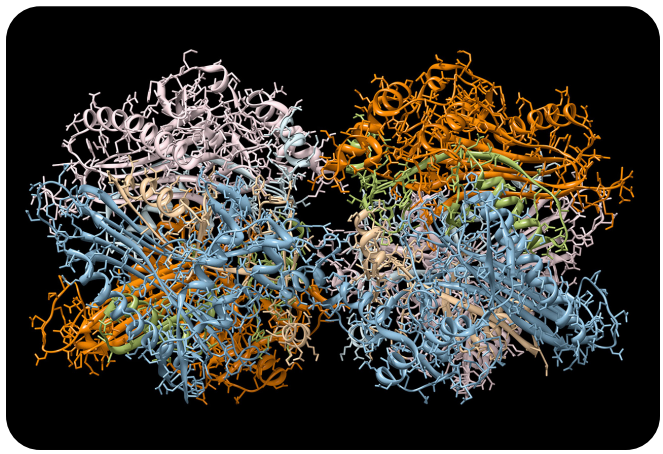
WHITE PAPER

Bioluminescence resonance energy transfer (BRET) to monitor protein-protein interactions

Introduction

Proteins perform their cellular functions primarily as components of complexes. Many human diseases can be traced to aberrant protein–protein interactions (PPIs), resulting either from the loss of an essential interaction or through the formation of an abnormal protein complex at an inappropriate time or location through interactions with endogenous proteins, proteins from pathogens, or both.

The ability to noninvasively image PPIs has important implications for a wide variety of biological research endeavors, including drug discovery and molecular medicine. In particular, the visual representation, characterization, quantification, and timing of these biological processes in living subjects can complement available *in vitro* or cell culture methodologies. In turn, such studies can accelerate the evaluation of novel drugs in living subjects that promote or inhibit active homodimeric or heterodimeric protein assembly, and could be employed to characterize known PPIs more fully in the context of whole-body physiologically authentic environments.



Bioluminescence resonance energy transfer (BRET)

For a long time, conventional biochemical assays like co-immunoprecipitation, gel-filtration chromatography, sandwich enzyme-linked immunosorbent assay (ELISA), etc., have been used in the investigation of PPIs. These *in vitro* assays though successful, do not suffice as imaging probes because they: (i) are essentially endpoint measurements, (ii) fail to provide spatio-temporal information on specific PPIs, (iii) require mechanical, chaotropic, or detergent- based cell lyses, which may alternate PPIs in some cases, (iv) are insensitive to transient interactions that regulate certain cellular processes, and (v) have little or no utility for *in vivo* imaging in live cells or subjects. To overcome these limitations, non-invasive imaging approaches such as bioluminescence resonance energy transfer (BRET) have been developed over the last decade, which allow the study of PPIs in their native environment and are capable of providing a unified platform that can be translated from cell culture- based assays to the imaging of live subjects.

revvity

Biophysical basis of BRET

BRET is an intrinsic phenomena occurring in the organisms Renilla reniformis and Aequorea victoria. Exploiting the underlying principles of BRET from nature, literatures have demonstrated BRET biosensor applications since 1999. The BRET phenomenon that follows the Förster resonance energy transfer (FRET) principle occurs between two proximally situated chromophores – a bioluminescent donor such as a luciferase protein and a fluorescent protein (FP) acceptor with overlapping emission and excitation spectra, respectively. Following donor excitation upon substrate addition, part of the electronic excitation energy of the donor is dissipated due to random collisions with other molecules, while the remaining electronic relaxation energy is transferred to the acceptor molecule through non-radiative dipole–dipole coupling. Upon excitation, the acceptor molecule emits its photonic energy at its characteristic wavelength. This results in a decrease in donor emission paralleled by an increase in acceptor emission. The strict dependence of BRET on the inter-chromophoric distance (1–10 nm) makes it an appropriate "molecular yardstick" for determining PPIs. This is true, since the average protein radius is ~5 nm, which means that a positive BRET signal will only be detected if the two proteins come within ~10 nm of each other, a distance that is an indicator of direct interaction between the two proteins. Due to the enzymatic nature of the donor molecule, no external illumination is required, and thus this assay offers high signal to background ratio and thereby excellent sensitivity. However, absence of a BRET signal does not necessarily mean that the two target proteins do not interact with each other. Lack of a signal can also be accounted for by an unfavorable orientation between the donor and acceptor dipoles.

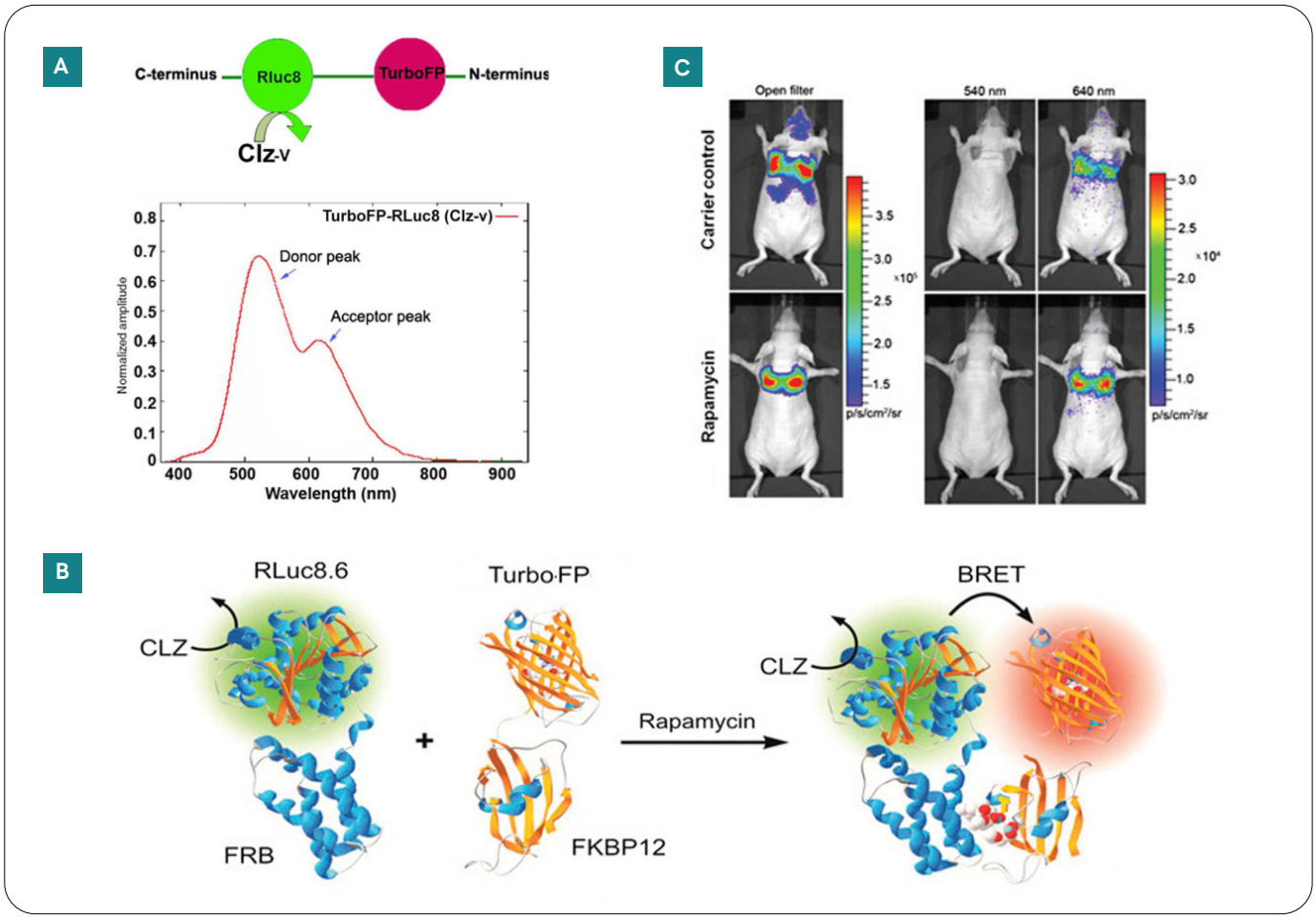


Figure 1. Bioluminescence resonance energy transfer performance in deep tissue imaging. (A) Generation of a BRET pair using mutant Renilla luciferase protein. TurboFP-RLuc8 pair using the Clz-v substrate. The chart represents normalized amplitude vs. wavelength measured from purified protein with Clz-v substrate. (B) Schematic illustration of the most successful BRET format tested for monitoring the rapamycin induced FRB-FKBP12 association. (C) Representative bioluminescence images of nude mice with accumulated mammalian cells in the lungs which stably over-express FRB and FKBP12 interacting partners fused to RLuc8.6 and TurboFP, respectively. The mice were injected i.v. with Clz substrate and sequentially imaged using open, donor, and acceptor filters. (adapted from 2. DE A, *et al.* Front.. Endocrinol. 2013; 4 (131):1-6.)

Expansion of BRET assay formats

In the past few years, improvements in various components of BRET such as luciferases, FPs, substrates, and instrumentations have contributed to the remarkable expansion in the range of BRET platforms available. Armed with these BRET vectors, the progress of molecular imaging of live cells, animals, and plants with varied applications has been made possible. With the advent of engineered RLuc variants with an elevated photon output and/or a red- shifted Em max, new BRET systems in combination with FPs in the orange and red regions of emission spectra were developed (Figure 1). More recently, the development of the small, bright, and stable Nanoluciferase (NanoLuc; Nluc) and its use in NanoBRET has vastly broadened the potential applications of BRET assays. This small (19 kDa) luciferase subunit Nanoluciferase (NanoLuc; Nluc) was derived from a larger multi-component luciferase isolated from the deep sea shrimp *Oplophorus gracilirostris*. In conjunction with its complementary substrate furimazine, Nluc's small size and superior luminescence profile has led to its rapid uptake in research and its use as a luciferase donor in BRET assays has resulted in the creation of the new BRET methodology termed NanoBRET. Alternatively, water soluble hydrofurimazine substrate may be considered for prolonged light production and improved sensitivity for BRET imaging.

Adoption of BRET assay as live cell HTS screening

Considering that the time requirement for PPIs vary case by case, improvements in signal sustainability can greatly expand the utility of BRET assays for real-time monitoring of events from live cells. The fast oxidation of coelenterazine substrate that results in rapid decays of donor emission is a limiting factor. Therefore, live cell coelenterazine such as Enduren or BRET2 compatible DeepBlueC substrate derivatives such as protected BDC compounds were developed by chemically synthesizing the oxidation site protected compounds with ester or ether groups. As cellular enzymes like esterases cleave the bonds, the nascent substrate molecule becomes available for the luciferase reaction. Thereby, in the process a much slower oxidation kinetics is seen resulting in a significantly longer lasting light signal. Use of such modified live cell substrates have eased the process for adapting BRET as a HTS format. Unlike the Rluc-Clz flash light kinetics, Nanoluc-Furimazine enzyme- substrate provides sustained light emission and therefore naturally competes as an alternative BRET donor.

Case study: A novel STAT3 phospho-BRET sensor to study STAT3 pathway dynamics

STAT3 is a key oncogenic signaling molecule primarily activated by pY705 phosphorylation leading to its homodimerization and nuclear translocation, where it acts as a potent transcription factor for many key target genes involved in oncogenic functions such as cell survival, proliferation, apoptosis, differentiation, angiogenesis, and cytokine functions. STAT3 is reported to be overexpressed in many cancer cell lines and primary tumors such as breast, prostate, head and neck, lung, gastric as well as several hematological malignancies. Literature clearly suggest that more than 50% of breast cancer cases are positive for STAT3 overexpression, which plays a pivotal role in disease progression. Pertaining to its essential role as an oncogenic player, identifying drugs through virtual screening of inhibitor libraries is a mainstay approach. Considering the importance of STAT3 as an oncogenic candidate, a highly sensitive protein phosphorylation biosensor using BRET was designed for deciphering live cell STAT3 dimerization kinetics. Furthermore, a high-throughput screening (HTS) assay based on this sensor was demonstrated to determine the inhibitory action of drugs against the STAT3 pathway.

For successfully developing a BRET-based STAT3 phosphorylation sensor, careful selection of an appropriate acceptor pair with the NanoLuc (Nluc)-donor is essential. Hence, three red fluorescent proteins, i.e. mOrange (ExMax 548 nm/EmMax 562 nm), TagRFP (ExMax 555 nm/EmMax 584 nm) or TurboFP (ExMax 588 nm/EmMax 635 nm) were selected and individually fused to Nluc protein on either the N- or C-terminus with a separation of 12 aa. flexible GGS linker (Figure 2).

After obtaining a valid BRET pair using Nluc and TurboFP (emission maxima beyond 600 nm) combination *in vitro*, the potential of this pair to detect PPIs in deep tissues by performing *in vivo* BRET is demonstrated (Figure 3). Due to the drastic difference in attenuation of signal for short wavelength emission, the bleed through subtracted corrected BRET ratios obtained for *in vivo* BRET were three-fold higher as compared to *in vitro* BRET. This shows the ability of Nluc-TurboFP pair for sensitive detection of protein interactions even with deep tissue imaging in living subjects. Precisely, the STAT3 phospho-BRET molecular sensor was designed by fusing STAT3 with either Nluc donor or TurboFP635 acceptor protein. As the Nluc-STAT3 and TurboFP-STAT3 fusions are phosphorylated and form

dimers, BRET signal increases indicating proximity driven transfer of resonance energy from the donor to the fluorescent acceptor molecule. Therefore, by adding furimazine substrate, a precise measurement of STAT3 activation can be obtained from live cell environment (Figure 4).

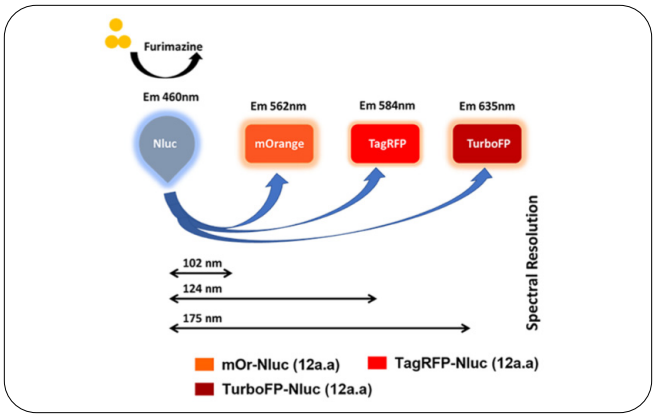


Figure 2. Development of Nanoluc based BRET system: A. diagrammatic representation of spectral separation between Nluc (NanoLuc) emission and excitation maximum of different fluorophores in presence of furimazine substrate. (from 5. Dimri S, *et al.* Am J Nucl Med Mol Imaging 2019; 9(6):321-334.)

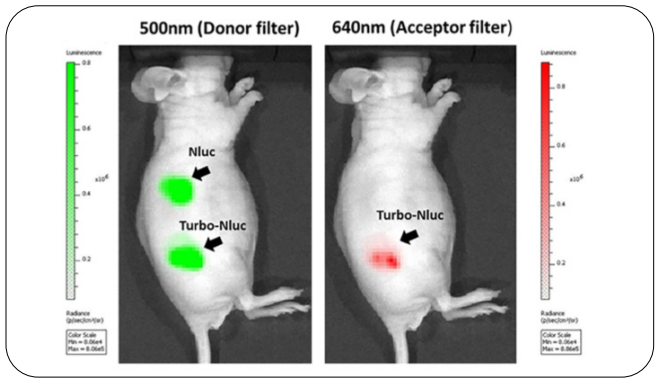


Figure 3. Representative image of nude mice implanted with HT1080 cells (3 × 106) stably expressing Nluc alone (top) and TurboFP-Nluc fusion protein (bottom) for *in vivo* BRET. Donor emission was collected at 500 nm filter and acceptor at 640 nm with 60 sec integration time per filter using furimazine as substrate. (from 5. Dimri S, *et al.* Am J Nucl Med Mol Imaging 2019; 9(6):321-334.)

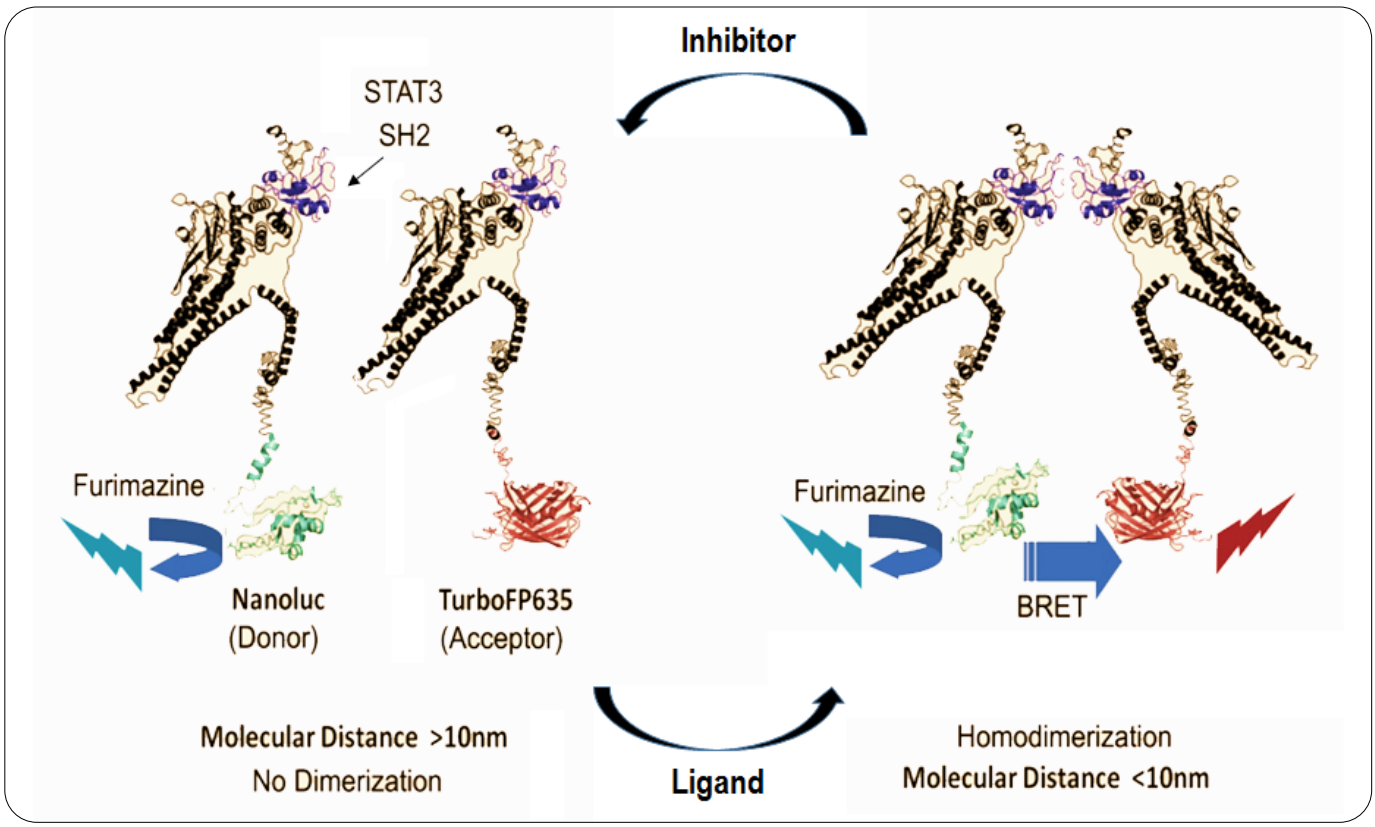


Figure 4. Schematic representation of STAT3 BRET sensor. STAT3 is fused with Nanoluc donor and TurboFP635 acceptor. In the presence of ligand as STAT3 dimerizes, the energy released from catalysis of furimazine substrate by Nanoluc enzyme is successfully transferred to TurboFP635 acceptor protein located in the closest proximity (<10 nm). As a result, TurboFP635 is excited and we get two resultant peaks, one in donor filter and other one in acceptor filter (640 nm). While in the absence of ligand, donor and acceptor molecules are further apart from each other, preventing the energy transfer to the acceptor protein and thus resulting in only donor signal. 3D model of full length STAT3 was developed using i-Tasser in-silico protein structure prediction software. The best model with highest c-score was selected for schematic representation of the STAT3 BRET sensor with modelled N-terminal domain.

The STAT3 Phospho-BRET biosensor can detect STAT3 activation and dimerization in different cancer cell lines and with multiple ligands and is compatible for HTS of different STAT3 inhibitors (Figure 5). For this, BRET assays in MCF7 cells expressing genetically encoded STAT3 phospho-BRET sensor were performed. A random screen of known and unknown STAT3 inhibitor compounds either in absence or presence of EGF yield significant results. Out of the seven compounds tested, four [MS-275, Niclosamide, Stattic, and Curcumin against EGF] show significant attenuation in STAT3 activity despite having EGF stimulation. Surprisingly, of the inhibitory compounds identified, Curcumin (74% inhibition) and niclosamide (80% inhibition) were more potent than the well-known STAT3 inhibitor, Stattic (22% activation), (Figure 5). The reason for higher potency of both niclosamide and curcumin could be attributed to their ability to inhibit STAT3 activation by blocking multiple pathways that directly or indirectly activate the STAT3 molecule, while Stattic is more specifically a SH2 domain binder.

Future developments: multiplexed BRET

Considering the complexities of signaling networks, modern biology demands measurement tools beyond the simple PPI assays. One might be interested in monitoring two or more concurrent dependent/independent PPI events within the same cell using spectral measurement of color-coded target proteins. With the series of BRET systems that are now available, one can recruit either a single/dual luciferase system with appropriate acceptor FPs, which can facilitate BRET multiplexing of three to four candidate proteins. The ease with which this objective can be achieved and the requirement of only a single substrate, makes it a highly attractive option for co-lateral protein interaction studies. Moreover, identification of new quantum efficient luciferase luciferin pairs that do not cross-react with D-luciferin, coelenterazine, or furimazine substrates will further broaden the scope of multiplexed BRET.

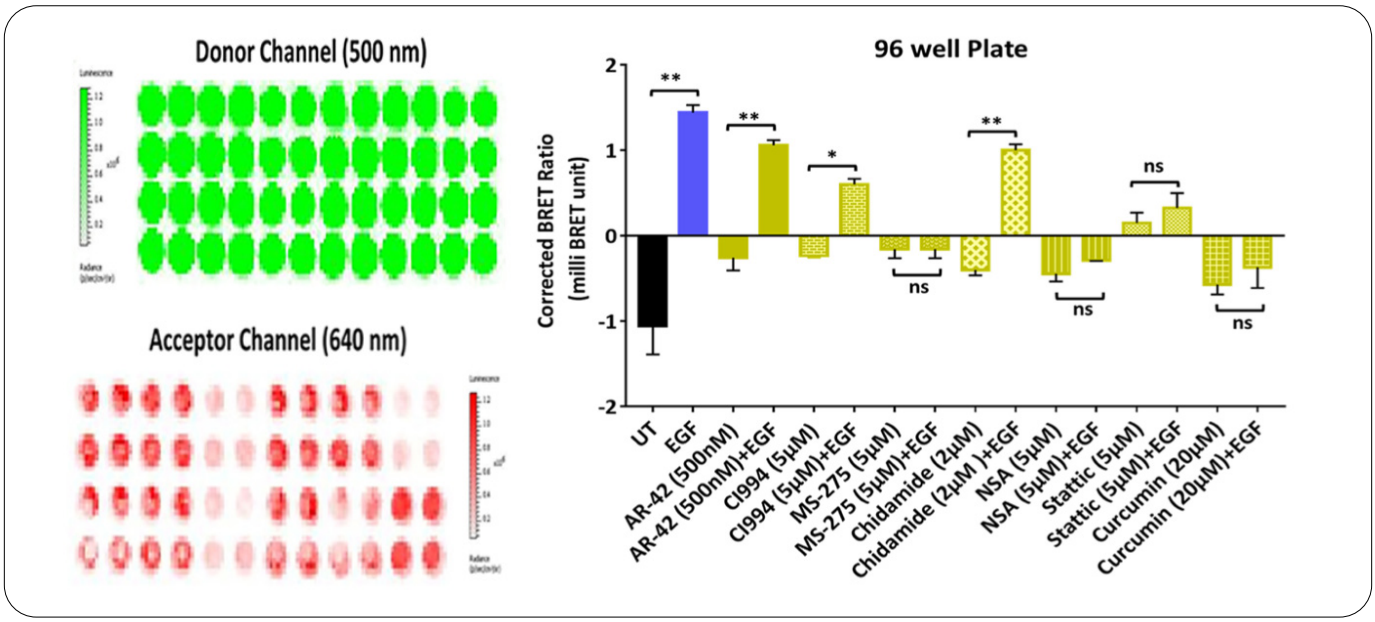


Figure 5. The STAT3 Phospho-BRET biosensor is HTS compatible for STAT3 pathway inhibitor screen: Representative well plate image of compound library screen using Phospho STAT3 BRET sensor in 96 black well plate, at specific donor and acceptor channels. Right graph represents corrected ratios calculated for each drug concentration with or without EGF (100 ng) (from 5. Dimri S, *et al*, Am J Nucl Med Mol Imaging 2019; 9(6):321-334.

Summary

Bioluminescence-based live cell assays are becoming increasingly attractive in biological applications as they are rapid, fairly sensitive, cost effective, and easy to perform. Some are even acquiescent to high-throughput systems and offer several advantages in comparison to other *in vitro* systems. BRET has been utilized for developing diverse live cell-based assays, many of which have now been adapted in small animal research for tracking specific protein functions, phosphorylation, and protease activation events as well as screening genetic and chemical modulators.

By making this technology versatile, their scope for BRET based molecular imaging of biological events from living cells and subjects will continue to expand.

References

1. Massoud TF, *et al.*, Reporter gene imaging of protein–protein interactions in living subjects., Curr Opin Biotechnol. 2007, 18(1): 31–37.
2. DE A, *et al.*, Evolution of BRET biosensors from live cell to tissue-scale in vivo imaging. Front. Endocrinol. 2013; 4 (131):1-6.
3. Schaub FX, *et al.*, Fluorophore-NanoLuc BRET Reporters Enable Sensitive *In Vivo* Optical Imaging and Flow Cytometry for Monitoring Tumorigenesis. Cancer Res. 2015; 75(23): 5023–5033.
4. Dale NC, *et al,* NanoBRET: The Bright Future of Proximity-Based Assays. Front. Bioeng. Biotechnol. 2019, 7:56.
5. Dimri S, *et al,* Dynamic monitoring of STAT3 activation in live cells using a novel STAT3 Phospho-BRET sensor. Am J Nucl Med Mol Imaging 2019; 9(6):321-334.
6. Dimri S, *et al,* Noncanonical pS727 post translational modification dictates major STAT3 activation and downstream functions in breast cancer. Experimental Cell Research, 2020 396 (112313).
7. Levi J. *et al* Bisdeoxycoelenterazine derivatives for improvement of bioluminescence resonance energy transfer assays, J Am Chem Soc. 2007;129(39):11900-1.

Authors

Asad Shahzada, Product Specialist, *In Vivo* Imaging Revvity

Ron Koop, Applications Scientist, *In Vivo* Imaging Revvity

Abhijit De Molecular Functional Imaging Lab, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre
Navi Mumbai, India