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# NanoBRET—A Novel BRET Platform for the Analysis of Protein–Protein Interactions

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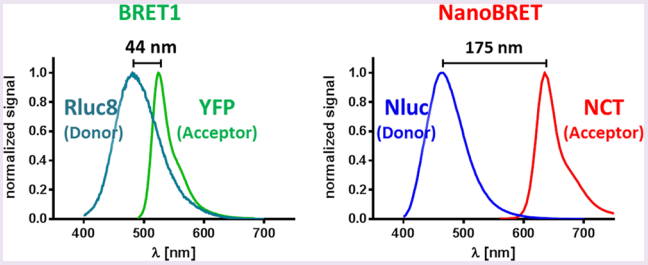
Supporting Information

**ABSTRACT:** Dynamic interactions between proteins comprise a key mechanism for temporal control of cellular function and thus hold promise for development of novel drug therapies. It remains technically challenging, however, to quantitatively characterize these interactions within the biologically relevant context of living cells. Although, bioluminescence resonance energy transfer (BRET) has often been used for this purpose, its general applicability has been hindered by limited sensitivity and dynamic range. We have addressed this by combining an extremely bright luciferase (Nanoluc) with a means for tagging intracellular proteins with a long-wavelength fluorophore (HaloTag). The small size (19 kDa), high emission intensity, and relatively narrow spectrum (460 nm peak intensity) make Nanoluc luciferase well suited as an energy donor. By selecting an efficient red-emitting fluorophore (635 nm peak intensity) for attachment onto the HaloTag, an overall spectral separation exceeding 175 nm was achieved. This combination of greater light intensity with improved spectral resolution results in substantially increased detection sensitivity and dynamic range over current BRET technologies. Enhanced performance is demonstrated using several established model systems, as well as the ability to image BRET in individual cells. The capabilities are further exhibited in a novel assay developed for analyzing the interactions of bromodomain proteins with chromatin in living cells.




Dynamic behaviors in cells are mechanistically entwined with a vast interplay of transient interactions occurring between intracellular proteins. Protein–protein interactions (PPI) are thus fundamental to understanding cellular physiology and are attractive as pharmaceutical targets. Although numerous methods are available for the discovery of protein interactions, most notably through analysis by mass spectrometry, it remains challenging to study the dynamics of these interactions in a cellular context. Resonance energy transfer has been an attractive approach for this purpose, where excited-state energy is transferred from one fluorophore to another when brought into close proximity.[1] The rigorous distance constraint of energy transfer restricts proximity detection to roughly the physical span of a typical protein (~5 nm), making this approach well suited for the crowded molecular space within cells.

Energy transfer is most often achieved using autofluorescent proteins (e.g., YFP), which can be linked to the interacting proteins through genetic fusions. The method allows visualization of subcellular location and interaction dynamics by microscopy imaging but often requires relatively high levels of expression for reliable measurements. This can make analysis at physiological concentrations challenging within the cells and may lead to artifacts of protein behavior. The intensity of the illuminating light source also causes problems with phototoxicity, photobleaching of the fluorophores, direct excitation of the acceptor fluorophore, and background fluorescence from the sample. These issues make sensitive and reliable measurements difficult, particularly for kinetic processes.

Although FRET is generally recognized as requiring an external light source, such as a laser, to generate the excited state of the donor, the energy can be supplied instead by a chemical reaction. For BRET (bioluminescence resonance energy transfer), using a luciferase as the donor sidesteps many of the deficiencies associated with direct illumination of the sample.[2−4] Furthermore, quantitative measurements even at very low expression levels in cells should be possible due to the extraordinary sensitivity typical of luciferase assays. Yet despite these predictable advantages, BRET has not been widely adopted largely due to limited sensitivity and dynamic range of the presently available methods.

Current methods routinely utilize Renilla luciferase (Rluc) as the energy donor, more recently relying on the Rluc8 mutant to provide higher light intensity.[5] In the most commonly used BRET format, BRET1, Rluc is typically matched with a yellow fluorescent protein (YFP) to achieve efficient energy transfer by

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significant overlap of its excitation spectrum with the bioluminescence. However, the spectral proximity of Rluc and YFP (~55 nm peak separation) produces a substantial measurement background by the extension of donor signal into the acceptor channel. The high background increases assay noise, which diminished dynamic range and sensitivity.

An alternative BRET, referred to as BRET2, was developed to increase spectral separation between the luciferase and the fluorescent acceptor.[6] The system utilizes a substrate analog, bisdeoxycoelenterazine, to shift the luminescence to much shorter wavelengths (400 nm peak from 480 nm), and GFP2 or GFP10, which exhibit excitation and emission maxima of 395 and 510 nm, respectively. Although the large Stokes shift of GFP2/GFP10 improves spectral resolution (115 nm), this advantage is largely negated by the very low quantum yield and poor luminescence stability.[7] The recent adoption of Rluc8[8] for BRET2 has improved performance, but light intensities are still far below other BRET configurations.[9]

The recent development of the Nanoluc luciferase (Nluc) offers an opportunity to further improve the performance of BRET assays.[10] Nluc is an engineered protein (19 kDa), which uses a novel coelenterazine derivative (furimazine) as its substrate. Its small size and high physical stability are desirable for use as a fusion tag, imparting minimal influence when tethered onto other proteins. Although it is only about half the size of Rluc8 (36 kDa), it produces sustained luminescence with much greater intensity, allowing very small quantities to be accurately quantified. Moreover, its bioluminescence spectrum is narrower than Rluc8,[10] permitting better spectral discrimination with acceptor fluorophores. To provide distinction from the other configurations of BRET, we refer to energy transfer using Nluc as the donor as NanoBRET.

With Nluc available as a more effective BRET donor, we focused on matching this with an optimal fluorescent acceptor. The HaloTag fusion tag allowed us to rapidly evaluate a range of fluorophores for this purpose. HaloTag (HT) is a two component system consisting of a stable protein (36 kDa) which can be genetically tethered onto other proteins and a chloroalkane ligand that can be chemically coupled onto fluorophores or other molecules of interest.[11] The chloroalkane irreversibly links to the HaloTag through rapid formation of a covalent bond. By using this modular system, we found that long-wavelength fluorophores were preferred for BRET measurements by providing better spectral separation from the luciferase donor. Best performance was achieved with a chloroalkane derivative of nonchloro TOM (NCT) dye, which provides peak light emission at 635 nm. Thus, peak emission from the donor and acceptor were spectrally separated by 175 nm, significantly greater than provided even by the BRET2 system.

The high luminescence intensity of NanoBRET allows detection of protein interactions at low levels comparable to endogenous physiological conditions. The assay exhibits a linear dynamic range extending over several orders of magnitude and provides greater sensitivity than Rluc8/YFP by typically more than an order of magnitude for analogous molecular configurations. The strong performance of NanoBRET was utilized to develop the first assays for quantifying the binding dynamics of bromodomain protein with chromatin in living cells. Furthermore, the high intensity of NanoBRET also raises the potential for quantifying energy transfer in individual cells by microscopy imaging.

## RESULTS AND DISCUSSION

**Optimization of the Fluorescent Acceptor.** Genetically fusing HaloTag directly onto Nluc (Nluc-HT) offered a convenient means for providing a fixed geometry between the donor and acceptor. Fifty-seven different chloroalkane−fluorophore conjugates were synthesized and screened in HEK293 cells transiently expressing the Nluc-HT fusion. By using a cell-based assay, we were able to evaluate the fluorophores for their ability to permeate cellular membranes and produce a BRET signal within the intracellular environment. Each fluorophore was evaluated using a set of four long-pass filters (cut-on 570, 590, 610, and 630 nm) for the acceptor emission, where the BRET response was determined by comparing measurements made both in the presence and absence of the fluorophore. The HaloTag TMR Ligand (590 nm emission peak) was used as a positive control, having been previously shown to bind efficiently and stoichiometrically to HaloTag in living cells. From these experiments, a nonchloro TOM (NCT) ligand was identified as providing the greatest signal over the background. The NCT ligand has an excitation maximum of 595 nm with an emission maximum of 635 nm when bound to HaloTag (Supporting Information Figure S1a). Labeling of HaloTag fusion proteins in living cells is rapid with saturation occurring in less than 10 min after the addition of 250 nM HaloTag NCT ligand (Supporting Information Figure S1b). No obvious cytotoxicity was exhibited by the NanoBRET expression constructs in combination of NCT ligand and furimazine (the principal reagents required for NanoBRET) for periods of up to 24 h (Supporting Information Figure S2).

The Förster equation predicts that the greatest energy transfer will occur with maximal overlap between the emission spectrum of the donor and excitation spectrum of the acceptor. The HaloTag Oregon Green Ligand (512 nm peak emission) has greater overlap, and the NCT ligand has less overlap, with Nluc than does the HaloTag TMR Ligand (Supporting Information Figure S1). Accordingly, in the Nluc-HT fusion, more energy is transferred from Nluc to the Oregon Green Ligand, and less to the NCT ligand, compared to the TMR Ligand (Figure 1A). However, since the decline in energy transfer efficiency is substantially smaller compared to the decrease in Nluc light intensity across the same spectral range, the signal to background ratio increases substantially. Thus, the highest signal to background was achieved with the NCT ligand, even though it does not support the most efficient energy transfer (Supporting Information Figure S3). Moreover, the modest reduction in transfer efficiency is more than compensated by the increased light intensity of Nluc. The specific activity of Nluc is about 150-fold greater than Rluc, and 30-fold greater than Rluc8. Signal decay kinetics are comparable between Nluc and Rluc8, with NanoLuc showing a slightly improved decay profile in biological buffers (Supporting Information Figure S4a−c).

In BRET measurements of molecular interactions, since energy transfer occurs when the donor is occupied by an acceptor, assay sensitivity can be specified as the lowest detectable degree of occupancy. This can be modeled using the Nluc-HT fusion, where fully bound to a fluorophore represents 100% occupancy and fully bound with a nonfluorophore represents 0% occupancy. Because the covalently bound HaloTag ligands cannot be exchanged once attached, different ratios of the "occupied" and "unoccupied" fusion protein can be combined to create fractional occupancy. Thus, a series of


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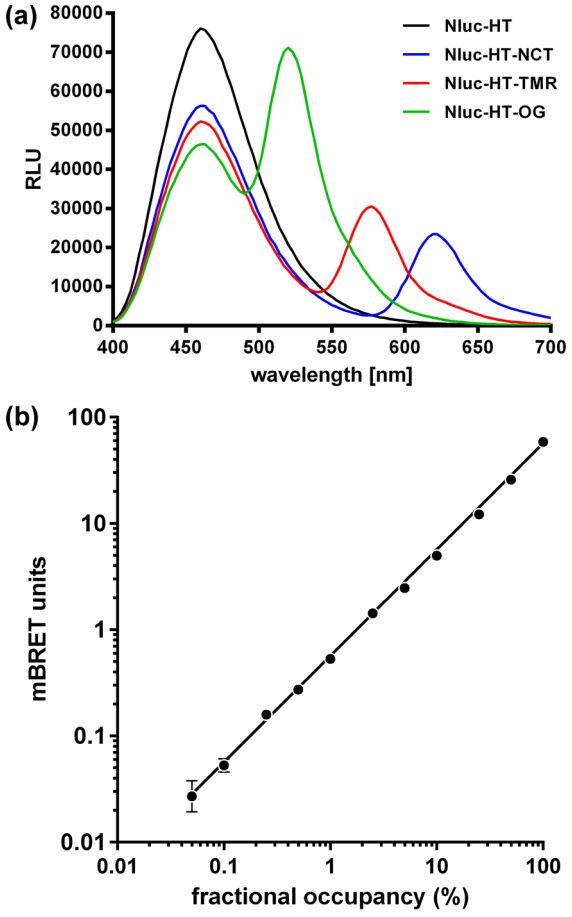


**Figure 1.** Comparison of different acceptors for NanoBRET. (a) BRET spectra for Nluc-HT (black) and Nluc-HT conjugated to HaloTag Oregon Green (green), HaloTag TMR (red), or HaloTag NCT (blue) ligands. (b) Correlation of BRET with fractional occupancy. Differing degrees of occupancy were created by combining Nluc-HT-NCT (fully acceptor occupied) with Nluc-HT-PEG-biotin (unoccupied donor) in defined ratios at a constant donor concentration. Fractional occupancy (%) indicates the amount of acceptor-occupied Nluc-HT relative to the total amount of Nluc-HT in the sample. Shown are the means ± SD ($n$ = 7) of a representative experiment. The results were plotted as mBRET units.

stable compositions can be readily generated that hold the total amount of Nluc constant while accurately varying the portion occupied by an acceptor. By this method, NanoBRET exhibits linearity with donor occupancy extending beyond 3 orders of magnitude (Figure 1B; see Supporting Information Figure S5 for uncorrected acceptor/donor channel emission ratios). The smallest degree of measurable occupancy can be determined using a statistical metric of discrimination from the unoccupied donor. A commonly used metric is Z′-factor, where quantitatively reliable discrimination is attained for values above 0.5. An evaluation of Z′-factor values relative to fractional occupancy indicates that the NCT ligand provides about 20-fold greater sensitivity than the Oregon Green Ligand and about 4-fold over the TMR Ligand (Table 1).

Since autofluorescent proteins are most commonly used as the acceptor in BRET assays, their performance was also evaluated in NanoBRET. TurboYFP is a fast maturing YFP variant derived from *Phialidium* sp., with brightness comparable to Venus and spectral properties similar to the Oregon Green Ligand. TagRFP is derived from *Entacmaea quadricolor* and has

**Table 1. Sensitivity Limits for NanoBRET Acceptors**

| | Z′-factor[a] | | | | |
|---|---|---|---|---|---|
| | acceptor fluorophore | | | | |
| fractional occupancy (%) | NCT | TMR | OG | YFP | RFP |
| 0.05 | 0.46 | <0 | <0 | <0 | <0 |
| 0.10 | 0.65 | <0 | <0 | <0 | <0 |
| 0.25 | 0.78 | 0.57 | <0 | <0 | <0 |
| 0.50 | 0.85 | 0.71 | <0 | <0 | <0 |
| 1.0 | 0.92 | 0.81 | 0.046 | <0 | <0 |
| 2.5 | 0.95 | 0.90 | 0.78 | <0 | <0 |
| 5.0 | 0.96 | 0.93 | 0.88 | 0.23 | 0.21 |
| 10 | 0.98 | 0.95 | 0.90 | 0.75 | 0.65 |
| 25 | 0.98 | 0.97 | 0.96 | 0.89 | 0.82 |
| 50 | 0.98 | 0.98 | 0.98 | 0.92 | 0.91 |
| 100 | 0.99 | 0.99 | 0.97 | 0.97 | 0.93 |

[a]The Z′-factor was determined for each value of % occupancy for Nluc-HT-OG, Nluc-HT-TMR, and Nluc-HT-NCT and for Nluc-turboYFP and Nluc-TagRFP (using Nluc-HT-PEG-biotin as the unoccupied donor).

spectral properties similar to the TMR Ligand.[12−14] These were directly fused genetically to Nluc, and fractional occupancy was established as before, using Nluc-HT as an unoccupied donor. Despite the spectral similarity with the fluorescent HaloTag ligands, assay sensitivity was substantially diminished (Table 1). This may be explained in part by having lower quantum yields relative to the HaloTag ligands, inefficient chromophore maturation as well as differences in geometries of these fusions relative to Nluc-HT.[15] In addition, the chromophore in autofluorescent proteins may also be influenced by intracellular conditions, as suggested by an apparent linkage we observed between energy transfer and expression levels for TurboYFP and TagRFP (Supporting Information Figure S6). Both exhibited decreased energy transfer with lower expression while HaloTag provided nearly constant energy transfer. BRET is expected to be independent of concentration, so long as the orientation between the donor and acceptor remains fixed, since the ratiometric values are normalized to donor intensity. This was the case for these fusions when measured as purified proteins (Supporting Information Figure S7).

**Performance of NanoBRET for Quantifying Protein Interactions.** Having established HaloTag-NCT as an optimal energy acceptor for Nluc, this combination should enhance the performance of BRET for analyzing intracellular protein−protein interactions (PPI). Furthermore, labeling HaloTag with NCT as the energy acceptor is straightforward, requiring only the addition of the tagged fluorophore to the culture medium prior to making measurements (Figure 2). We evaluated this by benchmarking performance to the commonly used pairing of Rluc8 with YFP. For this purpose, we selected the rapamycin-induced interaction between FKBP12 and Frb (Rapamycin-binding domain of mTOR) as the biological model. The Frb/FKBP interaction model has been frequently employed for the evaluation of new PPI analytical methods due to its simplicity and robustness.[16]

Because the efficiency of energy transfer can be affected by geometric orientation, it is important to examine all eight possible configurations of donor and acceptor fused onto the interacting proteins. Specifically, these are the donor and acceptor fused to each of the proteins at either terminus of the polypeptide. For all configurations of Nluc/HT-NCT and Rluc8/YFP fused to FKBP and Frb, induction of PPI by


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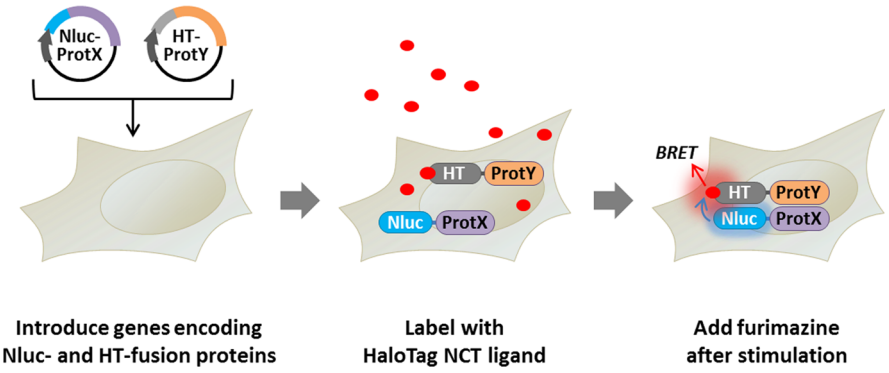


**Figure 2.** Schematic of NanoBRET for detecting protein−protein interactions.

rapamycin led to an increase in energy transfer. As expected, the different configurations of the fusions show considerable variation in their relative response to rapamycin (Figure 3a, see Supporting Information Figure S8a,b for uncorrected acceptor/donor channel emission ratios). For all configurations of Rluc8/YFP, the response was lower than for the corresponding configuration of Nluc/HT-NCT and varied by 4 to 70 fold between analogously oriented pairs. The most efficient configurations of Nluc/HT-NCT and Rluc8/YFP (Figure 3a, bars with hatch pattern), respectively, exhibited a 7-fold difference in response to rapamycin.

The donors and acceptors used in these comparisons are structurally distinct, possibly resulting in some differences in their relative geometry when fused to FKBP and Frb. Nonetheless, it is likely that NanoBRET consistently exhibits greater response dynamics due to its greater spectral separation between the donor and acceptor, narrower emission spectrum of Nluc, and higher quantum yield of NCT ligand. By either choice of donor and acceptor, the EC50 values were essentially unchanged and in agreement with previously published data (Figure 3b, see Supporting Information Figure 9A−D for raw RLU values and uncorrected acceptor/donor channel emission ratios), indicating that the pharmacology of rapamycin was not influenced by the reporter fusions.[16] In addition, NanoBRET measurements of this stable interaction remained relatively constant for 60 min both in the presence and in the absence of rapamycin, indicating the ability to continuously monitor intracellular PPIs over an extended period of time (Supporting Information Figure S10).

As molecular interactions are subject to concentration, having appropriate expression levels of the donor and acceptor is important for achieving biologically relevant data. Because regulatory proteins in particular tend to be present at low levels in cells, energy transfer must be measurable at low expression levels. Furthermore, unphysiologically high expression can lead to biological artifacts such as ligand independent signaling, improper recruitment of other cellular components and mis-folding or mis-localization of the proteins. To explore the capacity for detecting PPI at physiologically relevant levels, we measured rapamycin-induced BRET over a titrated range of donor and acceptor expression plasmids, combined in equal amounts and serially diluted with carrier DNA. For both Nluc/HT-NCT and Rluc8/YFP, a negative correlation between expression level and rapamycin response was evident, possibly due to competition between the introduced fusions and their endogenous counterparts at lower transfection amounts. Western blots for detection of FKBP showed that fusions to either acceptor were present in the sample at amounts comparable to the endogenous protein near the middle of the titration range (Supporting Information Figure S11A,B). Consistency in expression of FKBP fused to either acceptor, over the titrated range of DNA, was also confirmed by flow cytometry analysis of FKBP-HaloTag and FKBP-turboYFP expression (Supporting Information Figure 12A,B).

However, there was a marked difference in signal separation between the BRET pairs, especially at low levels of fusion protein. The NanoBRET pair exhibited complete data separation between rapamycin treated and untreated samples for the entire range of DNA dilutions (Figure 4a, Supplemental Figure 12c), whereas the Rluc8/YFP pair showed convergence to a single value at much higher analyte levels (Figure 4b, Supplemental Figure 12d). A Z′-factor analysis was used to estimate the capacity for reliably distinguishing a rapamycin-induced interaction from an untreated control.[17] The NanoBRET based assay maintained good sensitivity ($Z' > 0.65$) even at concentrations of FKBP-HT well below the endogenous FKBP. In comparison, the Rluc8/YFP pair exhibited decreased assay sensitivity even for analyte amounts well above endogenous levels. These results show that NanoBRET can provide better assay sensitivity and response dynamics even at analyte levels comparable to or below physiological concentrations within cells. The ability to perform such measurements at low concentrations of reporter may be particularly significant for technically challenging cell types, such as stem cells and primary cells, which are often difficult to transfect or have limited capacity for expressing transgenes.

**Analysis of Bromodomain Interactions.** The role of PPI in many disease relevant signaling pathways has motivated interest in compounds that target these interactions for therapeutic purposes.[18] NanoBRET provides a means for exploring the action of these compounds within the intracellular environment. This is exemplified by characterizing compounds able to inhibit binding of bromodomain proteins to chromatin, which reveals the importance of the cellular context and how biochemical studies can sometimes be misleading. The binding of bromodomain proteins to acetylated histones is one of the key mechanisms for epigenetic regulation of transcriptional activity. The ability to block binding by specific bromodomain proteins is an emerging area of drug therapy for a variety of cancers, which has been most elegantly shown for the BET family members, BRD2, BRD3, and BRD4.[19−21]

We show that the interaction between BRD4 and chromatin can be monitored with NanoBRET by expressing Nluc-BRD4 in mammalian cells with human histone H3.3-HT fusions (Figure 5A). Treatment of the cells with increasing doses of known inhibitor I-BET151, which competes for binding to



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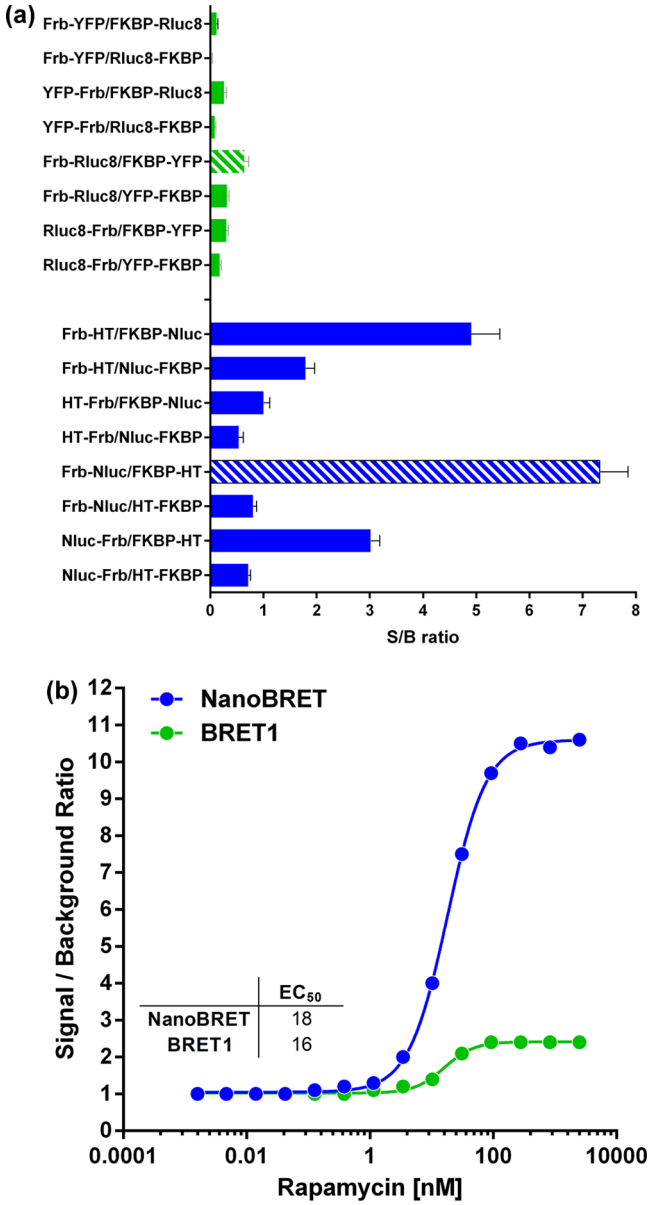


**Figure 3.** Comparative analysis of BRET1 and NanoBRET using the Frb/FKBP model system. (a) Optimization of donor and acceptor orientation for the Frb/FKBP interaction. BRET ratios were determined on HEK293 cells transiently transfected with the indicated Frb and FKBP fusion proteins at a donor/acceptor ratio of 1:4, each measured using untreated and rapamycin treated (1 $\mu$M, 15 min) samples. The optimal configuration for either BRET1 or NanoBRET is indicated by the hatch pattern. (b) HEK293 cells were transiently transfected with either Frb-Nluc/FKBP-HT or Frb-RLuc8/FKBP-turboYFP at a donor/acceptor ratio of 1:4. The cells were plated in a 384-well plate and left untreated ($n$ = 3) or treated with the indicated concentration of rapamycin ($n$ = 3) for 15 min before determining the BRET ratio.

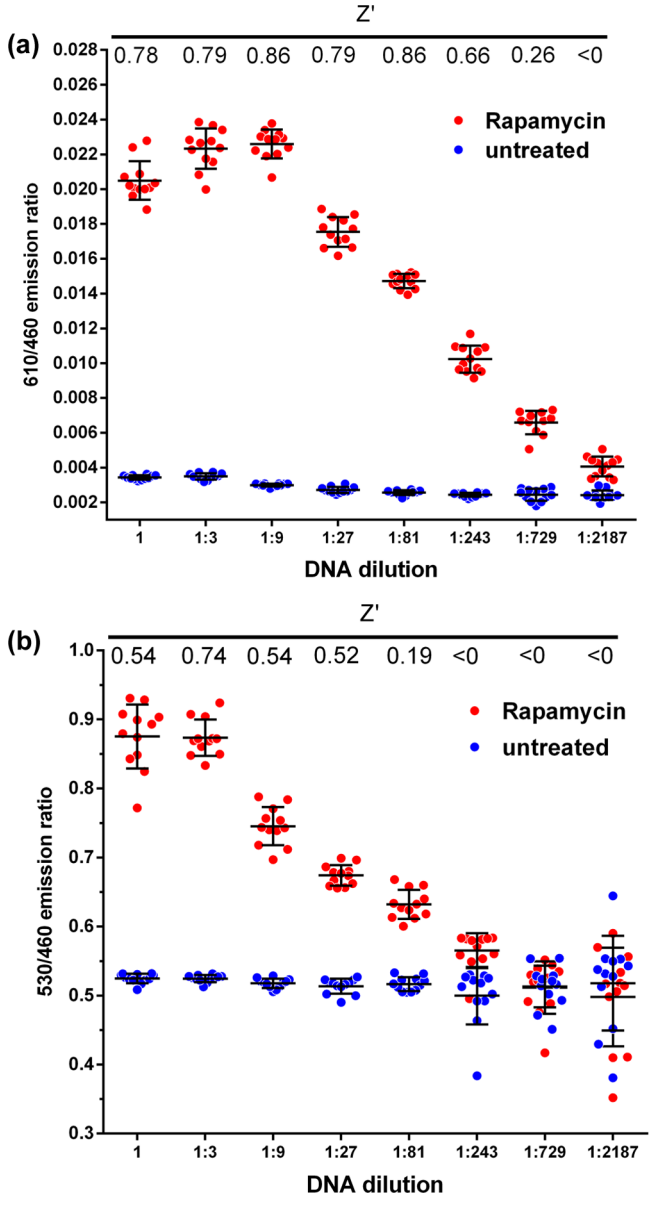


**Figure 4.** Effect of expression level on assay sensitivity for detecting the interaction of FKBP with Frb. HeLa cells were transfected with a serial dilution of expression constructs for (**a**) Frb-Nluc and FKBP-HT or (**b**) Frb-Rluc8 and FKBP-TurboYFP (1:1 ratio) and plated in thre 96-well plates. BRET ratios were determined for untreated or rapamycin treated samples (1 $\mu$M rapamycin, 15 min). Shown is a representative experiment with all individual data points ($n$ = 12) as well as mean ± SD plotted against DNA dilution used for transfection.

acetyl lysine, showed a dose dependent decrease of BRET signal with an IC50 close to published values (Figures 5A, see Supporting Information Figure S13A for uncorrected acceptor/donor channel emission ratios).[22] In similar studies with a non-BET family bromodomain protein, CBP, we do not see any displacement from chromatin with I-BET151, rather a slight increase we believe is due to increased occupancy of CBP after BET family displacement (Figures 5B, see Supporting Information Figure S13B for uncorrected acceptor/donor channel emission ratios). Similar results in both systems were obtained using histone H4-HT as the acceptor partner in place of histone H3.3-HT (Supporting Information Figure S13C and D). These results demonstrate that the action of I-BET151 can be observed by NanoBRET for specific pairs of bromodomain proteins and histones in living cells.

**NanoBRET for Cellular Imaging.** The ability to image BRET within individual cells has previously been restricted by the relatively low photon flux provided by bioluminescence. However, the bright luminescence of Nluc combined with recent advances in cellular imaging technologies is beginning to change this situation. We recently demonstrated that Nluc



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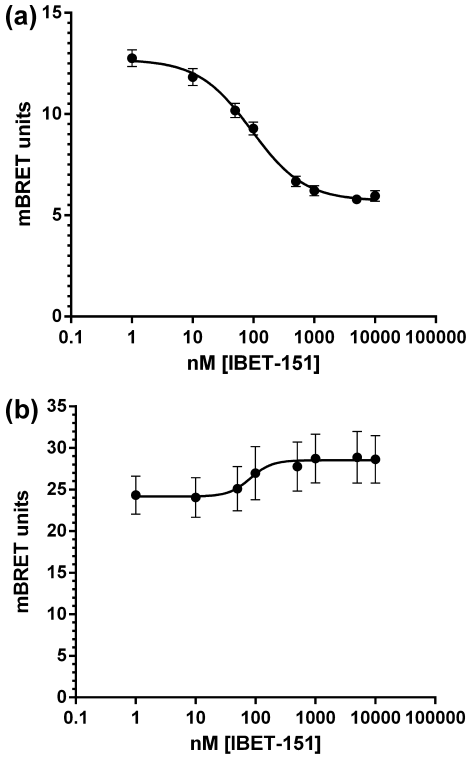


**Figure 5.** NanoBRET assays for bromodomain protein interactions with chromatin. NanoBRET assays were developed to measure the binding of Nluc-BRD4 (a) or Nluc-CBP (b) to Histone H3.3-HT. HEK293 cells were transfected with the indicated combination of constructs. The BRET ratio for each sample was determined following treatment for 18 h with the indicated concentrations of I-BET151. Shown are the means ± SEM of three independent experiments performed in quadruplicate.

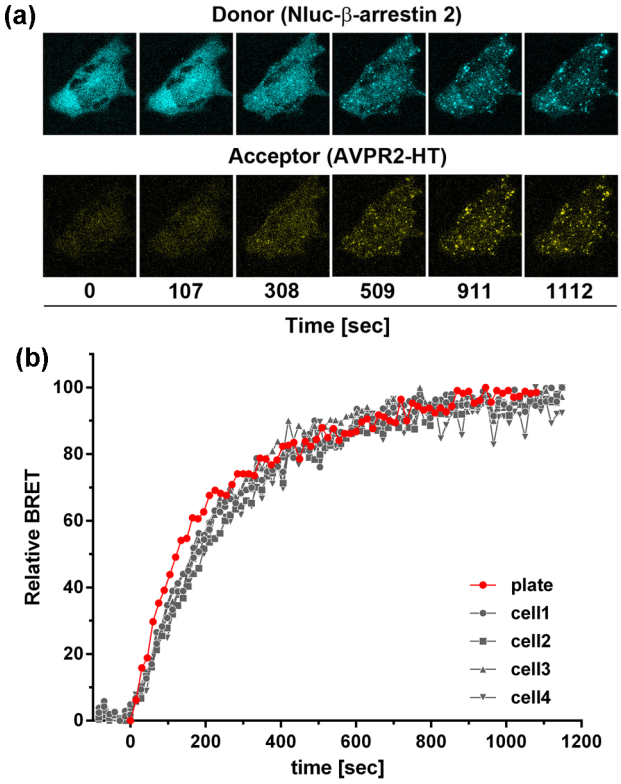


**Figure 6.** Real time imaging of ligand induced interaction of AVPR2-HT with Nluc-$\beta$-arrestin2 interaction. HeLa cells were transfected with expression constructs for AVPR2-HT and Nluc-$\beta$-arrestin2. Sequential acquisition of images was initiated under unstimulated conditions. After establishing a baseline for seven acquisition cycles, 1 $\mu$M arginine vasopressin was added to the sample by injection followed by acquisition cycles (13.4 s per cycle) covering approximately 20 min. (a) The panel shows pseudocolored images of the donor (cyan) and acceptor (yellow) channel at indicated time points following treatment with AVP. The images are representative of the results obtained in five independent experiments (scale bar = 10 $\mu$m). (b) Kinetic of Nluc-$\beta$-arrestin2 recruitment following stimulation with AVP measured by imaging or in plate reader. The imaging data represent five individual regions of interest (ROI), each representing a single cell. The data shown for the plate based assay format are the results of a representative experiment performed in triplicate. In order to compare results obtained from different analytical platforms, the data were plotted as relative change in BRET normalized to the maximum BRET ratio obtained for each data set.

enables visualization of subcellular translocation events within living cells with subsecond temporal resolution.[10] In this study, the well-established ligand-induced recruitment of $\beta$-arrestin 2 (ARRB2) to vasopressin receptor 2 (AVPR2) was used as a model for visualizing protein interactions.[23]

In the absence of a ligand, Nluc-ARRB2 showed the anticipated uniform distribution in cells (Figure 6A). A weak signal was also observed in the acceptor channel, which can be attributed to "bleed-through" of the Nluc luminescence. The addition of arginine vasopressin (AVP) induced a substantial signal increase in the acceptor channel, accompanied by the appearance of a vesicular pattern in both detection channels, which indicates association of Nluc-ARRB2 with AVPR2-HT, followed by trafficking into clathrin-coated pits (Figure 6A, Supporting Information Figure S14A).[24] The sequential imaging with short exposure times allowed the recording of arrestin recruitment kinetics in individual cells. Although transient transfection leads to variable expression levels between cells, the relative response kinetics were found to be similar and corresponded well with data obtained for a cell population in a 96-well plate format (Figure 6B, see Supporting Information Figure S14B and C for uncorrected acceptor/donor channel emission ratios).

In addition to the vasopressin receptor model, NanoBRET imaging was successfully applied to the rapamycin-induced interaction of FKBP with Frb, and the EGF-induced recruitment of grb2 to the EGF receptor (Supporting Information Figure S15A and B). The sustained high luminescence output of Nluc allows continuous monitoring of protein−protein interactions for up to 2 h and also improves spatial and temporal resolution. The enhanced feasibility for imaging of PPI in living cells afforded by NanoBRET may be of particular use for signaling events that are confined to specific subcellular compartments, especially if only a small subset of the donor-tagged protein is engaged in the interaction of interest.

**Conclusions.** BRET has been used for nearly 15 years to analyze protein−protein interaction in living cells. Benefiting from the capacity of luminescent chemistries to elucidate intracellular events, BRET assays are further advantaged by their ratiometric format for enhancing data precision and robustness. By representing acceptor proximity in relation to an available donor, the ratiometric normalization provides automatic correction for variable cell numbers or gene expression, inconsistent reagent potency, inadvertent luciferase inhibitors, and so forth. Yet, despite these advantages, usage of


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this technology has remained largely confined to relatively specific areas of investigation, most notably the analysis of cell surface receptor interactions. The main reason may be the comparatively limited sensitivity and dynamic range provided by current BRET systems.

With the recent development of Nluc, which emits bright, stable, and spectrally narrow luminescence, we saw an opportunity to design a BRET platform with substantially improved capabilities. This was achieved by pairing Nluc with a spectrally well separated acceptor, thus effectively reducing the background caused by "bleed through" of the donor signal into the acceptor channel. Our results indicate that NanoBRET (Figure 2) provides a reliable platform for analyzing protein−protein interactions at low expression levels in living cells, which is important for maintaining appropriate interaction dynamics. This applies not only to the tagged proteins subject to measurement but also to the many untagged endogenous proteins whose contributing influence comprises the physiological environment. The increased sensitivity of NanoBRET should be useful in combination with genomic editing technologies such as TALEN or CRISPR, allowing protein expression to be directed by the corresponding endogenous gene locus.[25]

Due to the delicate energy relationships associated with transient protein interactions, the influence of synthetic molecules on these interactions may be particularly reliant on the intracellular environment. Drug development founded on biochemical approaches may not adequately encompass the essential elements necessary to recapitulate a living molecular process. We have demonstrated that NanoBRET can deliver precise quantitative measurements of dynamic interactions as they occur within cells. Moreover, the technology delivers sufficient sensitivity to reveal these interactions occurring even to relatively small extents (i.e., at low fractional occupancies). The high emission intensity of NanoBRET also opens possibilities for imaging protein interactions within individual cells, and we speculate that the red-shifted emission of the acceptor may be suitable for imaging in living animals. In addition, linking fluorophores onto other types of molecules may prove useful for revealing a broader range of intracellular interactions.

The technical challenge of working with isolated full-length proteins also promotes the common usage of protein fragments as surrogates. Such simplified assays may not capture the multidomain and multifunctional nature of intracellular proteins and the many cellular factors which regulate their interactions. This is clearly demonstrated in our examples using NanoBRET to monitor interactions with chromatin. These interactions are highly modulated and dictated by the chromatin environment, including nucleosome positioning, presence of other transcription factors and transcription machinery, and the array of modifications present at any given time on the various histones. This environment cannot be replicated in a biochemical setting and thus highlights the necessity for monitoring such interactions within cells. NanoBRET configured with histones as energy acceptors should provide the ability to monitor interactions not only of bromodomains, as demonstrated here, but other chromatin interacting proteins as well.

## MATERIAL AND METHODS

**Fractional Occupancy Assay.** A 400 pM dilution of recombinant Nluc-HT, Nluc-turboYFP, and Nluc-tagRFP protein was prepared in PBS + 0.1% BSA (Promega). The Nluc-HT solution was split in separate samples and labeled for 180 min at RT with 5 $\mu$M of either the indicated fluorescent HaloTag ligands (Oregon Green, TMR, and NCT) or the nonfluorescent HaloTag PEG-biotin ligand. To prepare solutions representing different degrees of occupancy, the stock solutions of Nluc-HT-ligand, Nluc-TurboYFP, and Nluc-tagRFP were combined with Nluc-HT-PEG-biotin (as unoccupied donor) at defined ratios and a total concentration of 2 nM in PBS + 0.1% BSA. The percentage of acceptor occupied donor relative to total donor is referred to as fractional occupancy. BRET measurements were performed in white 96-well plates by combining 50 $\mu$L of the donor/acceptor mixtures with 50 $\mu$L of 20 $\mu$M furimazine in PBS. All measurements were performed at RT on a Thermo Scientific Varioskan Flash plate reader equipped with a 460/80 nm band-pass filter (donor) and 530 nm (HaloTag Oregon Green Ligand/TurboYFP), 590 nm (HaloTag TMR Ligand/Tag RFP), or 610 nm (HaloTag NCT Ligand) long-pass filters (acceptor) using an integration time of 0.5 s.

**Live Cell Labeling with HaloTag NCT Ligand.** For labeling of cells with the HaloTag NCT ligand, we developed two alternative protocols based on the needs of experimental PPI models (protocol use is also indicated in the description of the PPI models). *Rapid labeling protocol*: Cells were labeled by replacing the growth medium with labeling medium (Opti-MEM supplemented with 250 nM HaloTag NCT ligand) followed by incubation for 60−90 min at 37 °C. *Overnight labeling protocol*: Cells were labeled by replacing the growth medium with labeling medium (Opti-MEM supplemented with 4% FBS and 100 nM HaloTag NCT ligand) followed by incubation for 12−18 h at 37 °C. All HaloTag NCT ligand dilutions were performed using a stock solution of 5 mM HaloTag NCT ligand in DMSO. Subsequent treatment and processing of samples was performed as outlined in assay specific experimental procedures. Removal of excess ligand is not required since no unspecific bystander BRET was observed between the HaloTag NCT ligand and Nluc in biochemical or cell based assays for HaloTag NCT ligand concentrations of up to 500 nM.

**Frb/FKBP Interaction Assays.** HEK293 cells were transiently transfected with expression constructs for the indicated Frb and FKBP fusions and plated into a white 96-well tissue culture plate (Corning Costar #3917) at $2 \times 10^4$ cells/well in 100 $\mu$L volume. After 24 h of incubation, the growth medium was replaced by 50 $\mu$L Opti-MEM (Life Technologies). HaloTag containing samples were labeled by the addition of 50 $\mu$L of Opti-MEM containing HaloTag NCT Ligand at a concentration of 250 nM followed by incubation for 90 min at 37 °C. Interaction of Frb and FKBP was induced by the addition of rapamycin at the indicated concentration. After 15 min of incubation at 37 °C, furimazine (NanoBRET) or Coelenterazine h (BRET1) was added to each sample to a final concentration of 10 or 20 $\mu$M, respectively. All measurements were taken at RT using a Thermo Scientific Varioskan Flash plate reader using the filter setting as outlined above.

## ASSOCIATED CONTENT

### Supporting Information

Detailed experimental protocols, supplementary figures, and tables. This material is available free of charge at The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00143.

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### Notes

The authors declare no competing financial interest.



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