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Luciferase-Induced Photouncaging: Bioluminolysis

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ABSTRACT: Bioluminescence resonance energy transfer (BRET) has been widely used for studying dynamic processes in biological systems such as protein-protein interactions and other signaling events. Aside from being used as reporter, BRET can also act as a switch to turn on functions in living systems. Herein, we report the application of BRET to perform a biorthogonal reaction in living cells: releasing functional molecules via energy transfer to a coumarin molecule, a process termed bioluminolysis. An efficient BRET from Nanoluc-Halotag chimera protein (H-Luc) to a coumarin substrate yields the excited state of coumarin, which in turn triggers hydrolysis to uncage a target molecule. Compared to the conventional methods, this novel uncaging system requires no external light source, and shows fast kinetics ($t_{1/2}$ < 2 min). We applied this BRET uncaging system to release a potent kinase inhibitor, ibrutinib, in living cells, highlighting its broad utility in controlling the supply of bioactive small molecules *in vivo*.

Bioluminescent enzymes, collectively known as luciferases, have been broadly used as reporters in biological applications ranging from cell-based assays to *in vivo* imaging.[1-4] Luciferases catalyze the oxidation of chemical substrates to generate photons, with or without recourse to cofactors.[5] Compared to conventional fluorescence imaging, bioluminescence imaging is ultrasensitive, shows high signal-to-noise ratio and does not cause phototoxicity. Bioluminescence resonance energy transfer (BRET) thus attracted significant attention to study dynamic processes in biological systems such as protein-protein interactions, signaling events and as metabolite or drug sensors.[6-12] The vast majority of BRET applications have focused on using the photons generated by a luciferase as a signal reporting on an event. More recently, there has been a shift to extend the utility of BRET from a reporter function to an effector function. BRET has been utilized to control neuronal activity,[13-14] gene expression,[15] and a photo-switchable fluorescent protein.[16] A BRET-induced chemical reaction can also be used to change the function of bioactive molecules, and we reported a BRET induced ruthenium-photocatalyzed reaction.[17] While these precedents highlight novel applications, extension of the scope of chemistries that can be performed with bioluminescence is empowering. Herein we report the first example of direct photouncaging of small molecules with BRET in living cells, a process referred to as bioluminolysis (Figure 1).

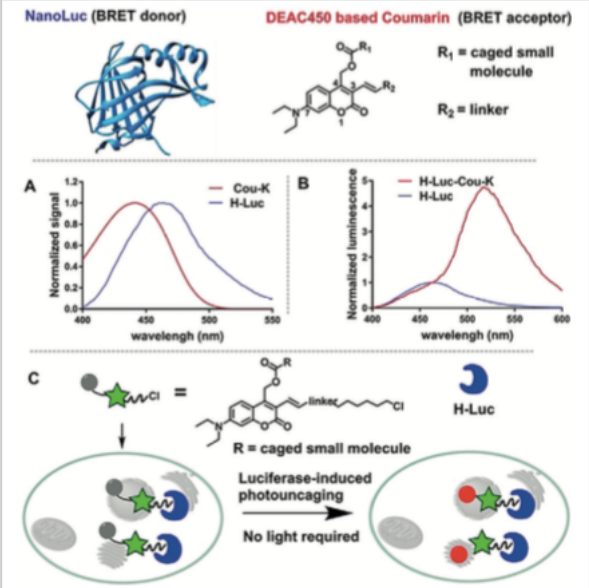

Figure 1. BRET-induced photolysis to release small molecule, bioluminolysis. A) Emission spectrum of **H-Luc** (blue, bioluminescence spectra) and DEAC450-based coumarin **Cou-K** (red, absorption spectra; see Figure S1 for structure of different coumarins used in this study). B) BRET of the H-Luc **Cou-K** conjugate (spectrum normalized to H-Luc emission). C) Schematic of targeted bioluminolysis (H-Luc = blue, coumarin = green, caged molecule = grey, uncaged molecules = red). Only the NanoLuc portion of the H-Luc is shown in the cartoon of H-Luc.

To spatiotemporally control small molecule activities in living systems, functional molecules are often caged by using photolabile protecting groups. Upon light irradiation, photoltic unmasking of the small molecule can quickly reveal a particular biological function.[18] Thus, light controlled release of bioactive molecules has enabled significant advances in biological studies and holds promise in clinical application. However, the majority of the available photocaging groups absorb in the UVA or UVB region.[19-20] Applications of this technology in

living tissue is limited by poor light penetration and phototoxicity of high intensity irradiation. Bioluminolysis, in which photolysis is induced by bioluminescence energy transfer, eliminates the need for external light and combines the advantages of BRET and optochemical control.

To establish the bioluminescence platform we chose the Nanoluc-Halotag chimera protein (H-Luc) that was recently developed by the Johnsson group[21]. H-Luc yields more efficient BRET than a Nanoluc-Halotag fusion protein. The choice of photolabile chromophore was based on the following criteria: i), an absorption maximum close to the emission maximum of H-Luc (~ 460 nm); ii), a high photolysis quantum yield, and iii) a molecular architecture allowing for dual-modification (photocaging and conjugation). In this way, the chromophore can be tethered to H-Luc for efficient BRET, whilst at the other site the desired cargo molecule can be appended.

While a number of photolabile chromophores have been reported, a select few suited our criteria. The Ellis-Davies group developed 7-diethylaminocoumarins with extended π-conjugation (acrylamide) at the 3-position, referred to as DEAC450.[22] The chromophore has an absorbance maximum of 450 nm rather than 360-380 nm for traditional aminocoumarins, thus overlapping with the emission spectrum of H-Luc (Figure 1A). DEAC450 have been used to uncage small molecules such as cGMP, GABA, and glutamate, with uncaging quantum yield ranging from 0.39-0.78.[23-26] Recently, DEAC450 derivatives replacing the acrylamide at the 3-position with electron-rich styryl moieties were reported to yield rapid photolysis.[27] In addition, the photolysis was accompanied by a cyclization involving the styryl group resulting in a less bright, blue-shifted fluorophore. Both of these photolabile fluorophores were investigated for bioluminolysis.

We initiated our study with a coumarin linked to H-Luc via the photolabile bond. In this case, the photolysis should be accompanied by loss of BRET (Figure 2A). We synthesized **Cou-HT A** (abs: 450 nm, em: 530 nm, see Figure S1 for synthetic routes of all compounds) with the Halo-tag ligand linked via a carbamate to 4-benzilic position of the coumarin. As anticipated, the conjugation to H-Luc proceeded rapidly at μM concentrations (k = 1.6 x 10^5 $M^{-1}s^{-1}$). Upon addition of furimazine to the H-Luc-Cou-HT A adduct, a high BRET efficiency (E_{RET} = 0.81) between H-Luc (em: 460 nm) and Cou-HT A was observed (Figure 2B and C). However, the intensity of the BRET was rapidly reduced ($t_{1/2}$ < 2 min). When the same adduct (H-Luc-Cou-HT A) was irradiated with a 455 nm LED lamp (1 W), and an aliquot of the reaction was treated with furimazine to quantify the BRET intensity, we found a comparable reduction of BRET as a function of irradiation time (Figure S2), indicating that the bioluminescence-induced uncaging is highly efficient, even when compared to the conventional irradiation method. Furthermore, this uncaging process can also be visualized through luminescence imaging (Figure 2D) taken by digital camera. H-Luc-Cou-HT A complex initially emitted green light, and the color progressively changed to blue after furimazine addition, due to the coumarin release, which is consistent with the changes of BRET emission spectra (Figure 2B). The rate of the reaction was analyzed as a function of furimazine concentration indicating that it reaches a plateau at 10 μM of furimazine with a rate of 6 x 10^{-3} s^{-1} (Figure S3). A control experiment with the same coumarin core structure (Cou-HT B) but linked to H-Luc through an uncleavable bond did not show comparable reduction of BRET (Figure S4). Taken together, these results support the bioluminolysis mechanism. Given the quantum yield of NanoLuc (0.3),[28] the E_{RET} (0.81) and the coumarin quantum yield (0.4), the overall yield of bioluminolysis is estimated at 10%.

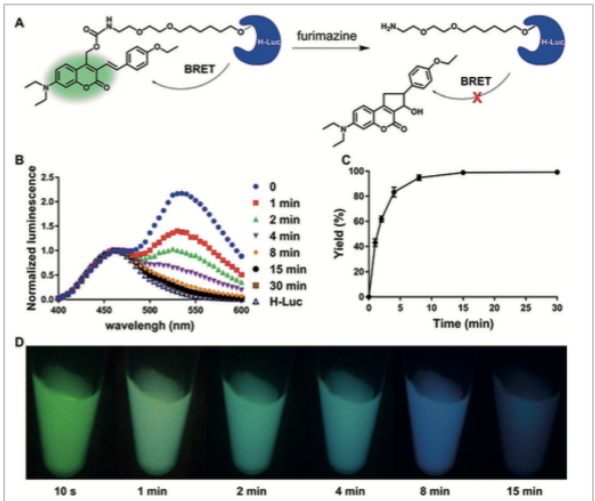

Figure 2. BRET-induced coumarin hydrolysis. A) Scheme of the bioluminolysis of H-Luc-**Cou-HT A** conjugate. B) Luminescence spectrum of H-Luc-**Cou A** conjugate (100 nM in HEPES buffer with 10 mg mL^{-1} BSA) over time following the addition of furimazine (50 μM final concentration). C) Reaction progress calculated based on the BRET ratio. D) Images of BRET-induced coumarin hydrolysis (same conditions as in (B)) captured using a DSLR camera at different time points, exposure time = 10–20 s, F value 14.1, ISO auto.

Encouraged by our initial observations that BRET could be used to cleave functional molecules via bioluminolysis, we next investigated the reaction in living cells. We tested the reaction in H-Luc expressing Hela cells. Cells treated with **Cou-HT A** for 15 min retain the molecule after washes since it is covalently linked to H-Luc. We first tested whether **Cou-HT A** could be imaged without photolysis. We found that 0.05-0.1% laser power (488 nm) was sufficient to detect the coumarin however did not result in significant photolysis, even after 10 min exposure (Figure S5). As shown in Figure 3, the signal of coumarin channel shows the labeling of coumarin with Halo-tag linker. After addition of furimazine, we observed a significant fluorescence decrease in 15 minutes, indicating that the coumarin was released from H-Luc through bioluminolysis. However, no significant changes are observed in the negative control with **Cou-HT B** (Figure 3), which is linked to H-Luc via an uncleavable bond.

These proof-of-concept experiments showed that BRET induced coumarin bioluminolysis can work both *in vitro* and in living cells. Therefore, we shifted our focus to the possibility of delivering biomolecule effectors using this system. The interaction of streptavidin and biotin is among the strongest noncovalent binding and it has been widely used in the detection and purification of biomolecules. Therefore, we sought to achieve BRET-opto-control of the binding between streptavidin and biotin fused H-Luc protein. We synthesized **Cou-biotin** (Figure 4A) for this purpose. We opted for the acrylate moiety with a PEG rather than the styryl moiety at the 3-position because the latter proved less soluble in the buffers used. H-Luc was labeled with **Cou-biotin** and incubated with streptavidin beads. Again, the BRET from H-Luc to **Cou-biotin** was very efficient (E_{RET} = 0.91) (Figure 4B), and quickly induced cleavage of the coumarin moiety, resulting in the H-Luc-Coumarin being released from the streptavidin beads. After 5 min, 42% of the bioluminescence signal of H-Luc was in the supernatant rather than on the beads (Figure 4C), and almost complete release of H-Luc protein from the streptavidin beads was achieved within 30 minutes. In a parallel experiment, the reduction of bioluminescence signal on streptavidin beads was observed by bioluminescence microscopy (Figure 4D-H). The signal was significantly reduced after 5 min, and the reaction was complete after 30 minutes. These experiments clearly show that BRET induced coumarin hydrolysis can reduce the interaction between streptavidin and H-Luc with fast dynamics, offering a possibility of controlling protein-protein interaction via bioluminolysis.

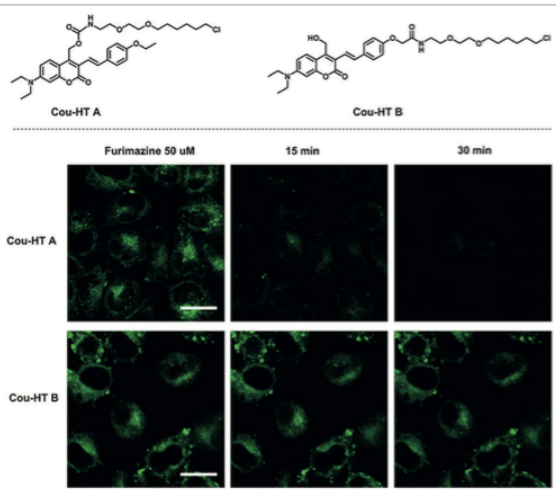

Figure 3. BRET-induced coumarin hydrolysis in live cells. Hela cells expressing H-Luc were treated with **Cou-HT A** or **Cou-HT B** (1 μM final concentration, 15 min), and cells were washed. Furimazine was then added (50 μM final concentration) and the cells were imaged at the indicated time points by fluorescence imaging (λ_{ex} = 488 nm laser and λ_{em} = 510–550 nm). Scale bar = 20 μm.

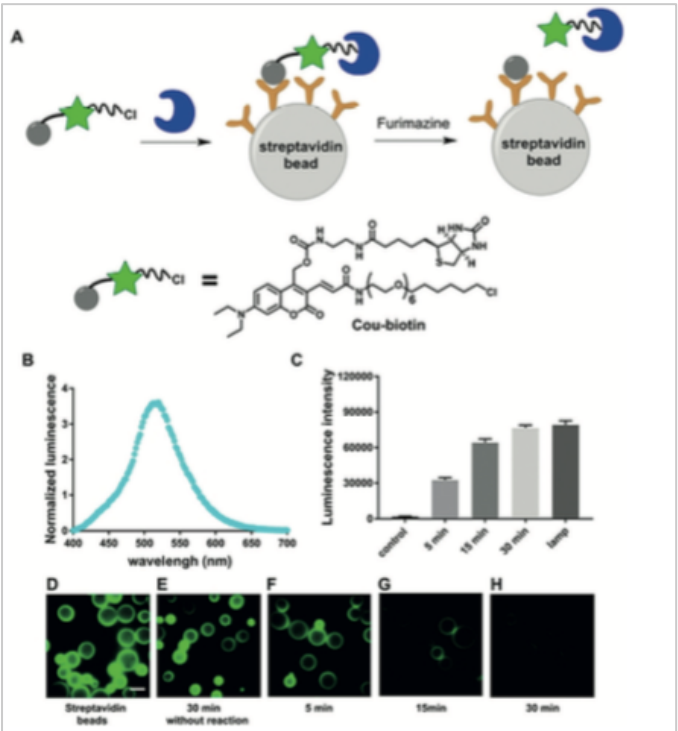

Figure 4. A) BRET-induced H-Luc release from streptavidin beads. B) The BRET from H-Luc to **Cou-biotin** C) Quantification of luminescence in the supernatant as a function of time after addition of furimazine (50 μM final concentration) and compared to a photolysis performed with 1 W LED lamp (455 nm). Bioluminescence intensity of H-Luc was measured by a plate reader. D) Bioluminescence imaging of photolyzed beads, E) beads were kept for 30 min before imaging to show stability of **Cou-biotin**, then 50 μM furimazine was treated for F) 5 min, G) 15 min, and H) 30 min. Scale bar = 20 μm. Bioluminescence images were recorded on an Olympus LV200 imaging system with exposure time of 2 s, no filter.

Next, we investigated bioluminolysis to uncage a bioactive molecule in cellulo. The model bio-effector we chose is a potent covalent kinase inhibitor that reacts with BTK and ErbB2 with an IC_{50} of 3-6 nM.[29] We designed and synthesized a coumarin caged ibrutinib with a Halo-tag linker (**Cou-ibrutinib**, Figure 5). This caging position was anticipated to abrogate the inhibitor-kinase interaction based on the fact that the aniline interact with the protein surface deep in the nucleotide binding pocket (PDB: 5P9J[30] and 5YU9[31]). The presence of a bulky caging group at this position would thus preclude drug binding. Biochemical assays with purified Erbb2 confirmed that the caged **Cou-Ibrutinib** was not functional (Figure S6). However, bioluminolytic release of ibrutinib would result in covalent inhibition of the kinase. In order to quantify target engagement by the release drug in cell, we used an **ibrutinib-Cy5** conjugate to label the kinase[32] (the use of non-sulfated cyanine dye afford cell-permeable probes).[33] Treatment of SKBR3, a breast cancer cell line expressing ErbB2, with **ibrutinib-Cy5** followed by a wash results in a strong Cy-5 stain arising from the covalent interaction of the drug with its target kinase. However, if ibrutinib was previously introduced by bioluminolysis of **Cou-ibrutinib**, and sufficient amounts were released to saturate the target, no labeling should be observed upon treatment with **ibrutinib-Cy5**.

Furthermore, the caged version of **Cou-ibrutinib** should not inhibit the labeling of the target kinase. H-Luc expressing SKBR3 cells were treated with Cou-ibrutinib (2 µM, 15 min) and subsequently with ibrutinib-Cy5. Indeed, the level of kinase labeling was comparable to the same cell treated directly with **ibrutinib-Cy5**. However, if furimazine was added (i.e. **Cou-ibrutinib**, 2 µM, 15 min; wash; furimazine, 30 min incubation; **ibrutinib-Cy5**) much lower Cy5 signal was observed (Figure 5, see Figure S7-8 for further control experiments and LC-MS characterization of **Cou-ibrutinib** photolysis). We also determined that furimazine does not interfere with kinase labeling by initially treating cells with furimazine, followed by **ibrutinib-Cy5**. We observed no difference in the fluorescence signal of **ibrutinib-Cy5** in cells treated with or without furimazine (Figure 5C). Collectively, these experiments demonstrate that bioluminolysis can be used to deliver effective doses of a bioactive molecules in target cells expressing H-Luc. While this experiment did not focus on subcellular localization, the ability to direct H-Luc to specific subcellular compartments suggest that drug could be delivered with unprecedented resolution in cell culture or even whole organisms. Interestingly, the signal in the coumarin channel also decreased as the reaction progressed. It was previously reported that coumarin modified with electron-rich donor styryl moieties at the 3-position yielded a photolysis product that cyclized (see Figure 2A for structure) thus blue shifting the fluorophore.[27] Monitoring of the photolysis of **Cou-ibrutinib** by LC-MS after irradiation with 455 nm LED lamp showed two distinct products for the coumarin moiety (same molecular weight), the expected alcohol and a second product with a blue-shifted absorption (Figure S8). The formation of this product was corroborated by the measurement of absorption and fluorescence spectra over time where the intensity of the 450 nM absorption is reduced over time resulting in a dramatic reduction of fluorescence at 520 nm (Figure S9).

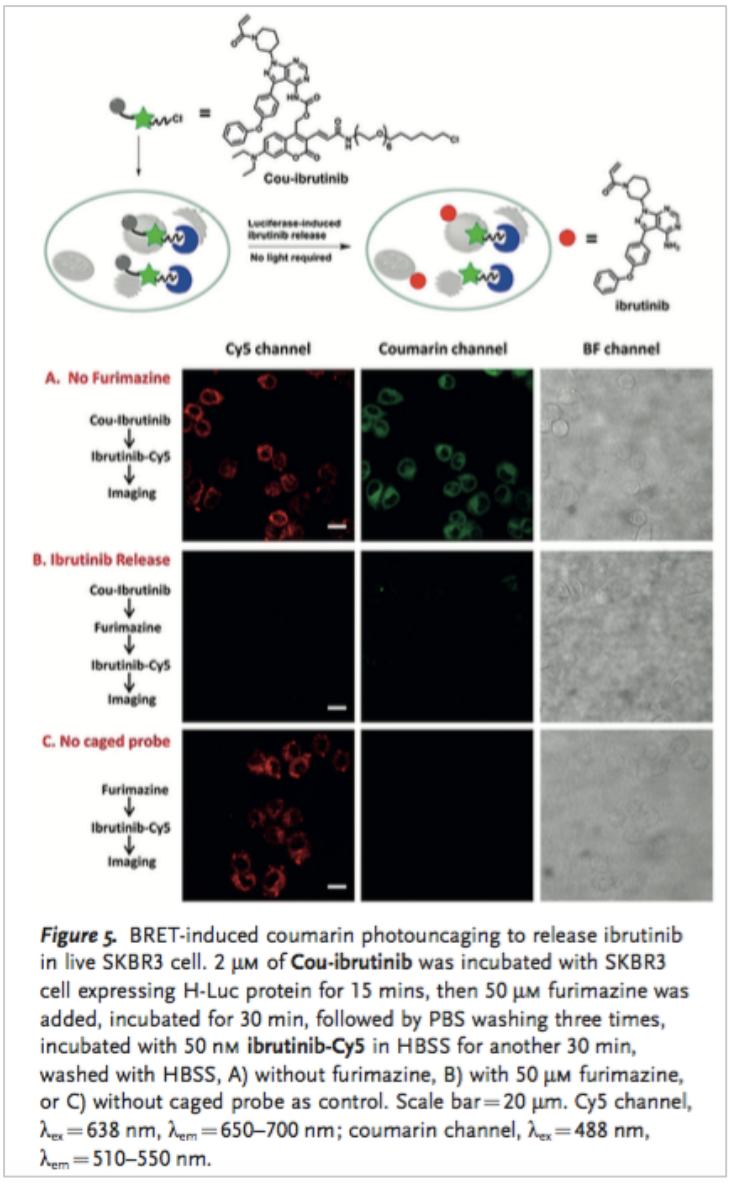

Figure 5. BRET-induced coumarin photouncaging to release ibrutinib in live SKBR3 cell. 2 µM of **Cou-ibrutinib** was incubated with SKBR3 cell expressing H-Luc protein for 15 mins, then 50 µM furimazine was added, incubated for 30 min, followed by PBS washing three times, incubated with 50 nM **ibrutinib-Cy5** in HBSS for another 30 min, washed with HBSS, A) without furimazine, B) with 50 µM furimazine, or C) without caged probe as control. Scale bar = 20 µm. Cy5 channel, λ_{ex} = 638 nm, λ_{em} = 650–700 nm; coumarin channel, λ_{ex} = 488 nm, λ_{em} = 510–550 nm.

In conclusion, we have developed a novel photouncaging system induced by luciferase without the requirement of external light. To the best of our knowledge, it is the first example of BRET-induced photolysis. This bioluminolysis was realized by the highly efficient conjugation of an appropriately functionalized coumarin to a Halotag-Nanoluc chimera protein (H-Luc). The reaction *in vitro* shows fast kinetics ($t_{1/2}$ < 2 min), comparable to photolysis by light irradiation. We could also demonstrate the BRET uncaging works efficiently in HeLa and SKBR3 cells. As the photouncaging reaction can be achieved with bioluminescence, instead of external light, the reaction does not suffer from limitations of light penetrability and phototoxicity. Since luciferase can be freely expressed in living systems, in principle this strategy offers a possibility of releasing bioactive small molecules with subcellular specificity and tissue specificity. The technology may also find therapeutic application with engineered cells that would express H-Luc in response to an antigen.

ASSOCIATED CONTENT

Supporting Information

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201907734.

Raw data has been deposited (https://doi.org/10.5281/zenodo.3374180).

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