologies). The expression vectors pFN31K,, pFC27K, pFN28K, pFC14K and PF5A-PGK were from Promega. *E. coli* strain KRX (Promega) [F´, *tra*D36, Δ*omp*P, *pro*A+B+, *lac*Iq, Δ(*lac*Z)M15] Δ*omp*T, *end*A1, *rec*A1, *gyr*A96 (Nalr), *thi*-1, *hsd*R17 (rk–, mk+), e14– (McrA–), *rel*A1, *sup*E44, Δ(*lac-pro*AB), Δ(*rha*BAD)::T7 RNAPolymerase] was from Promega. All chemicals and ligands used were from Sigman-Aldrich unless otherwise noted. All HaloTag ligands, coelenterazine h, and furimazine were from Promega. Dulbecco's modified eagle medium (DMEM), Opti-MEM®, Penicillin/Streptomycin and Trypsin-EDTA were from Life Technologies (Carlsbad). Fetal calf serum (FBS) was from HyClone (GE Healthcare). Microtiter plates were purchased from Corning.

Cell Culture and Transfection: HeLa and HEK293 cells were maintained in DMEM containing 0.3 mg/ml glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin and 10% fetal calf serum at 37°C in 5% CO_2. Transient transfections were performed using two different protocols as indicated in the results section. *Standard transfections* were carried out using Fugene HD transfection reagent (Promega) according to the manufacturer's instructions. *Reverse transfections* were carried by adding the DNA transfection mix to a cell suspension prior to seeding of cells into assay plates. Cells were harvested using Trypsin-EDTA and adjusted to $2x10^5$ cells per ml in growth medium. For each 96-well plate 500 μl 20x transfection mix was prepared by adding 5 μg DNA to 500 μl of Opti-MEM followed by addition of 15 μl of Fugene HD (Promega). Cell suspension and transfection mix were mixed at a ratio of 20:1 and plated

followed by incubation for 24 h. For serial DNA titrations the total DNA concentration was adjusted for each transfection to 50 ng DNA per well using the promoter-less pGEM3Z vector as carrier DNA.

Plasmid construction

All fusion protein expression vectors used for the protein-protein interaction models (supplementary table 1) in this study were generated using different members of Flexi vector family (see Promega website for vector sequences) designed for expression of fusion proteins in mammalian cells. The vectors for the generation of N and C-terminal Nluc and HaloTag fusions were from Promega .Vectors for the generation of Rluc8 and turboYFP fusions were generated by replacing the cDNA of Nluc in pFN31 or pFC32 with the cDNA of either Rluc8 or turboYFP. All mammalian expression vectors used in this study originate from a common vector (pF5a-CMV) and use the same CMV promoter to control expression. Different alphanumeric designations indicate the nature and positioning of the reporter gene used for generating the fusion (see supplementary table 1 for details, pFNC14 is identical to pFNC27 with the exception of a Neo^R expression cassette for stable cell generation). Length of the Gly-Ser based linker varied from 16 to 17 amino acids between gene of interest and reporter gene depending on reporter gene and positioning.

Nluc-HaloTag, NLuc-turboYFP and Nluc-tagRFP direct fusions were created in a modified pF5A vector containing a human PGK promoter. These direct fusions were generated using a short 4 amino acid long linker (GSSG) to keep donor and acceptor in close proximity.

All fusion constructs were generated using standard PCR or restriction cloning methods and were verified by sequencing.

Preparation of recombinant proteins: 50 ml cultures (LB) of E.coli (KRX) expressing the protein of interest were prepared by auto-induction[1,2]. Cells were harvested, suspended in 10 ml buffer (50 mM HEPES (pH 7.5), 500 mM NaCl, 10 mM Imidazole) containing protease inhibitor cocktail (Promega,

1Xfinal), lysozyme (Sigma, 0.5 mg/ml final) and RQ1 DNase (Promega, 250U) and incubated for 30 minutes on ice. The suspension was sonicated (Fisher #FB-705 with a microtip (#418-21) at amplitude 20 for 2 minutes (5 sec pulse)) on ice and clarified by centrifugation (15,000xg for 15 min at 4^{o}C). The cleared lysate was purified using HisLink resin (Promega) following the manufacturers recommendations.

Luciferase assay: 100 µl of 0.4 nM recombinant Nluc, Rluc or Rluc8 in PBS + 0.1% BSA (Sigma-Aldrich) were plated in a white 96-well plate (n=3). Immediately after addition of 100 µl PBS containing furimazine (Nluc) or coelenterazine h (Rluc/Rluc8) at a final concentration of 10 µM, the samples were analyzed in a GloMax Multi+ plate reader (Promega) using an integration time of 0.5 sec. For time course experiments luciferase activity was measured every 60 seconds for 1h.

Fluorescence and BRET Spectra: Spectral analysis was performed using a Tecan Infinite M1000 PRO plate reader (spectral scanning function, bandwidth 20 nm, 2 nm step size). The excitation and emission spectra of HaloTag Oregon green Ligand, HaloTag TMR ligand and HaloTag NCT ligand were measured at a concentration of 125 nM in PBS. Excitation spectra were obtained using a fixed emission at 560 nm (Oregon Green), 650 nm (TMR) or 680 nm (NCT). Emission spectra were obtained using a fixed excitation at 445 nm (Oregon green) or 510 nm (TMR, NCT). Luminescent emission spectra were measured for Nluc-HT alone or in presence of the indicated HaloTag ligand at a final Nluc concentration of 2 nM in PBS + 0.1% BSA. All spectra were normalized to their respective maxima (=1).

Bromodomain/Histone NanoBRET assays: HEK293 cells (8 x 10^5) were plated in each well of a 6-well plate and co-transfected with an acceptor vector, Histone H3.3-HT or Histone H4-HT, and one donor vector from the following list; Nluc-BRD4, Nluc-CBP. Twenty hours post-transfection cells were

collected, washed with PBS, and exchanged into media containing phenol red-free DMEM and 4% FBS in the absence (control sample) or the presence (experimental sample) of 100 nM HaloTag NCT Ligand. Cell density was adjusted to 2 x 10^5 cells/ml and then plated into white 96-well tissue culture plates (Corning Costar #3917). IBET-151 was then added directly to media at final concentrations between 0-10 μM and the plates were incubated for 18 h at 37 ^oC in the presence of 5% CO2. Furimazine substrate was added to both control and experimental samples at a final concentration of 10 μM. Readings were performed within 5 minutes using the CLARIOstar (BMG LABTECH) equipped with a 460/80 nm band-pass filter (donor, Nluc) and a 610 nm long-pass filters (acceptor, HaloTag NCT ligand) using an integration time of 0.5 sec. For data representation mBRET units were calculated (for definition of mBRET unit see data presentation section).

Western blotting: HEK293 cells were reverse transfected with a serially diluted mixture of Frb-Nluc and FKBP-HT or Frb-Rluc8 and FKBP-TurboYFP (DNA ratio 1:1, all samples were adjusted to the same total amount of DNA by adding carrier DNA) and plated in 6-well plates. After 24 h incubation in a tissue culture incubator the cell were harvested and washed once in PBS. Lysates were prepared by incubating the cell pellet in 100 μl mammalian lysis (Promega, 50 mM Tris-HCl, 150 mM NaCl, 1% Triton® X-100 and 0.1 % sodium deoxycholate, pH 7.5) buffer for 30 min on ice, centrifugation (10 min at 15000 rpm) and transfer of the supernatant to a fresh micro-tube. Samples were separated on a 4-12 % SDS PAGE and transferred to a PVDF membrane using the iBLOT system (Life Technologies) according to manufacturer's instructions. The blots were developed using an anti-FKBP12 mAb (Abcam) following manufacturers recommendations.

BRET Imaging: All imaging experiments were performed using the Olympus LV200 bioluminescence microscope (Olympus) equipped with a heated stage. HeLa cells were reverse transfected with

expression constructs for the indicated Nluc and HaloTag fusion proteins (DNA ratio 1:5) and were plated into 35 mm glass bottom dishes (MatTek Corporation) in 2 ml growth medium (DMEM supplemented with 10 % FBS) and incubated for 24 h in a tissue culture incubator. The growth medium was then replaced with labeling medium (Opti-MEM, supplemented with 250 nM HaloTag NCT Ligand) and incubated for 60 minutes (Frb-Nluc/FKBP-HT) or 4 h (Nluc-β-arr2/AVPR-HT, Nluc-grb2/EGFR-HT) at 37°C. The longer incubation period for the b-arr2/AVPR2 and grb2/EGFR models is required in order to reduce basal interaction levels by serum starvation. After replacement of labeling medium with assay medium (Opti-MEM supplemented with 10 μM furimazine) the sample was placed in the instrument. A suitable field of view was identified based on imaging of total luminescent signal of the donor fusion. All images were acquired using a 150x/1.4 UPLanSApo (Nluc-β-arr2/AVPR2-HT) or 60x objective (Frb-Nluc/FKBP-HT, Nluc-grb2/EGFR-HT) and Olympus CellSens software. To image BRET events, images of donor and acceptor emission were acquired sequentially using a 460/80 bandpass filter and a 590 nm long-pass filter respectively. After establishing a baseline for the sample in absence of stimulus, either 1 μM arginine vasopressin (AVP, Nluc-β-arrestin2/AVPR2-HT), 500 nM rapamycin (Frb-Nluc/FKBP-HT) or 100 ng/ml EGF (Nluc-grb2/EGFR-HT) was added by injection. Image acquisition continued for additional 60-80 cycles. BRET ratios for individual regions of interest representing individual cells were calculated using Image J software. Both channels were first converted into a single image stack followed by determination of the mean intensity for a ROI covering individual cells. Channel specific background values were obtained from a region of equal size without cells and subtracted for both channels. The acceptor/donor ratio was calculated using background corrected values for donor and acceptor and plotted against time.

***Data Presentation and Statistical Analysis*.** Data were analyzed using MS Excel 2010 and Prism 6 graphing software (GraphPad). The results are represented using two units, the acceptor / donor channel emission ratio and BRET ratio. The acceptor/ donor channel emission ration represents the ratio of the

raw signals measured for the acceptor and donor channels respectively. In this regard it is important to remember that the total signal measured in the acceptor channel consist of two unrelated components. The first component is the signal emitted by the acceptor fluorophore which results from resonance energy transfer between the donor and acceptor. The second component originates directly from the donor emission due to the overlap between donor spectrum and the filter setting used for the acceptor channel. Because the ratio of the donor emission measured in the donor and acceptor channels is constant, it can be subtracted from the raw acceptor/donor emission ratio to obtain the BRET ratio. For this reason it should be considered good practice to include a control sample that includes only the donor expression construct. The acceptor/donor emission ratio obtained from this "donor only" sample consists solely of donor luminescence and can be used to correct the measurements made in the acceptor channel.

Acceptor / donor channel emission ratio (A/D channel emission ratio)

The acceptor and donor channels are indicated by their respective filter wavelengths (e.g. 610/450 emission ratio) The A/D channel emission ratio is calculated by dividing the raw emission values measured for the acceptor and donor channel respectively:

$$A/D\ channel\ emission\ ratio = \frac{Acceptor\ channel\ emission}{Donor\ channel\ emission}$$

BRET ratio and milliBRET unit (mBRET):

BRET ratios are calculated by subtracting the A/D channel emission ratio of the donor only control from the A/D channel ratio of the sample using the following equation:

$$BRET\ ratio = \frac{Acceptor\ channel\ emission\ of\ sample}{Donor\ channel\ emission\ of\ sample} - \frac{Acceptor\ channel\ emission\ of\ donor\ only}{Donor\ channel\ emission\ of\ donor\ only}$$

mBRET units were calculated as follows:

$$mBRET\ unit = BRET\ ratio * 1000$$

Signal to background ratio (S/B ratio):

S/B ratios were calculated using A/D channel emission ratio values obtained for treated and untreated sample (control):

$$SB\ ratio = \frac{A/D\ emission\ ratio\ of\ treated\ sample - A/D\ emission\ ratio\ of\ control}{A/D\ emission\ ratio\ of\ control}$$

Z'factor

The Z' factor was calculated using the following equation[11]:

$$Z' = 1 - 3 \times \frac{(SD\ of\ sample + SD\ of control)}{(mean\ of\ sample - mean\ of control)}$$

Accession codes.

FKBP (NM_000801), Frb (amino acids 2019-2112 of MTOR, NM_004958), β-arrestin2 (ARRb2, NP_004304) ,Vasopressin V2 receptor (AVPR V2, NP_000045,) , CBP (Q92793), Bromodomain containing protein 4 (BRD4, NP_490597), Histone H3.3 (NM_002107), Histone H4(NM_175054.2).

Supporting References

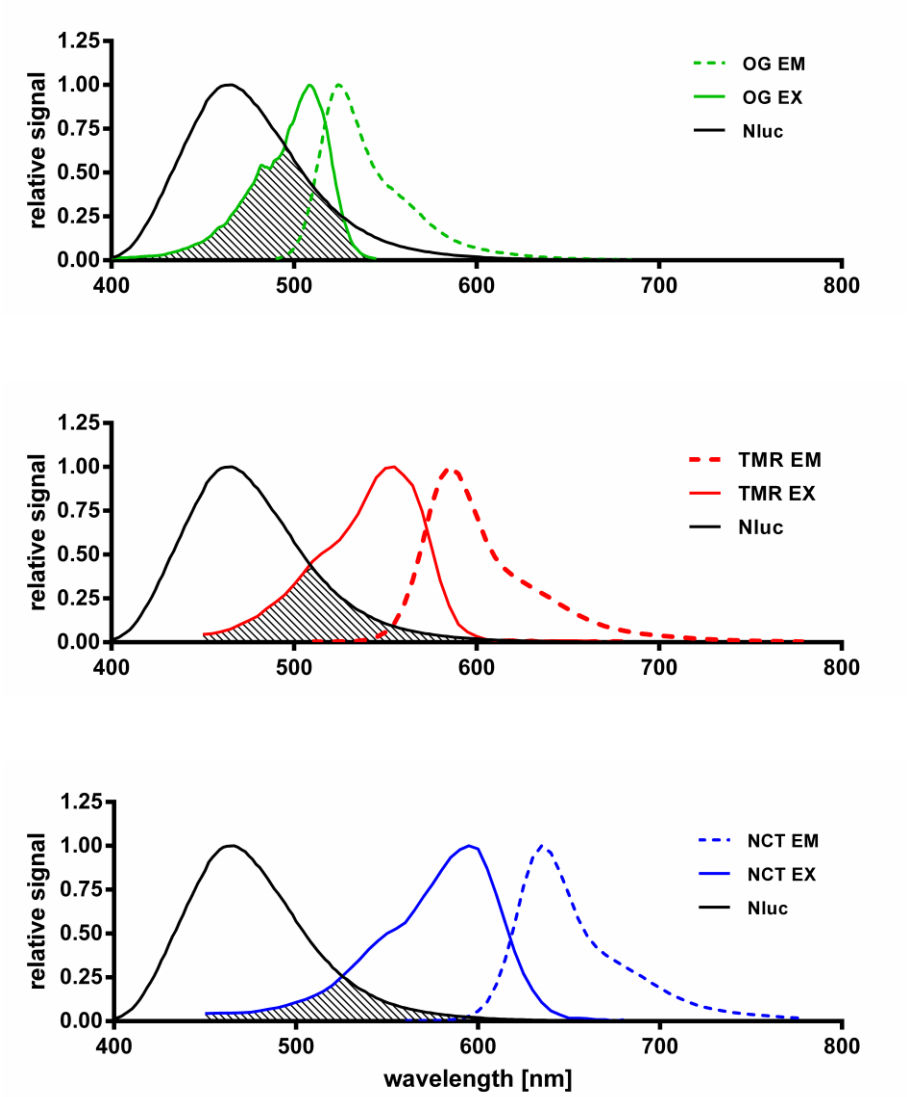
1. Hartnett, J., Gracyalny, J. and Slater, M.R. (2006) The Single Step (KRX) Competent Cells: Efficient cloning and high protein yields. Promega Notes 94, 27–30.

2. Schagat, T. et al.(2008) KRX autoinduction protocol: A convenient method for protein expression. Promega Notes 98, 16–18

Supporting Table 1: Expression constructs

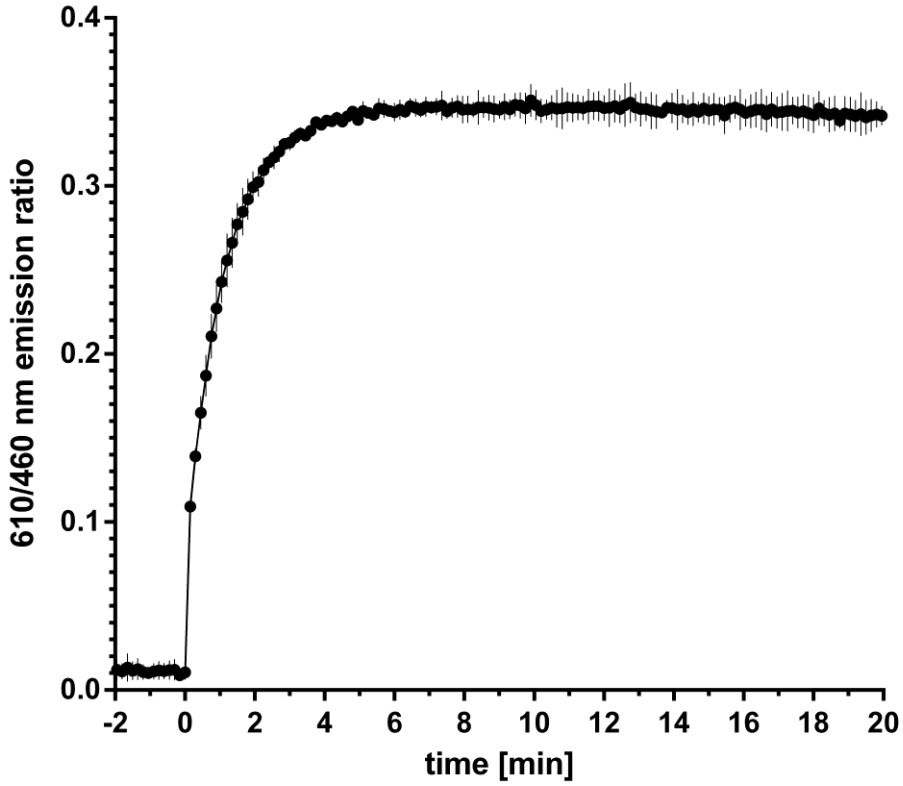
fusion tag (attachment point)	vector name	promotor	Fusion partner
HaloTag (C-terminal)	pFC27K	CMV	FKBP / Frb
HaloTag (N-terminal)	pFN28K	CMV	FKBP / Frb
Nluc / Rluc8 / turboYFP (C-terminal)	pFC31K	CMV	FKBP / Frb
Nluc / Rluc8 / turboYFP (N-terminal)	pFN31K	CMV	FKBP / Frb
HaloTag (C-terminal)	pFC27K	CMV	AVPR2
Nluc (N-terminal)	pFN31K	CMV	b-arrestin2
HaloTag (C-terminal)	pFC27K	CMV	EGFR
Nluc (N-terminal)	pFN31K	CMV	grb2
HaloTag (C-terminal)	pFC14K	CMV	Histone H3.3 / H4
Nluc (N-terminal)	pFN31K	CMV	Brd4, CBP
HaloTag (C-terminal)	pFC14K	CMV	Histone H3.3
Nluc / HaloTag/ turboYFP / tagRFP	pF5A	PGK	direct fusions between Nluc and HaloTag / turboYFP / tagRFP

Supporting Figure S1a:



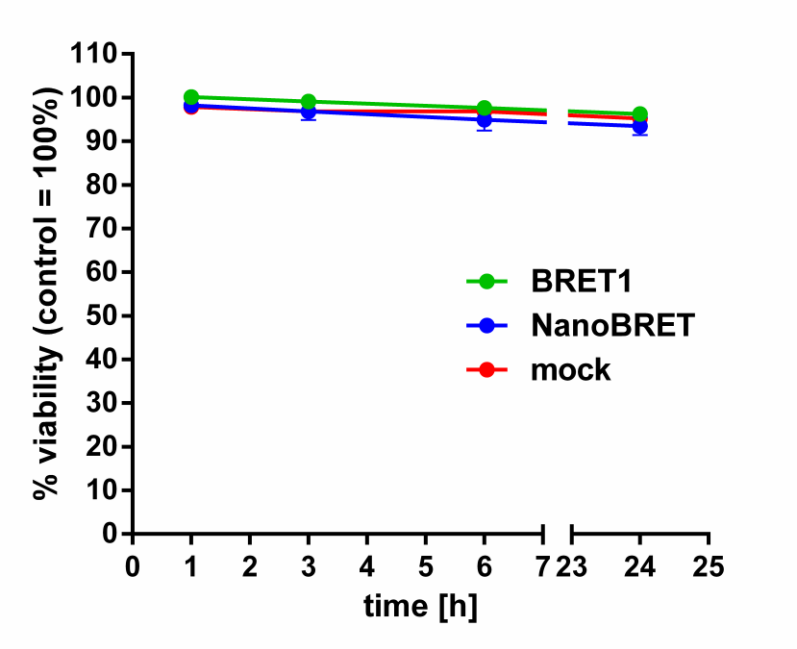
Normalized excitation (solid line) and emission (dotted line) spectra for Halotag Oregon Green (green), HaloTag TMR (red) and HaloTag NCT (blue) ligands. Each spectral profile also contains the emission spectrum for Nluc (black line).

Supporting Figure S1b:



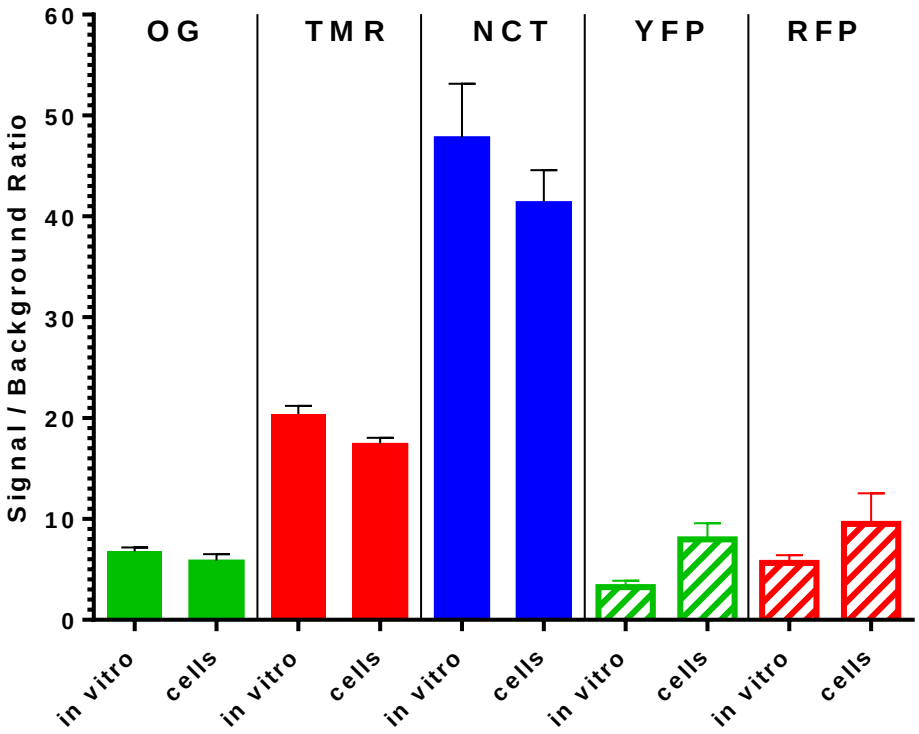
Kinetic of HaloTag NCT ligand labeling in cells: HEK293 cells were reverse transfected with Nluc-HaloTag fusion expression construct and plated in white 96-well tissue culture plates at a density of 20000 cells per well in 100 μl growth medium and incubated for 24h at 37°C. After replacing growth medium with 100 μl assay medium (Opti-MEM supplemented with 10 μM furimazine) the samples were placed in a BMG CLARIOstar multimode plate reader. In order to determine the labeling kinetics of HaloTag NCT ligand continuous BRET measurements were taken for a period of 22 minutes. After establishing the donor-only background baseline for 2 minutes, 100 μl labeling medium (Opti-MEM supplemented with 10 μM furimazine and 500 nM HaloTag NCT ligand) was added to each sample using on board injectors, followed by additional 20 minutes of BRET measurements. Shown are the means ± SD of a representative experiment performed in triplicate.

Supporting Figure S2



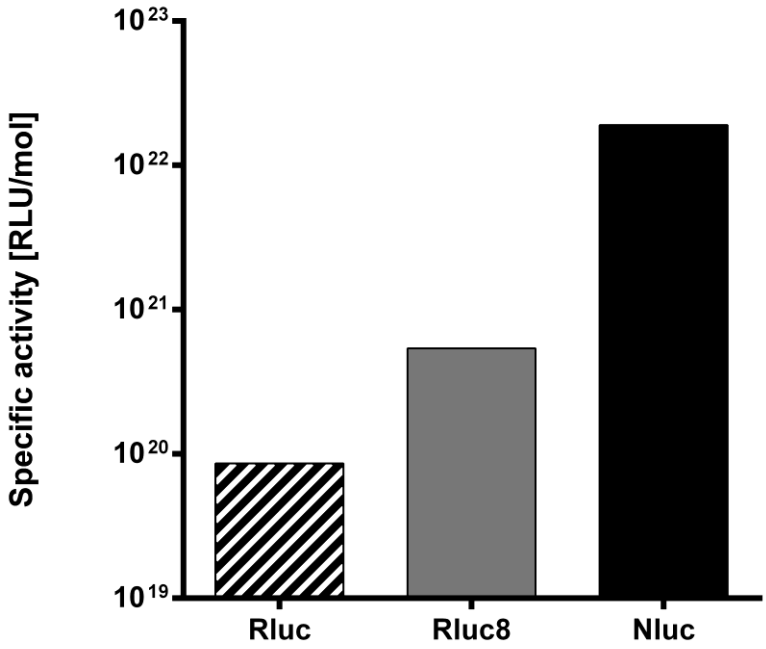
The effect of the BRET1 and NanoBRET assay systems on cell viability: HEK293 cells were left untreated (control) or reverse transfected with the expression constructs for Frb-Rluc8 and FKBP-turboYFP (BRET1), Frb-Nluc and FKBP-HT (NanoBRET) or carrier DNA (mock). Following transfection the cells were plated in 10 cm tissue culture dishes in 10 ml growth medium (DMEM supplemented with 10% FBS) and incubated for 24h. The cells were then harvested and plated in a black 96 well tissue culture plate in 100 µl growth medium at a density of 20000 cells per well and incubated for 24 h. The growth medium was then replaced with 100 µl Opti-MEM medium (control, mock) or Opti-MEM medium supplemented with either 10 µM coelenterazine h (BRET1) or 250 nM HaloTag NCT-ligand / 10 µM furimazine (NanoBRET). All media also contained CellTox Green reagent at a dilution of 1:500 (following manufacturer's recommendations). Changes in cell viability were then measured for 24h using the CellTox Green cytotoxicity assay (Promega) according to manufacturer instructions. All fluorescence measurements were performed on a BMG CLARIOstar multimode plate reader (BMG). The data are plotted as % viability of BRET1, NanoBRET or mock transfected cells relative to untreated cells (control). Shown are the means ± SEM of 4 independent experiments performed in triplicate.

Supporting Figure S3:



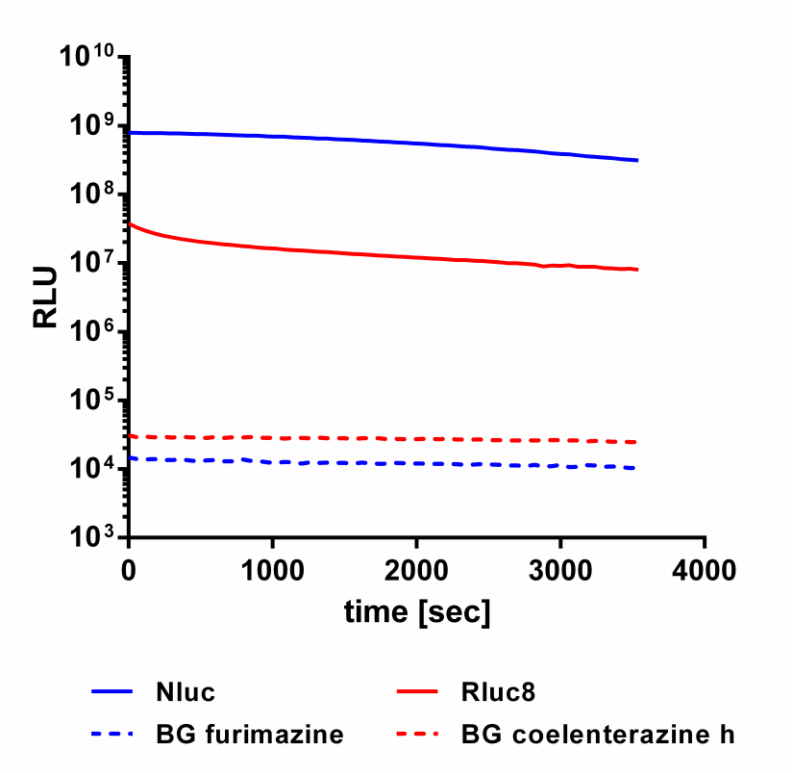
The dynamic range of different NLuc-acceptor combinations was determined in biochemical and cell based assay formats by comparing the BRET ratio of Nluc against indicated Nluc fusions. The donor channel was measured using a 450/80 bandpass filter. The acceptor channel was measured using either a 530 nm longpass (LP) filter (Nluc-HT-Oregon Green, Nluc-TurboYFP), 590 nm LP filter (Nluc-HT-TMR, Nluc-TagRFP) or a 610 nm LP filter (Nuc-HaloTag-NCT). The signal/background ratio was calculated as outlined in the material and methods section.

Supporting Figure S4A



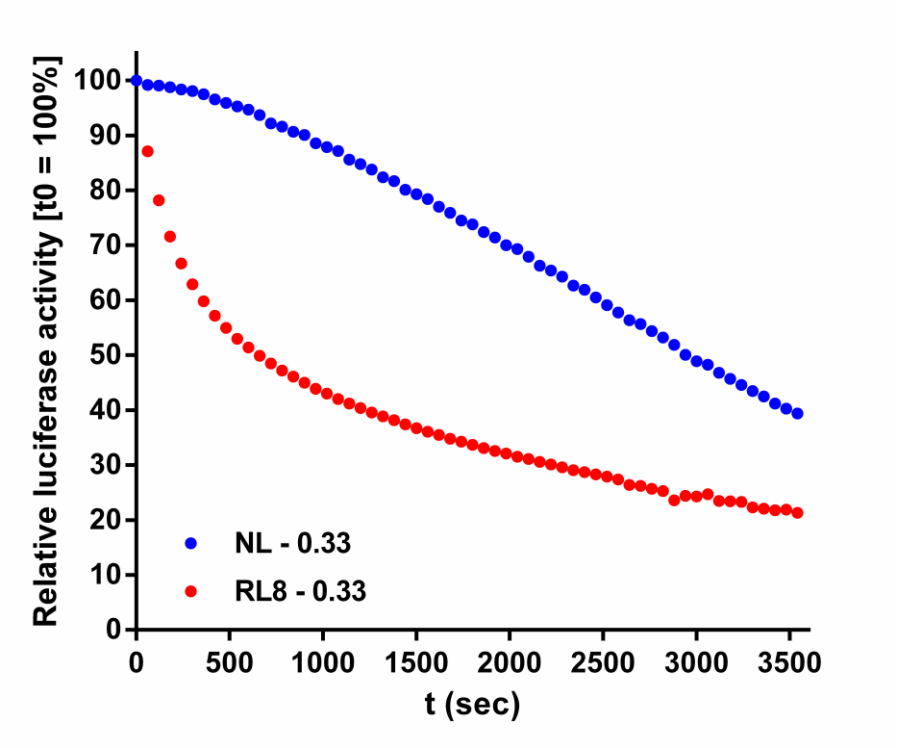
Specific Luciferase activity measured in vitro for Nluc, Rluc8 and Rluc recombinant protein in PBS + 0.1% BSA.

Supporting Figure S4B



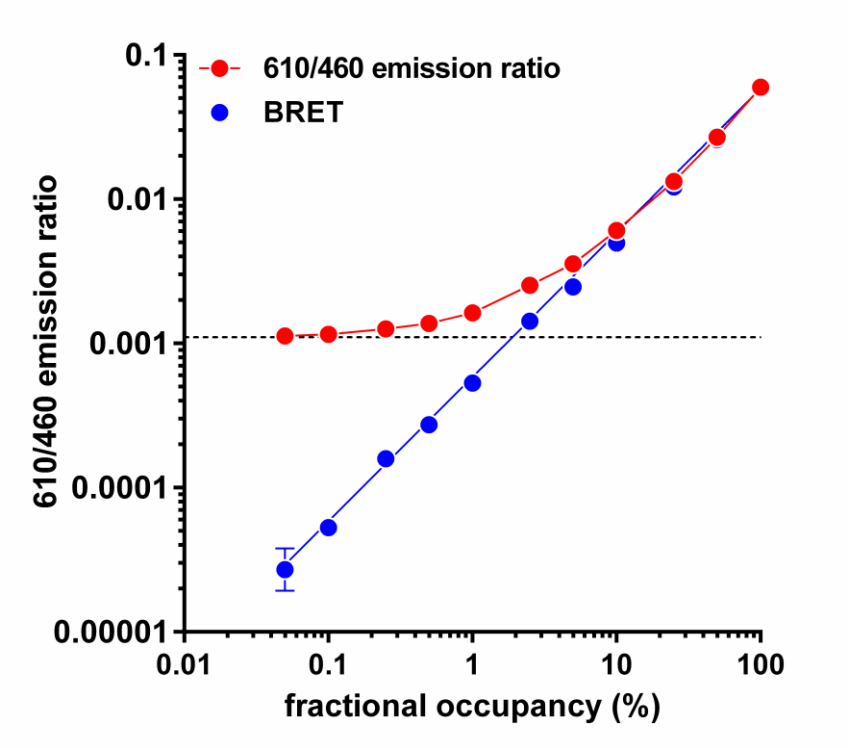
Comparison of Nluc (solid blue line) or Rluc8 (solid red line) kinetic in vitro. The dashed lines represent the chemoluminescent background signal emitted by samples containing 10 μM furimazine (dashed blue line) or coelenterazine h (dashed red line) in the absence of luciferase.

Supporting Figure S4C



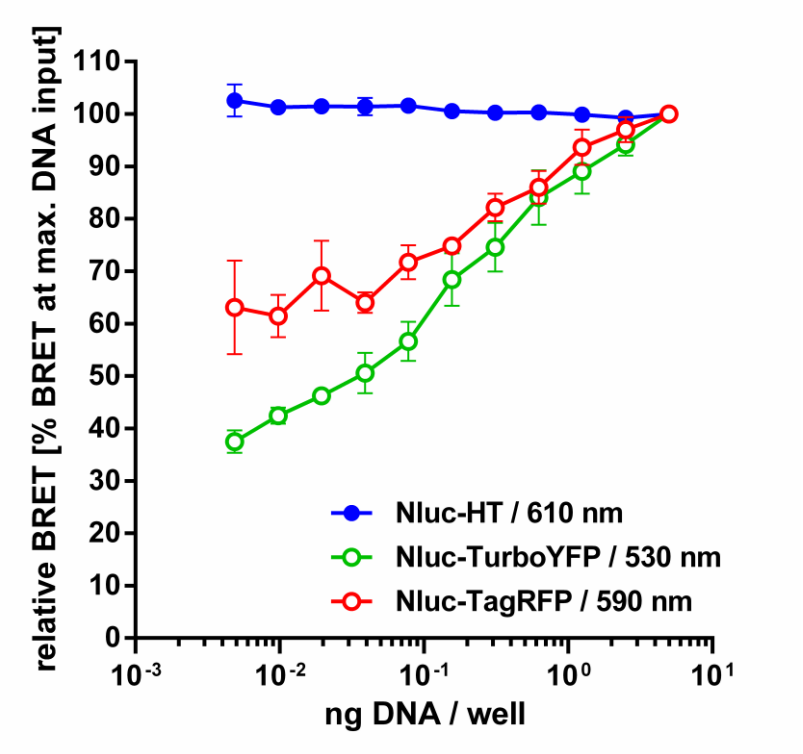
Normalized kinetic of Nluc (blue line) or Rluc8 (red line) activity (derived from data shown in figure S4B).

Supporting Figure S5



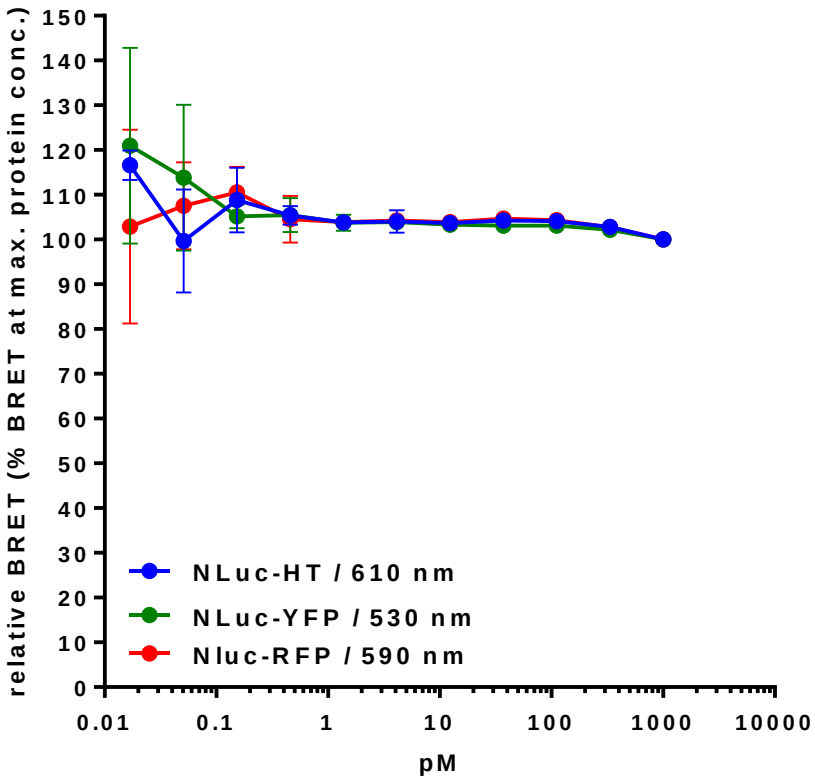
Comparison of acceptor/donor channel emission ratio (610/460 emission ratio) and BRET ratio of the fractional occupancy assay shown in figure 2B: The 610/460 emission ratio represents the ratio of the raw acceptor and donor channel emission signals. The apparent non-linear nature of 610/460 emission ratio (red line) is explained by the composite nature of the signal measured in the acceptor channel signal, which consists of the energy transfer dependent emission of the acceptor and the energy transfer independent “cross-over” emission of the donor due to the spectral overlap between donor and acceptor channel filter settings. The donor cross-over emission in the acceptor channel is directly proportional to total donor emission and adds therefore a constant to the acceptor/donor channel emission ratio. Subtracting the “donor only” emission ratio from the acceptor/donor channel emission ratio yields the actual BRET ratio (blue line) which exhibits the expected linear relationship between fractional occupancy and BRET.

Supporting Figure S6



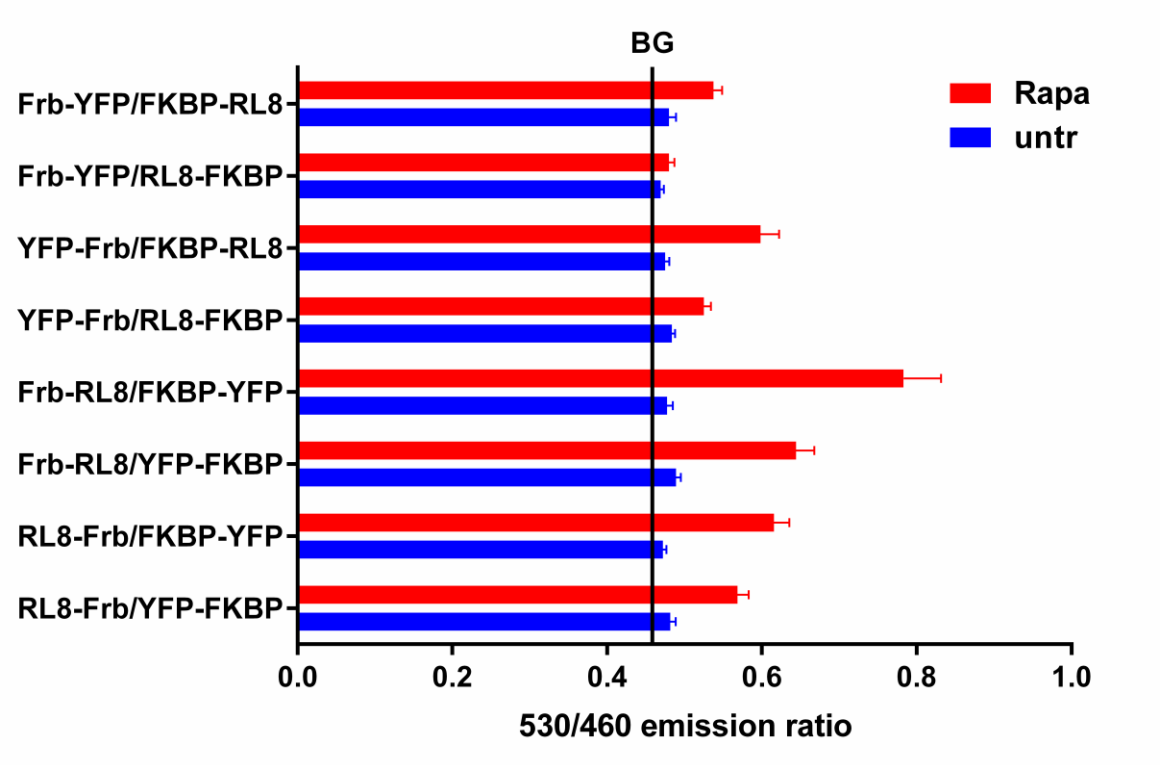
Effect of protein expression level on BRET for different Nluc-acceptor fusions: HEK293 cells were transiently transfected with a serial dilution of expression constructs for Nluc-HT, Nluc-TurboYFP and Nluc-TagRFP. The amount of expression construct used for transfection is shown as ng DNA per well of a 96-well plate. The relative BRET value is shown as percentage of BRET measured for the sample with the highest DNA input. The cut-on wavelength for the long-pass filters used for measuring the acceptor signal is indicated for each acceptor.

Supporting Figure S7



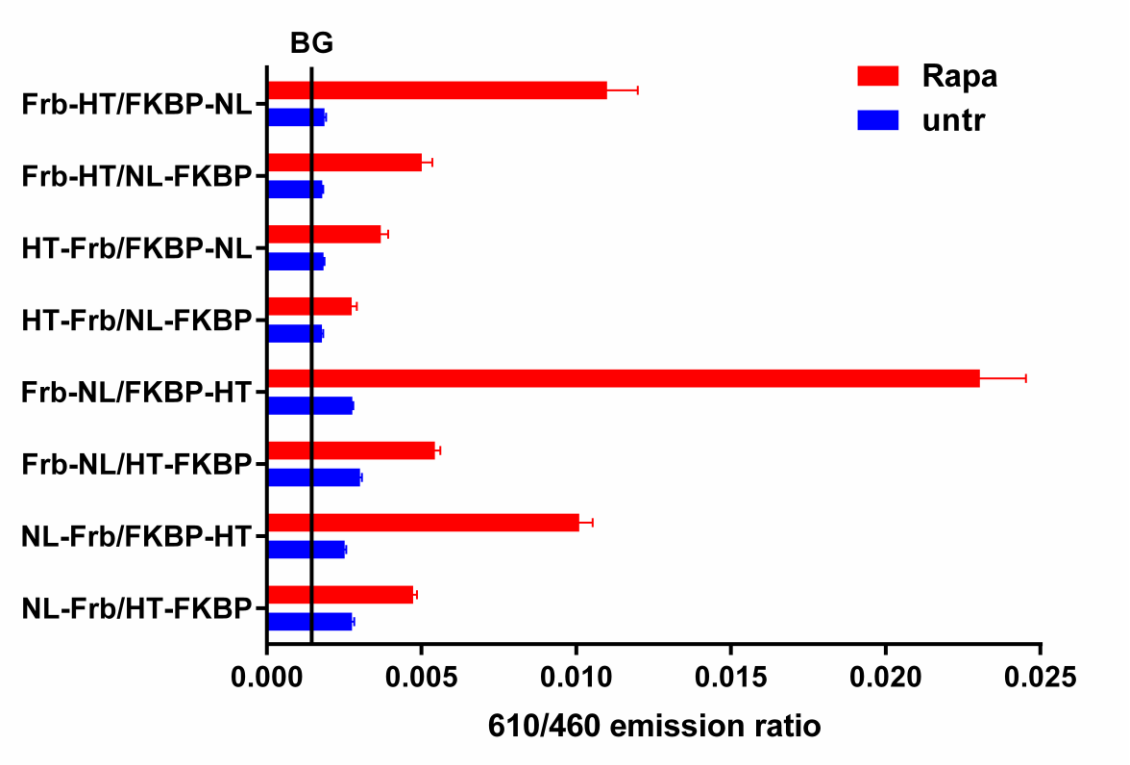
Effect of protein concentration on BRET for different Nluc-acceptor fusions: BRET ratios were determined for a serial dilution of the indicated recombinant Nluc fusion proteins (1 nM starting concentration). The relative BRET value is shown as percentage of BRET measured for the sample with the highest protein concentration. The cut-on wavelength for the long-pass filters used for measuring the acceptor signal is indicated for each acceptor.

Supporting Figure S8A



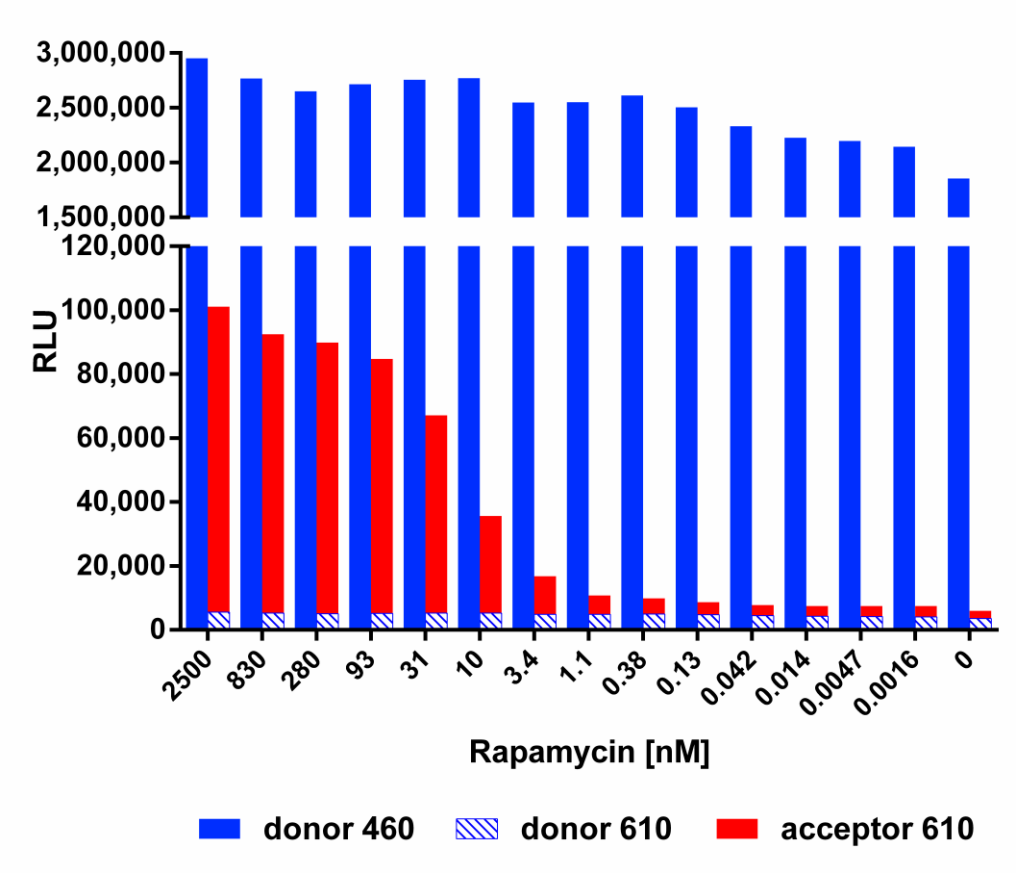
Acceptor/ donor channel emission ratios for untreated (blue) and rapamycin (red) treated BRET1 FKBP/Frb pairs which were used to calculate S/B ratios shown in figure 3A. The black line (BG) represents background caused by donor alone (as outlined above).

Supporting Figure S8B



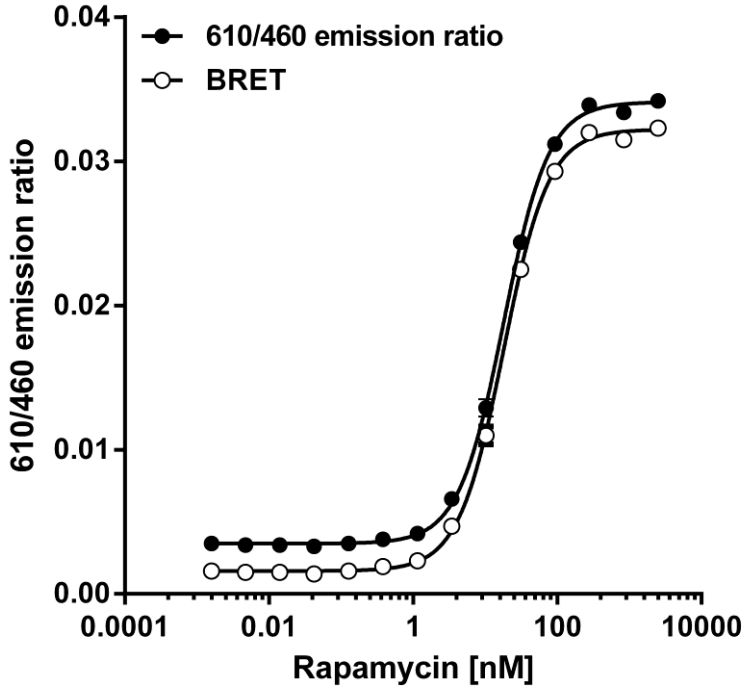
Acceptor/ donor channel emission ratios (610/460 emission ration) for untreated (blue) and rapamycin (red) treated NanoBRET FKBP-HT/Frb fusion pairs which were used to calculate signal to background ratios shown in figure 3A. The black line (BG) represents background caused by donor alone (as outlined above).

Supporting Figure S9A



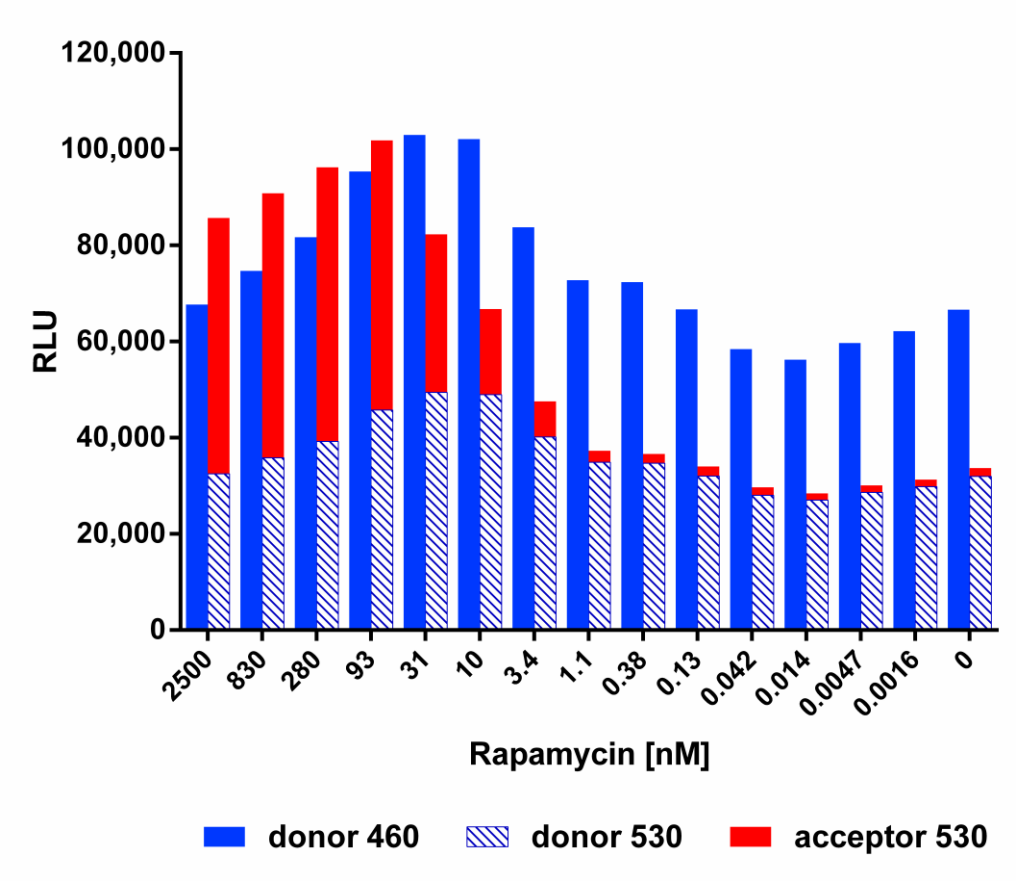
Raw donor (blue) and acceptor (striped-blue + red) channel RLU values measured for the FKBP-HT/Frb-Nluc interaction assay (Figure 3B). The striped-blue bars represent the RLU values for donor portion of the acceptor channel measurement which were calculated based on the 460/610 emission ratio of the donor-only control.

Supporting Figure S9B



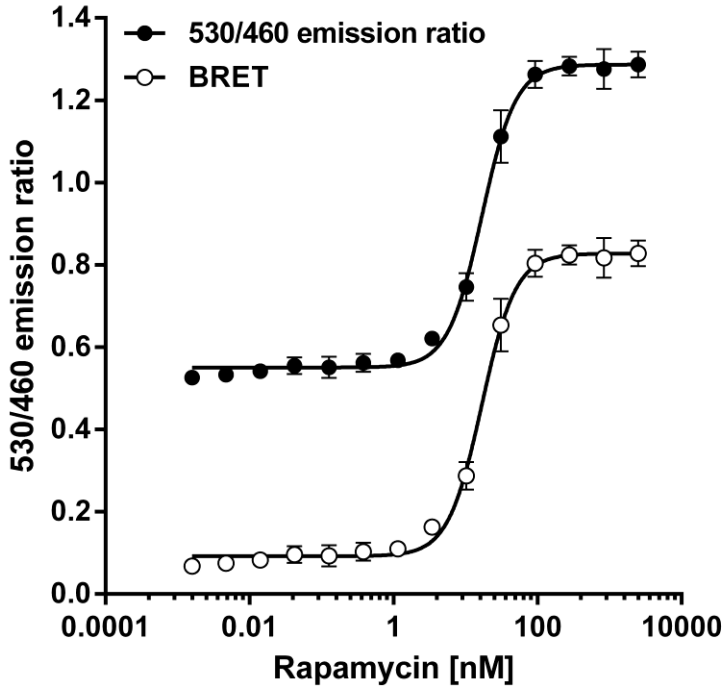
Comparison of acceptor/ donor channel emission ratio (610/460 emission ratio, black circles) and BRET ratio (open circles) for FKBP-HT/Frb-Nluc assay shown in figure 3B.

Supporting Figure S9C



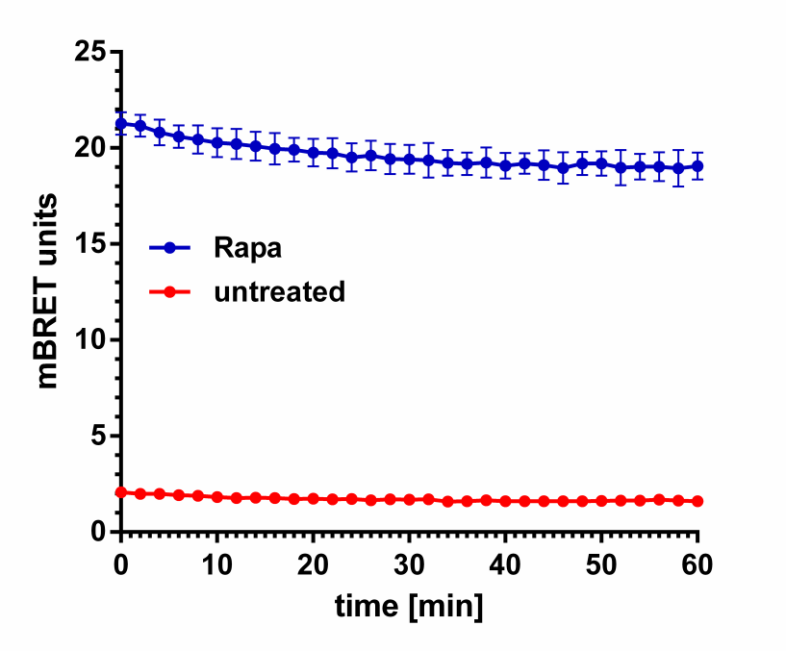
Raw donor (blue) and acceptor (striped-blue + red) channel RLU values measured for the FKBP-turboYFP/Frb-Rluc8 interaction assay (Figure 3B). The striped-blue bars represent the RLU values for donor portion of the acceptor channel measurement which were calculated based on the 460/530 emission ratio of the donor-only control. A direct comparison between figure S9A and S9C demonstrates the difference in the relative contribution of donor background to the total acceptor channel signal.

Supporting Figure S9D



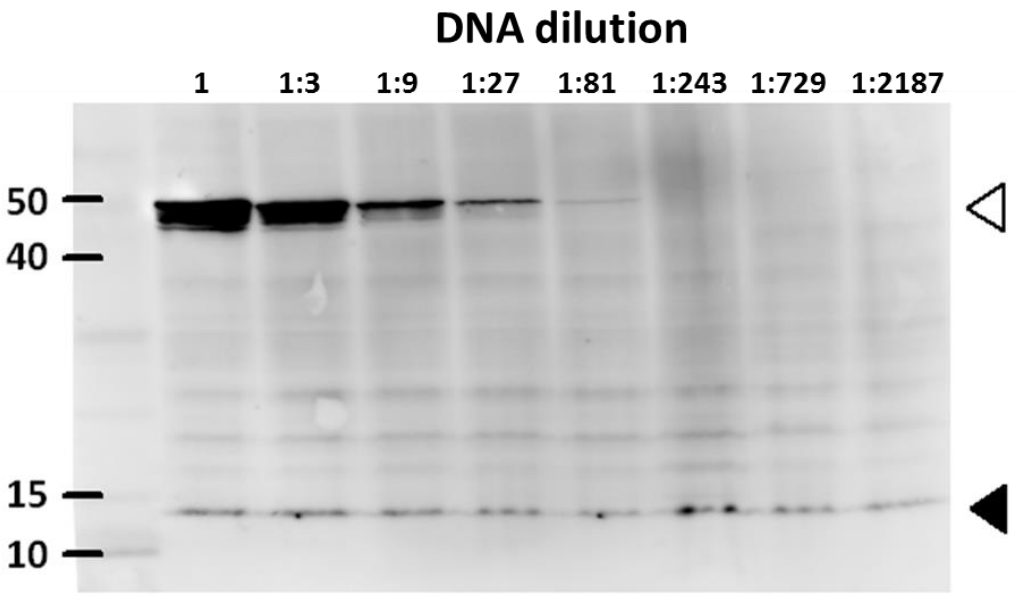
Comparison of acceptor/ donor channel emission ratio (530/460 emission ratio, black circles) and BRET ratio (open circles) for the FKBP-turboYFP/Frb-Nluc interaction assay shown in figure 3B.

Supporting Figure S10



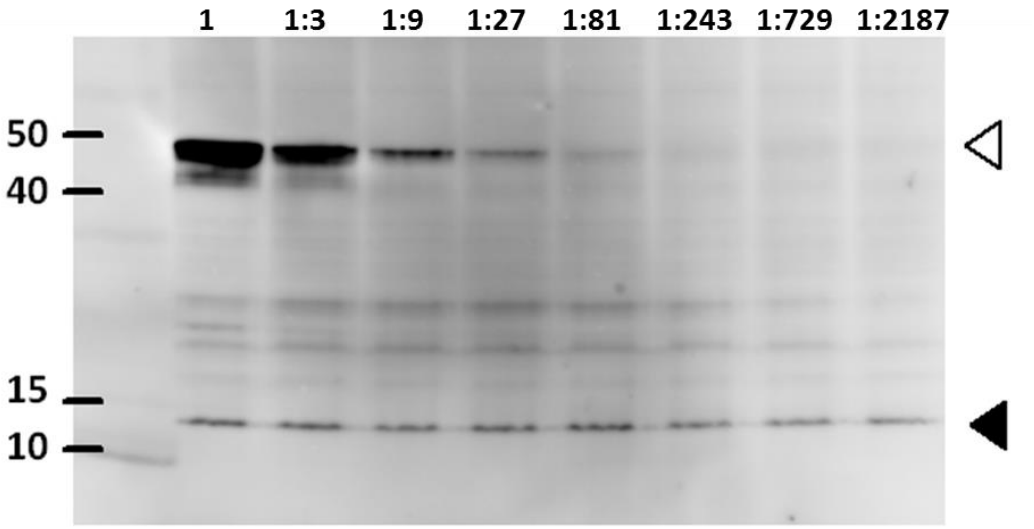
Time course of FKBP-HT/Frb-Nluc interaction assay: HEK293 cells were reverse transfected with FKBP-HT and Frb-Nluc, plated in a white 96-well and incubated for 24 h. After labeling with 250 nM HaloTag NCT ligand for 60 minutes the cells were left untreated or treated with 500 nM rapamycin for 10 minutes, followed by addition of furimazine at a final concentration of 10 μM. The samples were then measured at room temperature every 120 seconds for 60 minutes in a BMG Clariostar plate reader. Shown are the means ± SD (n=3) of a representative experiment.

Supporting Figure S11A



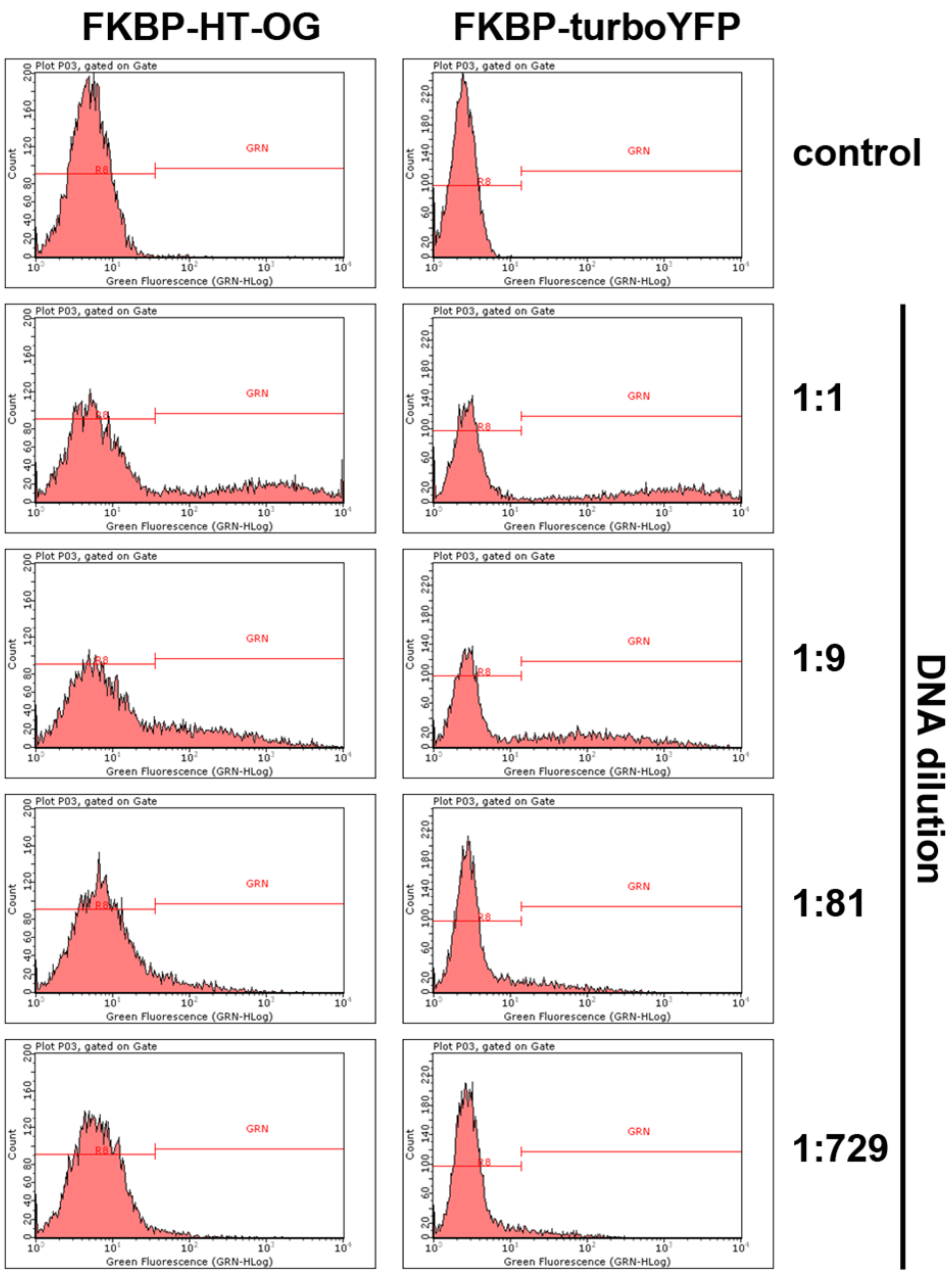
Western blot analysis of FKBP levels: Cell lysates of HEK293 cells transiently transfected with a serial dilution of DNA expression constructs for Frb-Nluc and FKBP-HT (DNA ratio 1:1) were prepared and analyzed by western blotting using a mouse anti FKBP12 antibody for detection of FKBP-HT fusion (◁) and FKBP wt protein (◀)

Supporting Figure S11B



Western blot analysis of FKBP levels: Cell lysates of HEK cells transiently transfected with a serial dilution of DNA expression constructs for Frb-Rluc8 and FKBP-turboYFP (DNA ratio 1:1) were prepared and analyzed by western blotting using a mouse anti FKBP12 antibody for detection of FKBP-turboYFP fusion (◁) and FKBP wt protein (◀)

Supporting Figure S12A



Flow cytometry analysis of FKBP-HaloTag and FKBP-turboYFP expression in HeLa cells transfected with a serial dilution of DNA for FKBP-HT/Frb-Nluc or FKBP-turboYFP/Frb-Rluc8.

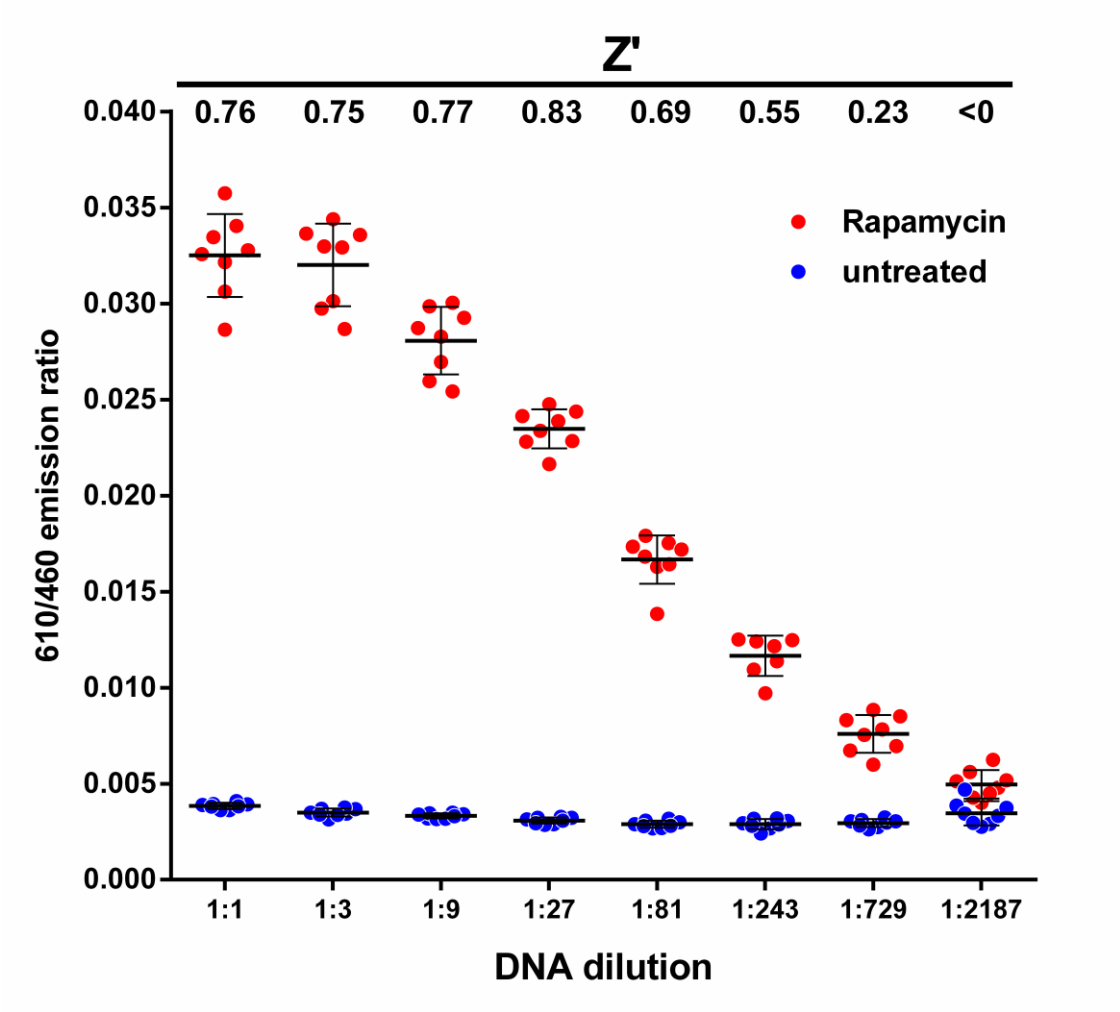
Supporting Table S12B

DNA Dilution	% FKBP-HT-NCT^{+}	% FKBP-turboYFP^{+}
1:1	29	35
1:3	30	37
1:9	26	36
1:27	19	22
1:81	10	14
1:243	5	8
1:729	3	3
1:2187	1	1

Percentage of FKBP-HaloTag and FKBP-turboYFP positive cells for each DNA dilution.

Figure S12A,B: Analysis of FKBP-HT/FKBP-turbo-YFP expression by flow cytometry
HeLa cells were reverse transfected with a serial dilution of expression constructs for Frb-Nluc and FKBP-HT or Frb-Rluc8 and FKBP-turboYFP (1:1 DNA ratio) and plated in 24 well plates. A mock transfected sample was also included as control for flow cytometry analysis. After incubation for 24h all NanoBRET samples were labeled for 60 min with 1 μM HaloTag Oregon Green ligand. HaloTag Oregon Green ligand was used instead of HaloTag NCT ligand in order to allow use of the 488 nm laser line for analysis of both NanoBRET and BRET1 samples. Excess HaloTag ligand was removed by two sequential washing steps with PBS. All cells were then harvested by trypsinization, washed twice in PBS and re-suspended in PBS + 2% FBS. Flow cytometry analysis of FKBP-HaloTag and FKBP-turboYFP expression was performed using a Guava EasyCyte HT flow cytometer (EMD Millipore) and InCyte acquisition software according to manufacturer's instructions. Shown are the results of a representative experiment.

Supporting Figure S12C



Supporting Figure S12D

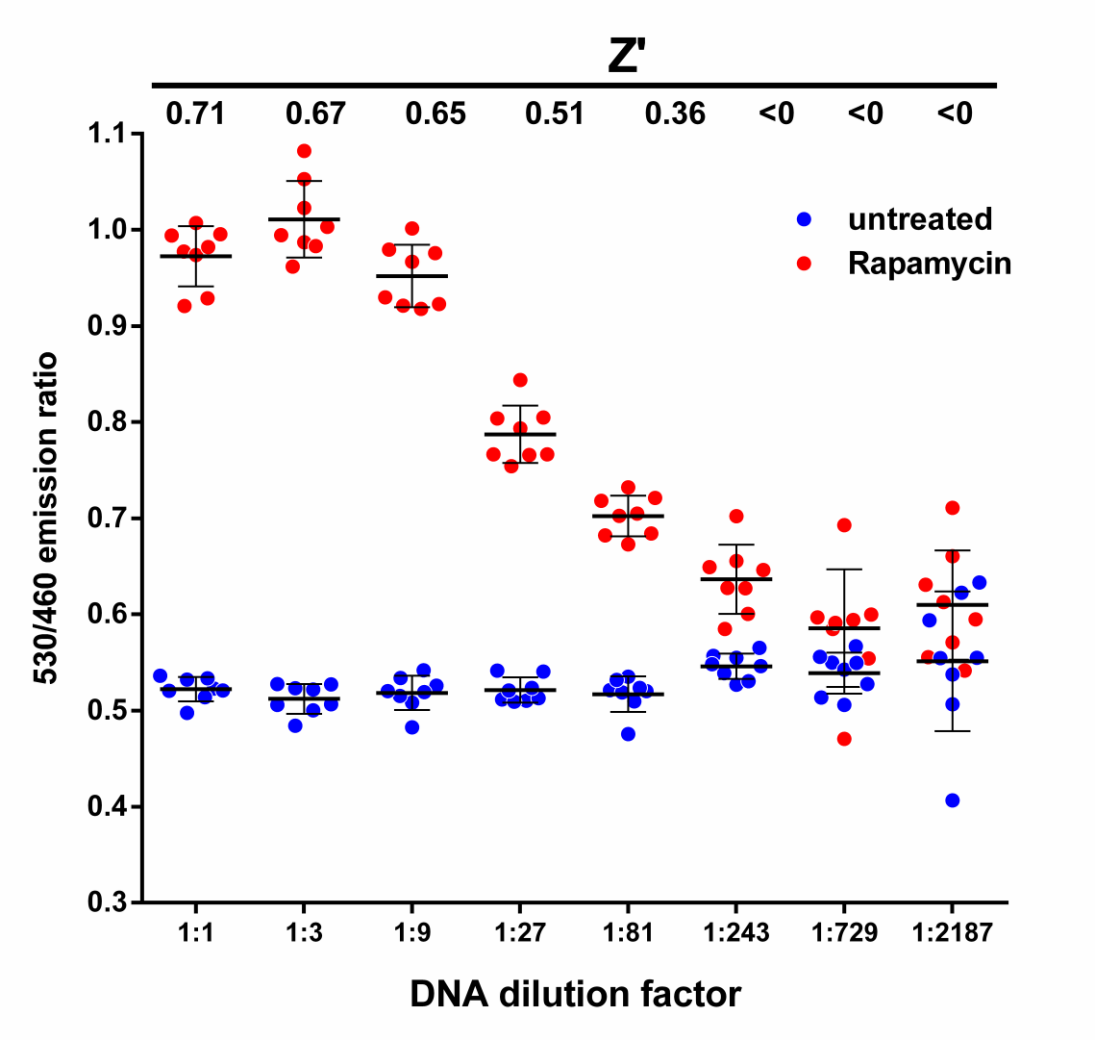
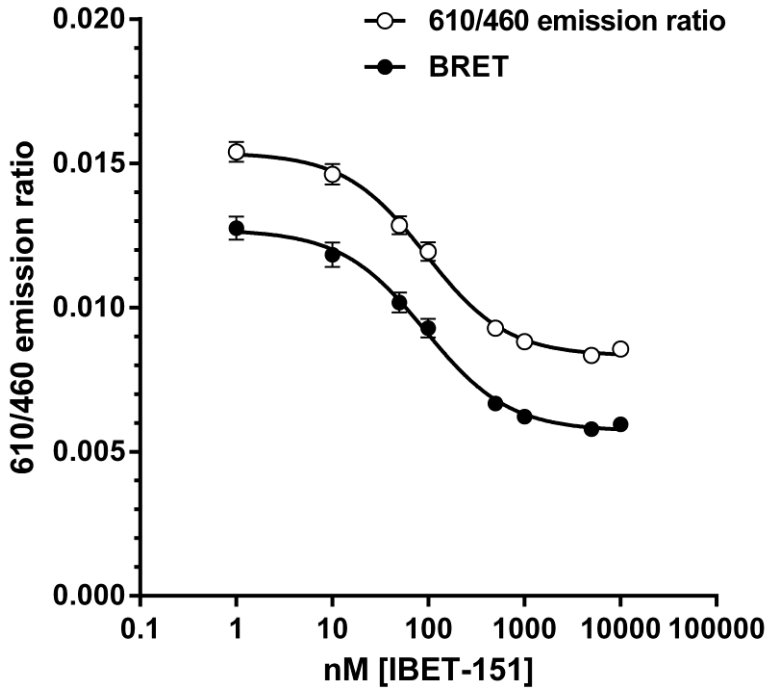


Figure S12C,D: NanoBRET and BRET1 assay for rapamycin induced interaction of Frb and FKBP

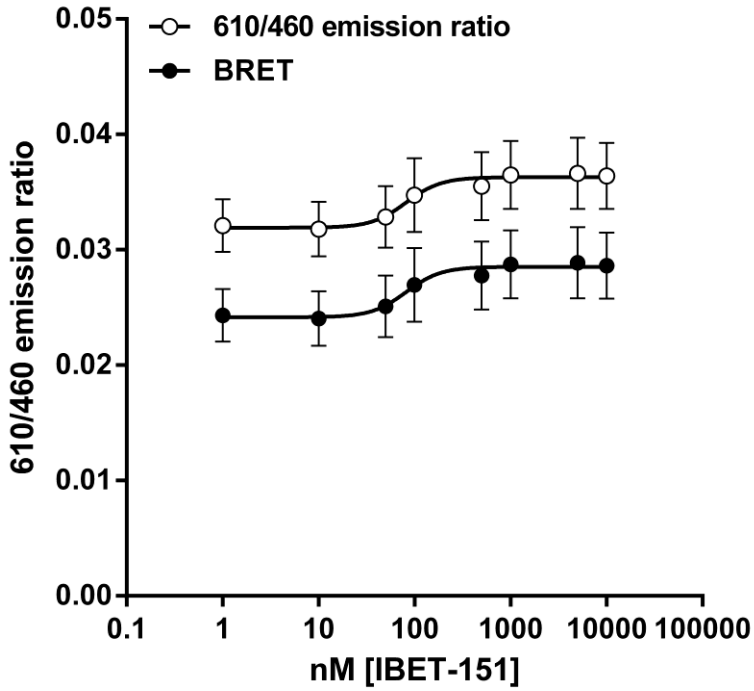
HeLa cells were reverse transfected with a serial dilution of expression constructs for Frb-Nluc and FKBP-HT (Figure S12C) or Frb-Rluc8 and FKBP-turboYFP (Figure S12D) at a DNA ratio of 1:1and plated in 96 well plates. Acceptor/donor channel emission ratios were determined for untreated or rapamycin treated samples (1 μM rapamycin for 15 minutes at room temperature). Shown are the results of a representative experiment with all individual data points (n=8) as well as mean ± SD plotted against DNA dilution used for transfection.

Supporting Figure S13A



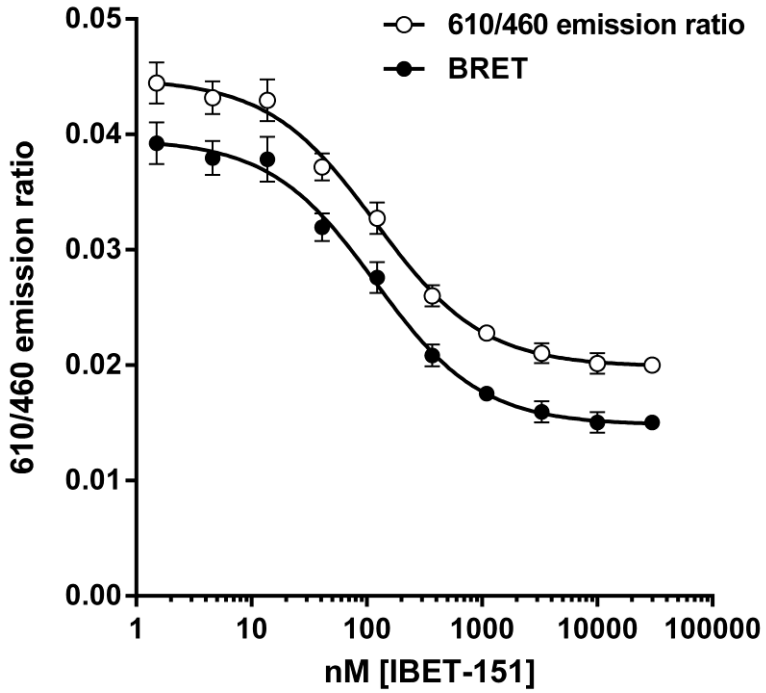
Comparison of acceptor/ donor channel emission ratio (530/460 emission ratio, open circles) and BRET ratio (black circles) for Nluc-Brd4 / Histone H3.3-HT interaction assay shown in figure 5A.

Supporting Figure S13B



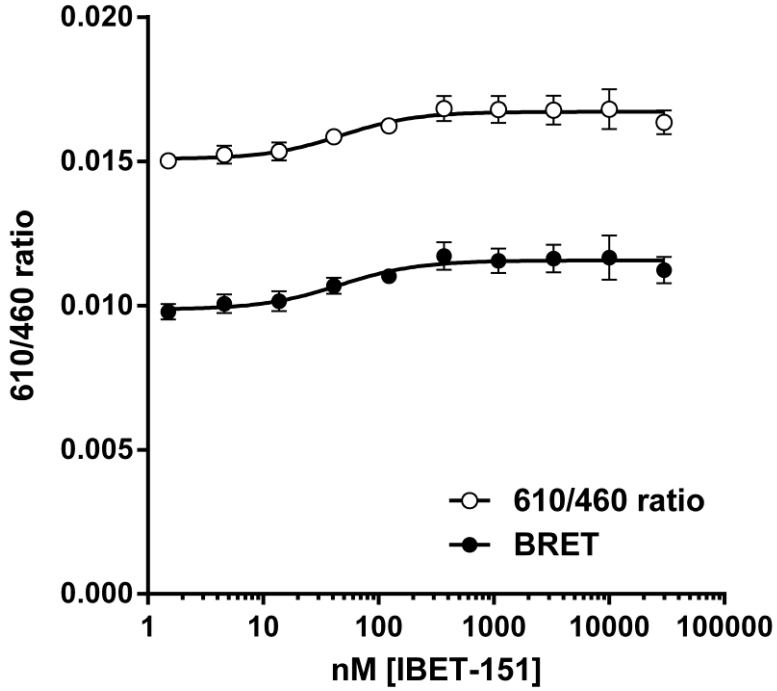
Comparison of acceptor/ donor channel emission ratio (530/460 emission ratio, open circles) and BRET ratio (black circles) for Nluc-CBP / Histone H3.3-HT interaction assay shown in figure 5B.

Supporting Figure S13C



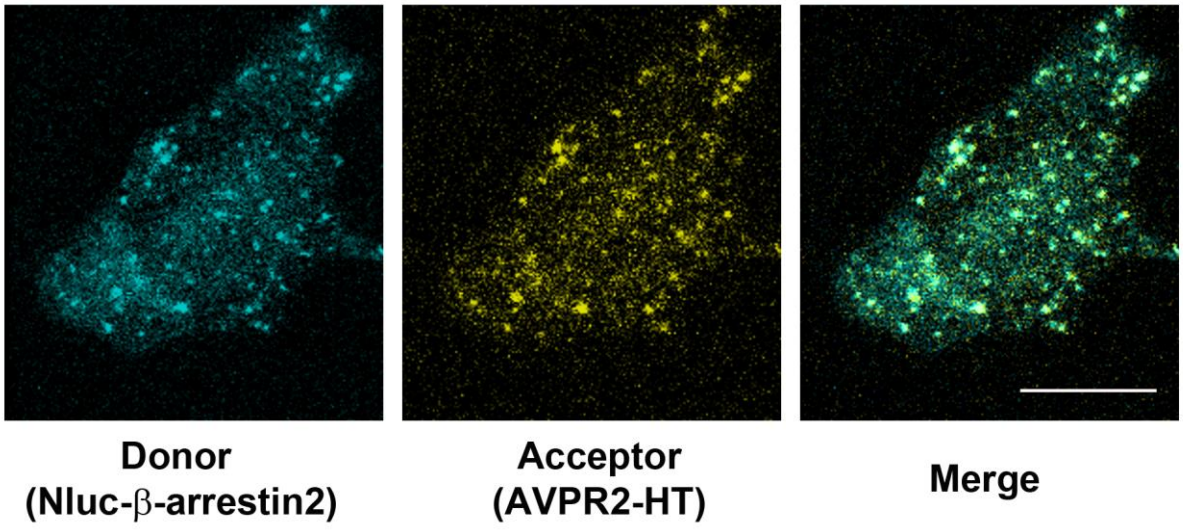
NanoBRET assay for measuring the interaction between Nluc-BRD4 to Histone H4-HT: The acceptor / donor channel emission ratio (610/460 emission ratio, open circles) and BRET ratio (closed circles) was determined for each sample following treatment for 18h with the indicated concentrations of I-BET151. Shown are the means ± SD (n=3) of a representative experiment.

Supporting Figure S13D



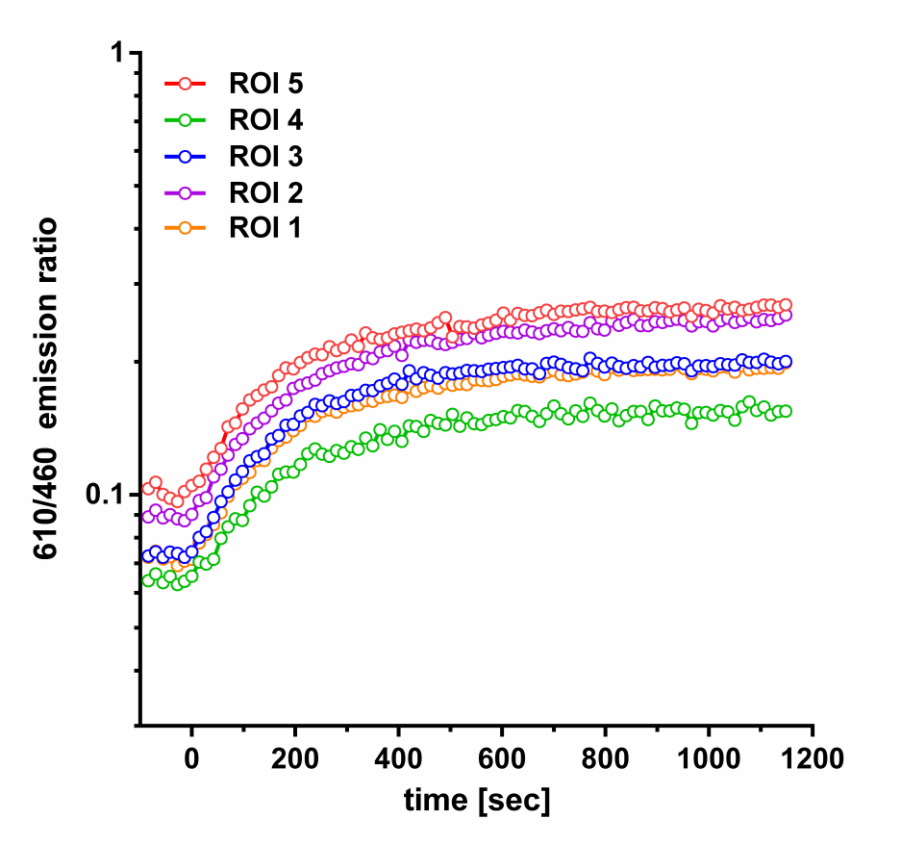
NanoBRET assays for measuring interaction between Nluc-CBP and Histone H4-HT: HEK293 cells were transiently transfected with the indicated combination of constructs. The acceptor / donor channel emission ratio (610/460 emission ratio, open circles) and BRET ratio (closed circles) was determined for each sample following treatment for 18h with the indicated concentrations of I-BET151. Shown are the means ± SD (n=3) of a representative experiment.

Supporting Figure S14A



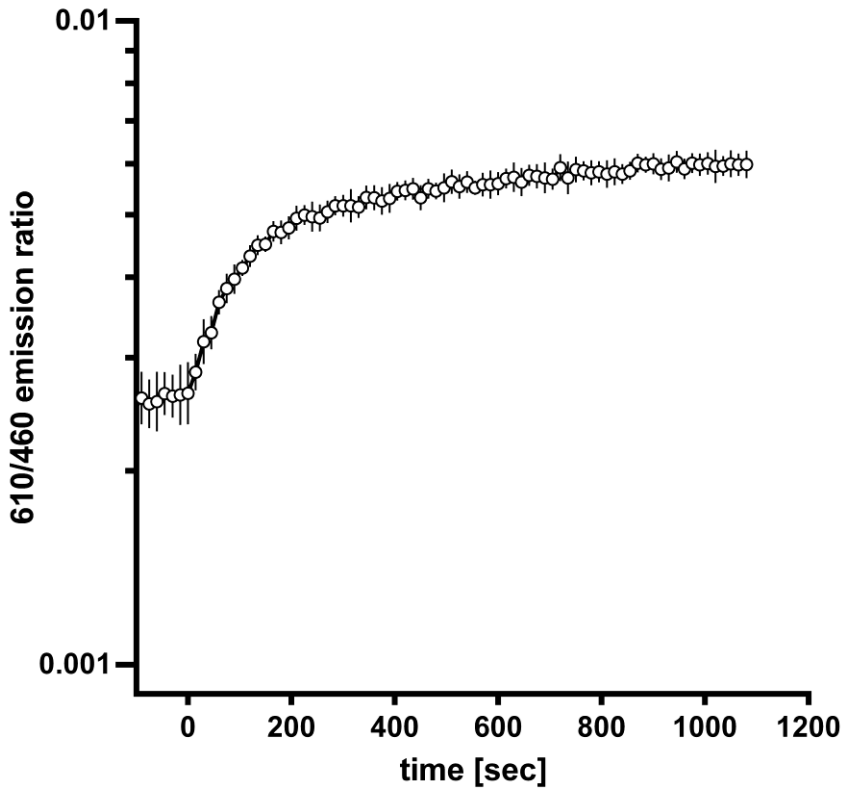
The panel shows pseudo-colored images of the donor (cyan) and acceptor (yellow) channel after treatment with 1 μM AVP. The images shown represent the 1112 sec time point in figure 6A. The merged image shows co-localization of Nluc-β-arrestin 2 and AVPR-HT after treatment with 1 μM AVP for 20 min.

Supporting Figure S14b



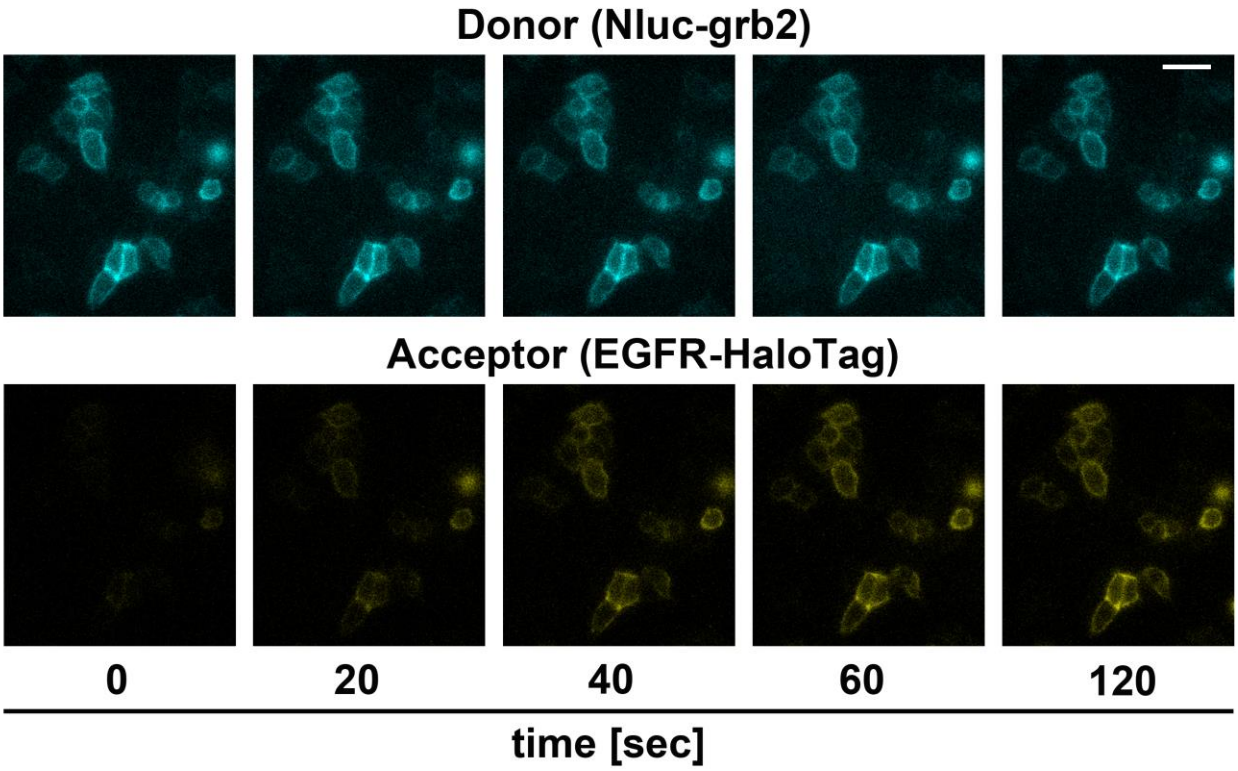
Acceptor/ donor channel emission ratio (610/460 emission ratio) for imaging-based kinetic measurement of Nluc-β-arrestin 2 recruitment to AVPR2-HT following stimulation with AVP as shown in figure 6b.

Supporting Figure S14c



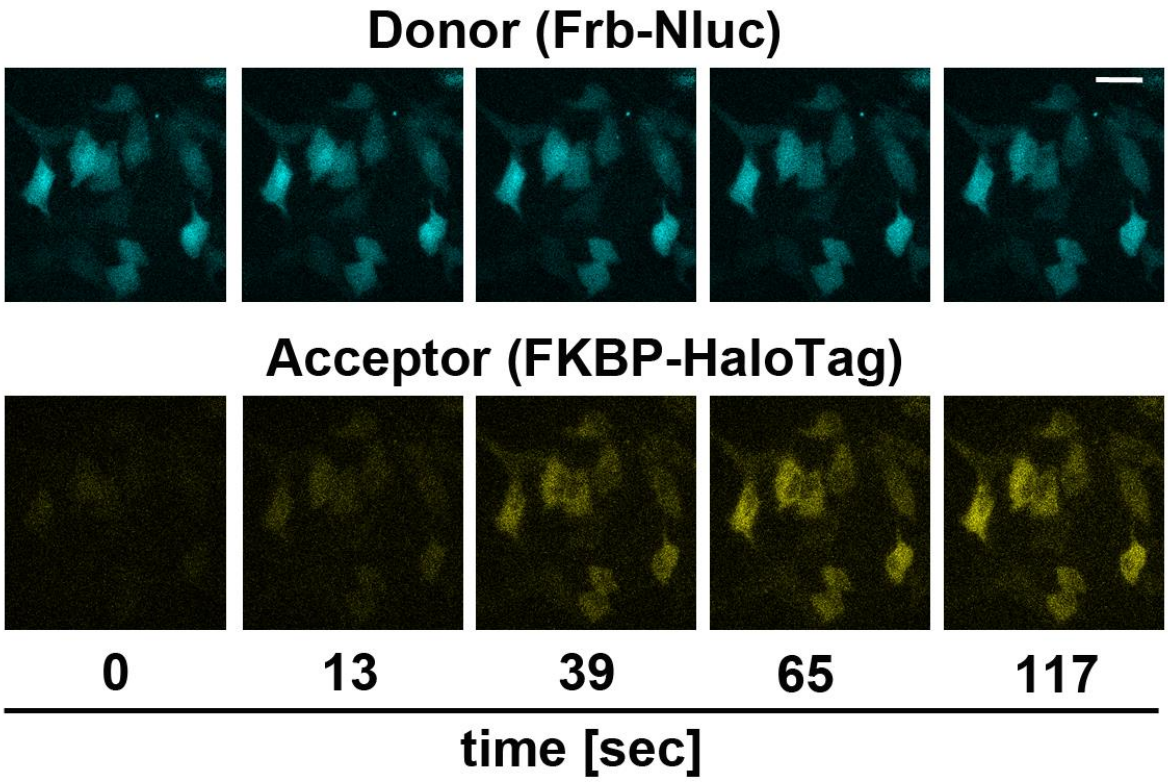
Acceptor/ donor channel emission ratio (610/460 emission ratio) for plate-based kinetic measurement of Nluc-β-arrestin2 recruitment to AVPR2-HT following stimulation with AVP as shown in figure 6b.

Supporting Figure S15a



Real time imaging of ligand induced interaction of EGFR-HT with Nluc-grb2. HeLa cells were reverse transfected with expression constructs for EGFR-HT and Nluc-grb2 and plated in 35 mm dishes. After 24 h of incubation the samples were serum starved and labeled with 250 nM HaloTag-NCT ligand for 4h. Sequential acquisition of images was initiated under unstimulated conditions. After establishing a baseline for 5 acquisition cycles (20 sec per cycle), 100 ng/ml of EGF was added to the sample by injection followed by 60 acquisition cycles covering approximately 20 minutes. The panel shows pseudo-colored images of the donor (cyan) and acceptor (yellow) channel at indicated time points following treatment with EGF. The images are representative of the results obtained in 3 independent experiments (scale bar = 25 μm).

Supporting Figure S15b



Real time imaging of rapamycin induced interaction of FKBP-HT with Frb-Nluc. HeLa cells were reverse transfected with expression constructs for FKBP-HT and Frb-Nluc and plated in 35 mm dishes. After 24 h of incubation the samples were serum starved and labeled with HaloTag-NCT Ligand for 4h. Sequential acquisition of images was initiated under unstimulated conditions. After establishing a baseline for 3 acquisition cycles (13 sec per acquisition cycle), rapamycin (500 nM final concentration) was added to the sample by injection followed by 69 acquisition cycles covering approximately 15 minutes. The panel shows pseudo-colored images of the donor (cyan) and acceptor (yellow) channel at indicated time points following treatment with rapamycin. The images are representative of the results obtained in 5 independent experiments. Scale bar = 25 μm