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Supplementary Information

Ligand-Activated BRET9 Imaging for Measuring Protein-Protein Interactions in Living Mice

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Suppl. Tables

Suppl. Table 1. Summary of the optical performance of different BRET probes.

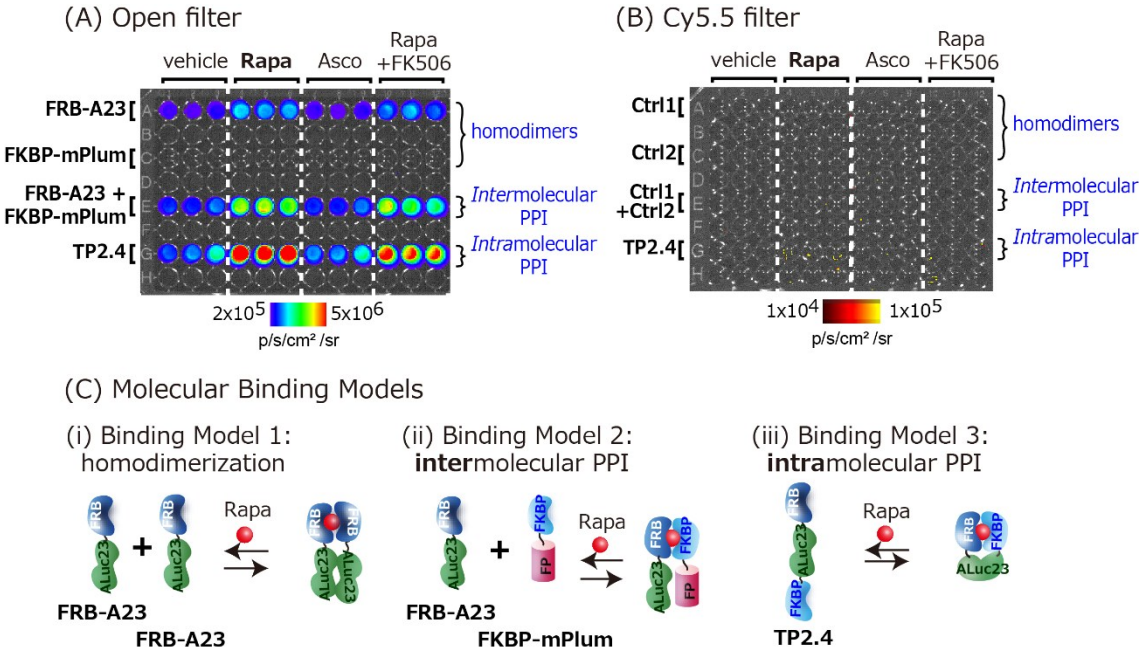
Probe category	Probes involved in PPI	Binding character	Stimulator	Signal-to-Background ratio (S/B ratio)***	Peak height (%) (608 nm/503 nm)
Control	FRB-A23 plus FRB-A23	**Inter**molecular homodimer	vehicle	1.0 (±0.2)*	
			Rapa	3.5 (±0.3)*	
			Asco	1.0 (±0.3) *	
			Rapa + FK506	3.0 (±0.6) *	
	FKBP-mPlum plus FKBP-mPlum	**Inter**molecular heterodimer	vehicle	1.0 (±0.2) *	
			Rapa	1.5 (±0.6) *	
			Asco	0.9 (±0.2) *	
			Rapa + FK506	1.3 (±0.2) *	
	FRB-A23 plus FKBP-mPlum	**Inter**molecular homodimer	vehicle	1.0 (±0.2) *	
			Rapa	3.5 (±0.2) *	
			asco	1.0 (±0.3) *	
			Rapa + FK506	3.1 (±0.7) *	
	TP2.4	**Intra**molecular heterodimer	vehicle	1.0 (±0.1)**	2.9 (±1.1)*
			vehicle	**1.0 (±0.0)*****	11.9 (±1.6)*
			Rapa	4.4*	
			Rapa	**1.0 (±0.0)*****	
			Rapa	3.1 (±0.1)**	
			Tem	2.9 (±0.1)**	
			Eve	3.2 (±0.2)**	
			Def	3.2 (±0.3) **	
			Resv	0.9 (±0.1) **	
v1 series	v1_mOrg	**Intra**molecular heterodimer	Rapa	2.3*	
	v1_mRasb	**Intra**molecular heterodimer	Rapa	2.4*	
	v1_mChe	**Intra**molecular heterodimer	Rapa	2.4*	
	v1_mNep	**Intra**molecular heterodimer	Rapa	2.5*	

	v1_mPlum	**Intra**molecular heterodimer	Rapa	2.5*	
v2 series	v2_mOrg	**Intra**molecular heterodimer	Rapa	2.2*	
	v2_mRasb	**Intra**molecular heterodimer	Rapa	3.4*	
	v2_mChe	**Intra**molecular heterodimer	vehicle	1.0 (±0.1) **	4.8 (±1.6)*
			vehicle	**1.0 (±0.1)****	**21.0** (±3.4)*
			Rapa	3.3*	
			Rapa	**3.0 (±0.3)****	
			Rapa	3.1 (±0.4)**	
			Asco	1.1 (±0.1)**	
			Rapa + FK506	2.3 (±0.2)**	
			Eve	2.3 (±0.3)**	
			Def	2.5 (±0.1)**	
			Tem	1.7 (±0.1)**	
	v2_mNep	**Intra**molecular heterodimer	Rapa	2.2*	
	v2_mPlum	**Intra**molecular heterodimer	vehicle		3.8 (±0.5) *
			Rapa	2.4*	13.2 (±1.4) *
v3 series	v3_mOrg	**Intra**molecular heterodimer	Rapa	1.4*	
	v3_mRasb	**Intra**molecular heterodimer	Rapa	1.6*	
	v3_mChe	**Intra**molecular heterodimer	Rapa	1.4*	
	v3_mNep	**Intra**molecular heterodimer	Rapa	1.6*	
	v3_mPlum	**Intra**molecular heterodimer	Rapa	1.5*	

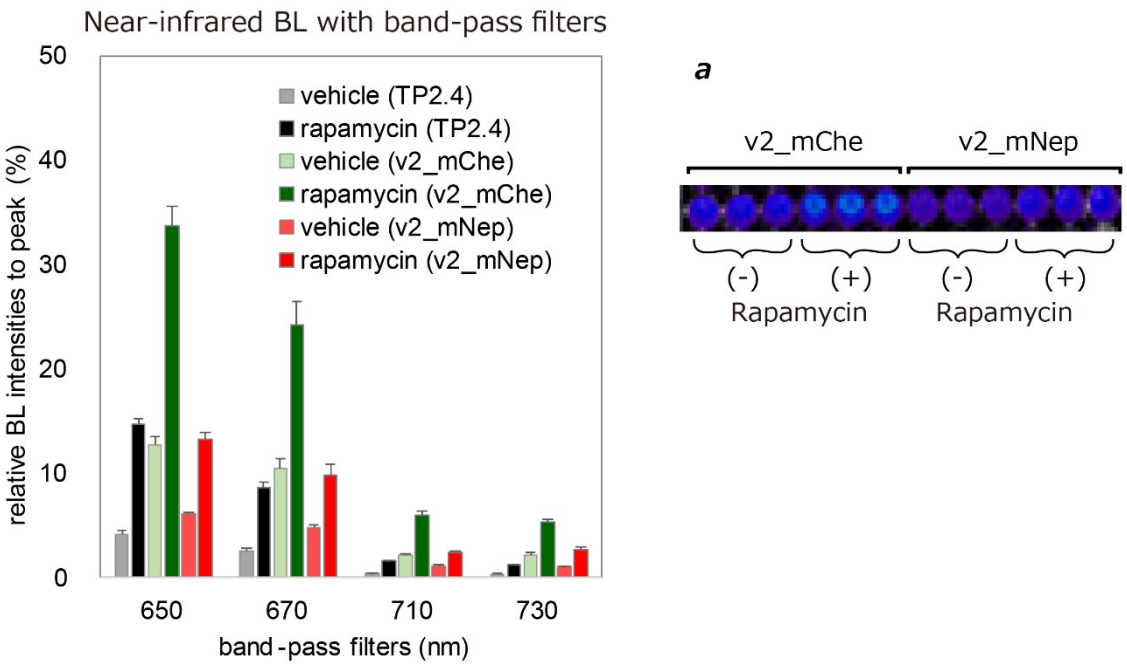
*Transiently transfected cells.
**Stably expressed cells.
***Xenografts in animals
****The parenthesis shows Standard Deviation (±SD) in two sigma.

Abbreviations: Rapa, Rapamycin; Def, Deforolimus; Tem, Temsirolimus; Eve, Everolimus; asco, ascomycin.

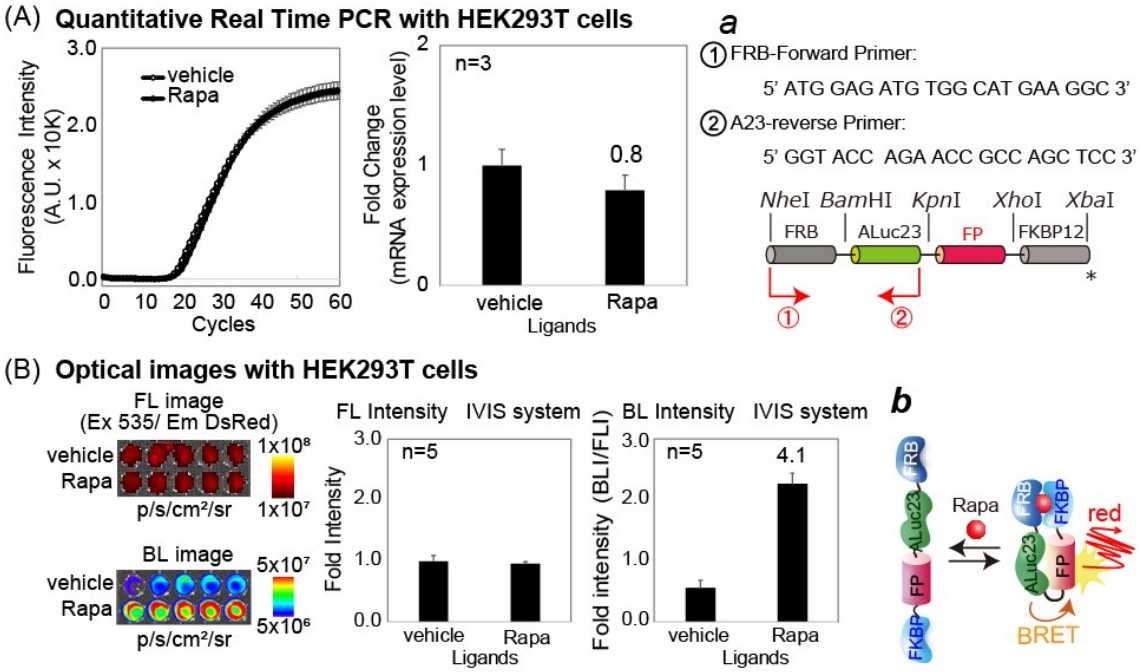
Suppl. Figures



Suppl. Figure 1. Difference in the absolute bioluminescence (BL) intensities according to molecular binding models. MDA-MB231 cells transiently transfected with pFRB-A23, pFKBP-FP, pTP2.4, or a mixture of pFRB-A23 and pFKBP-FP were stimulated with rapamycin, ascomycin, or the mixture of rapamycin plus FK506. (A) The open-filtered BL image ($n = 3$). (B) The Cy5.5-filtered BL image ($n = 3$). (C) Illustration of three molecular binding models: homodimerization, *inter*molecular and *intra*molecular PPIs.



Suppl. Figure 2. The relative BL intensities of the MDA-MB231 cells stably expressing the selected probes in the presence or absence of rapamycin in the far red (FR) region. The signs (+) and (-) mean the presence or absence of rapamycin, respectively ($n = 3$). The inset '*a'* shows the BL image that was obtained with a 670 nm band-pass filter, where v2_mChe shows superior BL intensities compared to v2_mChe. The inset '*a*' represents the represent BL images of v2_mChe and v2_mPlum in response to rapamycin.



Suppl. Figure 3. Characterization of the expression and degradation levels of v2_mChe after stimulation using rapamycin in HEK293T cells. (A) Determination of mRNA levels of v2_mChe using quantitative Real-Time PCR. Inset *a* shows the specific primers (① and ②) for the RT-PCR and their recognition sites inside the probe backbone. (B) Analysis of the FLI and BLI variations of v2_mChe after overnight stimulation of rapamycin. The overall results show that the BLI is solely enhanced by rapamycin-triggered PPI of v2_mChe. Inset *b* shows a schematic of the potential working mechanism.

Suppl. Experimental Procedures

Construction of a series of plasmids encoding BRET9 probes for measuring rapamycin-activated PPIs.

The cDNA constructs encoding BRET9 series probes were made by tandemly linking four different cDNA fragments encoding the following proteins: FRB (94 aa, PDB: 1AUE_A), artificial luciferase 23 (ALuc23, GenBank: MF817968), the FK506 binding protein (FKBP12) (109 aa, GenBank: AAP36774), and one of the FPs: i.e., mCherry, mNeptune, mOrange, mPlum, and mRasberry (Figure 1). For convenience, these FPs are here abbreviated to mChe, mNep, mOrg, mPlum, mRasb, respectively.

We obtained all the synthesized templates above by custom-ordering from Eurofin Genomics (Tokyo, Japan). We generated each cDNA fragment by PCR with an appropriate primer set to introduce unique restriction sites at the 5' and 3' ends. Specifically, we created *Nhe*I/*BamH*I or *Age*I/*BamH*I sites at the ends of the gene encoding FRB; we introduced *BamH*I/*Kpn*I sites at the ends of the gene encoding ALuc23; and similarly, *Kpn*I/*Xho*I or *Xho*I/*Xba*I sites at the ends of the gene encoding FKBP12; and *Xho*I/*Xba*I, *Kpn*I/*Xho*I, or *Nhe*I/*Age*I sites at the ends of the cDNA fragment encoding each FP. We digested the corresponding cDNA blocks with the respective restriction enzymes, purified, and then ligated into the corresponding restriction enzyme-digested pcDNA3.1(+) vector backbone (Invitrogen). We named the constructed plasmids: pBRET9_v1_FP, pBRET9_v2_FP, or pBRET9_v3_FP, where FP abbreviates each gene encoding a FP that was embedded in the plasmid backbone. The single-chain probes after expression were simply called v1_FP, v2_FP, or v3_FP, respectively.

We categorized the engineered BRET probes after expression into four different groups according to the molecular structures and named them v0-v3 series (Figure 1). As such, (i) the acceptor FP was placed at the C-terminal end of the probe in 'v1 series', (ii) the acceptor FP was located at the middle of the probe in 'v2 series', (iii) the acceptor FP was set at the N-terminal end of the probe in 'v3 series', and (iv) their controls were designated as 'v0 series'. We named every probe according to the following general designation: vX_Y, where "X" and "Y" indicated the category number and the abbreviation of each inserted FP, respectively. For

instance, the v2 series probes embedding mCherry and mPlum were named v2_mChe and v2_mPlum, respectively.

As a reference, we constructed two "control" pcDNA3.1(+) plasmids, which were fragmented forms of v2_mPlum (i.e., FRB-ALuc23-FKBP12-mPlum) (Figure 1(B)). We named the plasmids encoding the fragmented construct, pFRB-A23 and pFKBP-mPlum, respectively. We named the other "control" plasmid encoding FRB-ALuc23-FKBP12, pTP2.4. We designed this control plasmid to contain no FP. We ensured the fidelity of all the cDNA constructs by testing with a genetic sequence analyzer (Eurofin genomics).

Characterization of *inter*- or *intra*molecular PPIs.

As a control study, we studied the *inter*- or *intra*molecular PPI models shown in Figure 1(C). We cultured MDA-MB231 cells (obtained from ATCC HTB-26) in 6-well plates (Nunc) and incubated until 80% confluency. We transiently transfected these cells with a lipofection cocktail (TransIT-LT1, Mirus) dissolving (i) pFRB-A23, (ii) pFKBP-mPlum, (iii) pFRB-A23 plus pFKBP-mPlum, or (iv) pTP2.4. We then trypsinized cells, plated in a 96-well clear bottom microplate, and incubated overnight. We stimulated the cells with vehicle, 10^{-6} M rapamycin, 5 x 10^{-6} M ascomycin, or a mixture of 10^{-6} M rapamycin and 5 x 10^{-6} M FK506 for 5 h. Next, we lysed the cells with a commercial lysis buffer (Promega) according to the manufacturer's instruction. Lysates in the microplate were mixed with an aliquot of a PBS buffer (pH 5.7) dissolving native coelenterazine (nCTZ) (final concentration = 10 μg/mL). We immediately determined the corresponding optical intensities using an IVIS Lumina II system equipped with open and Cy5.5 band pass filters (Caliper). We analyzed the BL images using the analysis software Live Image 4.5.5 (Caliper).

Characterization of BRET9 series probes in the presence or absence of rapamycin.

We determined the differences in the absolute BL intensities before and after rapamycin addition using MDA-MB231 cells transiently expressing each one of the BRET9 series probes (Figure 2(A)). We plated the MDA-MB231 cells transiently expressing each BRET9 series probe in a 96-well clear bottom microplate, and incubated overnight. We then stimulated the cells with 10^{-7} M rapamycin for 5 h. Cell lysates were prepared and illuminated using the same method described above.

Determination of the bioluminescence spectra of v2_mChe and v2_mPlum in the presence of rapamycin.

We analyzed the spectral differences of the selected BRET probes, TP2.4, v2_mChe, and V2_mPlum in the presence of rapamycin (Figure 2(B)). We cultured MDA-MB231 cells in a 96-well clear bottom microplate (Nunc), and transiently transfected with pTP2.4, pBRET9_v2_mChe, or pBRET9_v2_mPlum. After 16 h, we lysed the cells in the microplate using an aliquot (40 μL) of a lysis buffer (Promega). We then determined the BL spectra of each lysate in the microplate after simultaneous injection of an aliquot (40 μL) of a PBS buffer (pH 5.7) dissolving nCTZ (final concentration = 10 μg/mL). We used a microplate reader equipped with a series of band-pass filters (Spark 10M, TECAN) with varying optical filters ranging from 398 nm to 653 nm in 15-nm increments (each filter has a 15-nm band pass window). The integration time per filter was one second.

Establishment of MDA-MB231 cells stably expressing the control and acting probes. We established MDA-MB231 cell lines stably expressing TP2.4, or v2_mChe for more quantitative evaluation of the BL intensities, and for use in animal studies (Figures 3 and 4). We constructed lentiviral vectors encoding TP2.4 or v2_mChe by first extracting and subcloning the cDNA constructs from the made pcDNA 3.1(+) vectors, using *Nhe*I/*Xho*I or *Nhe*I/*Xba*I restriction enzymes, and then inserting into a respective enzyme-digested lentiviral vector named pHAGE-UBI-dTomato-CMV-MCS. We confirmed the fidelity of the vectors using a genetic sequence analyzer (Applied Biosystems). We used three-vector transfection systems (pHAGE-UBI-dTomato-CMV-v2_mChe, VPR, and VSVG) with adoption of the calcium phosphate transfection method. We then used the concentrated pure virus after titration to generate various stable cell lines by transduction. After three continuous passages, these cells were FACS sorted for clonal populations of cells having uniform expression, and then used these for various experiments. Finally, we established two MDA-MB231 cell clones expressing v2_mChe, and named them v2_mChe clones #1 and #2, respectively. We also established one MDA-MB231 cell clone expressing TP2.4 through subcloning cDNA encoding TP2.4 into pHAGE-UBI-dTomato-CMV-MCS vector, and FACS sorting for confirming the clonal population.

Characterization of the expression and degradation levels of v2_mChe according to rapamycin. The expression and degradation levels of v2_mChe were determined in HEK293T (ATCC, Manassas, VA) cells expressing v2_mChe after five hours of stimulation with rapamycin (Suppl. Figure 4).

Human Embryonic Kidney-derived HEK293T cells were plated in two 6-well plates

(Nunc) and transiently transfected with pcDNA 3.1 vector encoding v2_mChe. Twenty-four hours later the cells were stimulated for 5 h with 10^{-6} M rapamycin and harvested after washing with PBS. The total mRNAs were isolated using a Qiagen RNA isolation kit following the manufacturer protocol. The mRNAs were further treated with DNase to eliminate any DNA contamination before use for cDNA synthesis by using a random primer mediated cDNA master mix from Quanta Biosciences. We used 1 μg of RNA for cDNA synthesis. The cDNA of 100 ng equivalent was used for target specific primers of v2_mChe (FRB-forward and ALU23-reverse) and hTERT, using SyBr Green based real time PCR using BioRad PCR machine (Suppl. Figure 4 (A)).

Similarly, the cells in the other 6-well microplate were separately trypsinized and sub-cultured into a 96-well black frame optical bottom microplate (Nunc). The cells were then stimulated with 10^{-6} M rapamycin for five hours. The culture media in the microplate were carefully decanted and the corresponding FL intensities from HEK293T cells were determined using IVIS Lumina II imaging system (Caliper) equipped with 535 nm excitation and DsRed emission filters. On the other hand, the BL intensities were quantified with the same IVIS Lumina II system (Caliper) after injection of the specific substrate, native coelenterazine.

Determination of the biosensorial properties of MDA-MB231 cells stably expressing v2_mChe clones #1 and #2.

We examined the overall biosensorial properties of the 4 MDA-MB231 cell lines stably expressing v2_mChe or TP2.4 (Figure 3, Suppl. Figure 5). First, we determined the dose-response curves according to the specific ligand, rapamycin (Figure 3(A)). We seeded the MDA-MB231 cells stably expressing v2_mChe clones #1 or #2 in 96-well clear bottom microplates, and incubated for 1 day. As a control, we separately prepared MDA-MB231 cells stably expressing v2_mChe, that were established through survival selection of the cells bearing pcDNA3.1(+) puromycin vector encoding v2_mChe using the culture medium supplemented with puromycin, and plated them in the same microplates. We then stimulated the cells with varying concentrations of rapamycin ranging from $10^{-9.5}$ M to $10^{-6.5}$ M, or the vehicle (PBS). After removal of the culture media, we lysed the cells using a lysis buffer (Promega) and determined the corresponding BL intensities using the IVIS Lumina II imaging system (Caliper) after injection of a PBS buffer dissolving nCTZ (final concentration = 10 μg/mL) (pH 5.7) using a multichannel micropipette. We analyzed the corresponding calibration curves using Prism 8.0.1 (GraphPad), and summarized the results in Figure 3(A).

As v2_mChe clone #2 showed relatively higher absolute intensities and S/B ratios compared to the other clones, we further determined the ligand selectivity of MDA-MB231 cells stably expressing v2_mChe clone #2 (Suppl. Figure 5(A)). We stimulated the MDA-MB231 cells in a 96-well clear bottom microplate with the following agonists or antagonists for 5 h: 10^{-6} M rapamycin (Rapa), 10^{-6} M Temsirolimus (Tem), 10^{-6} M Everolimus (Eve), 10^{-6} M Deforolimus (Def), 5 x 10^{-6} M ascomycin (Asco), or the mixture of 10^{-6} M rapamycin plus 5 x 10^{-6} M FK506 (Tacrolimus). After complete removal of the culture medium, we lysed the cells using a lysis buffer (Promega) and simultaneously injected with an aliquot of a PBS buffer (pH 5.7) dissolving nCTZ (final concentration = 10 μg/mL). We determined the corresponding BL images using the IVIS Lumina II imaging system (Caliper).

We also determined the corresponding BL spectra using the MDA-MB231 cells stably expressing TP2.4 or v2_mChe clone #2. We prepared the cells and lysed them, as described above. Further, we determined the BL spectra using the same method as that of Figure 2(B).

***In vivo* BL imaging of rapamycin recognition of v2_mChe in living mice.**

We subcutaneously (*s.c.*) implanted MDA-MB231 cells stably expressing v2_mChe and TP2.4 on either flank of living BALB/c nude mice (*nu/nu*) on Day 0 (5 x 10^6 cells in 50 μL PBS plus 50 μL medium growth factor Matrigel). We waited two weeks until the tumors grew to a size in the range 150 - 250 mm^3. On Day 14, we randomly divided the mice to acting and reference groups. The three mice in the acting group were stimulated with 100 μg rapamycin dissolved in 100 μL of saline with 20% PEG400 cocktail, and injected i.p., whereas the two mice in the reference group were injected with the same amount of the cocktail carrying vehicle (1% DMSO and 10% PEG400). We imaged the mice 16 h later.

As a reference for determining the tumor size of each xenograft in mice, we first obtained FL images using the Lago instrument (Spectral Instruments Imaging) under the setting of 535 nm excitation for dTomato signal and 610 nm emission acquisition. To obtain the BL images, the mice were anesthetized using standard gas anesthesia (2% isoflurane, with oxygen at 0.8 to 1.0 L/min) and were *i.v.* injected with 50 μg of nCTZ in 100 μL saline. The corresponding BL images of the tumor xenografts were captured in the prone position using the Lago instrument (Spectral Instruments Imaging). To quantify the number of emitted photons, we drew ROI over the tumors and generated the maximum photons per second per square centimeter per steradian (p/sec/cm^2/sr) using the specific software,

Aura (ver 2.2.0).

All the animal experiments were performed in accordance with Stanford University Institutional Animal Care and Use Committee guidelines (APLAC-26748) and by adherence to the NIH Guide for the Care and Use of Laboratory Animals.