Electric Suppl. Information

# Single-Chain Multicolor-Reporter Templates for Subcellular Localization of Molecular Events in Mammalian Cells

**Sung-Bae Kim [1,*], Ramasamy Paulmurugan [2], Nobuo Kitada [3], Sojiro A. Maki [3]**

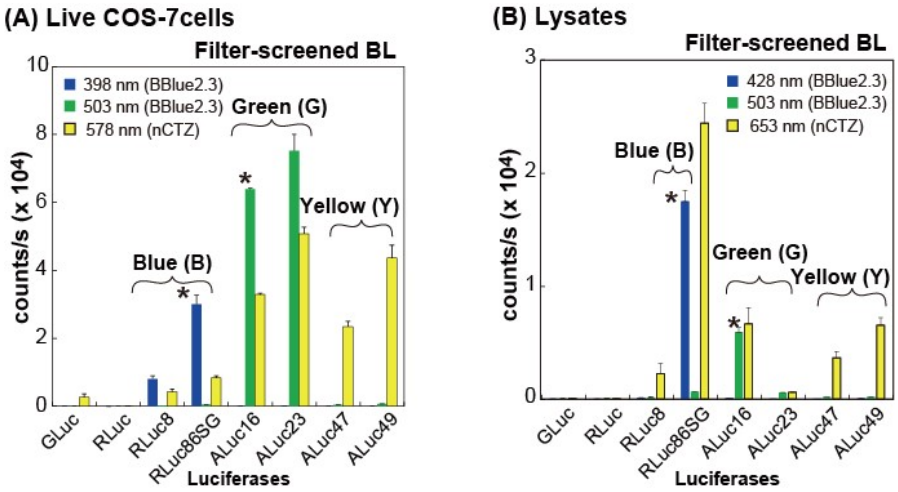
[1] Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8569, Japan

[2] Molecular Imaging Program at Stanford, Bio-X Program, Stanford University School of Medicine, Palo Alto, California 94304, United States

[3] Department of Engineering Science, Graduate School of Informatics and Engineering, The University of Electro-Communications, Chofu 182-8585, Japan

* Correspondence: kimu-sb@aist.go.jp

## Suppl. Figures




Suppl. Figure 1. (A) The absolute BL intensities of the live COS-7 cells at 398, 503, and 578 nm of the spectra according to the substrates. (B) The absolute BL intensities of the COS-7 cell lysates at 398, 503, and 578 nm of the spectra according to the substrates.

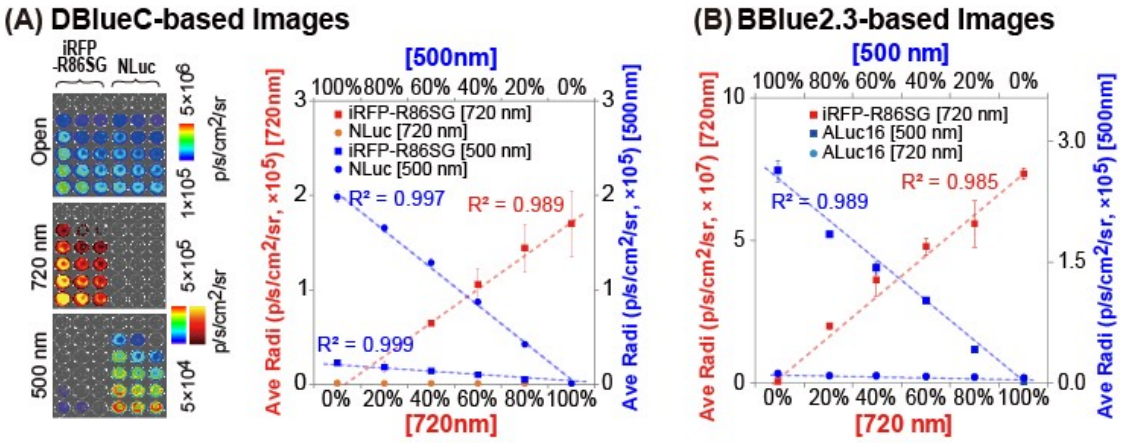



Figure S2. (A) The determination of the quantitative relationship between the BL intensities of live cells containing iRFP-R86SG or NLuc at green (500 nm) or at NIR (720 nm) spectral region in the presence of DBlueC substrate. The defined amount of live COS-7 cells expressing iRFP-R86SG were mixed with the corresponding amount of PBS at different ratios totaling 100%. Inset 'a' shows the optical images according to the filters. (B) The determination of the quantitative relationship between the BL intensities of live cells containing iRFP-R86SG or ALuc16 at green (500 nm) region or at NIR region (720 nm) in the presence of BBlue2.3.

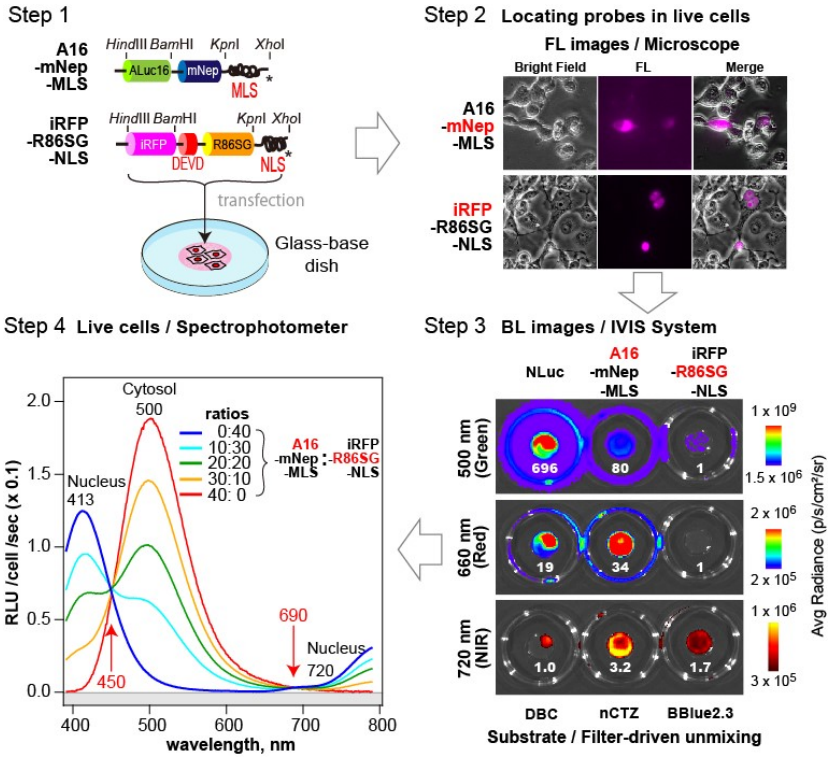



Figure S3. Consecutive monitoring of FL and BL signals and the optical filter- and substrate-driven unmixing in living mammalian cells. Step 1 illustrates transient transfection of the cells with the probes. Step 2 shows proper localization of the probes in the intracellular compartments of live cells. Step 3 exhibits the color variances of the BL signals according to the substrates. Step 4 indicates that the BL signals from the cells are quantitative and separable by BBlue2.3 substrate.

# Suppl. Methods

### 1. Reagents and materials

The native CTZ (nCTZ) was purchased from Nanolight (Pinetop, AZ, USA). We used CTZ analogue BBlue2.3 synthesized from our precedent study [1].

The cDNAs encoding various marine luciferases were custom-synthesized by Eurofins Genomics (Tokyo, Japan) or obtained from our previous studies, i.e., Gaussia luciferase (GLuc; AAG54095.1), Renilla luciferase (RLuc), Renilla luciferase 8 (RLuc8), R86SG, Artificial Luciferase 16 (ALuc16; GenBank MF817967), ALuc23 (GenBank MF817968), ALuc47 (GenBank MF817975), and ALuc49 (GenBank MF817976) [2 3].

The cDNAs encoding iRFP and mNeptune (mNep) were obtained from our previous studies [1 4]. On the other hand, constructs with membrane localization signal (MLS) peptide sequence and the

nucleus localization signal (NLS) sequence were custom-synthesized by Eurofins Genomics (Tokyo) according to the sequence information based on our previous studies [5] [6].

**2. Construction of mammalian expression vectors encoding various marine luciferases and BRET imaging probes**

The cDNA constructs encoding RLuc, RLuc8, and RLuc8.6-535SG (R86SG) were subcloned into a mammalian expression vector, pcDNA3.1(+). Separately, each cDNA construct encoding GLuc, Aluc16, Aluc23, Aluc47, and Aluc49, where the 3' ends were genetically tagged with a cDNA sequence encoding an ER retention signal "KDEL" through a polymerase chain reaction (PCR) based cloning to retain the expressed protein in the endoplasmic reticulum (ER) compartment, was subcloned into pcDNA3.1(+) vector.

The cDNA constructs encoding the BRET probes named A16-mNep-MLS and iRFP-R86SG-NLS were made through a series of PCR reactions introduced with unique restriction enzyme sites, HindIII/BamHI, BamHI/KpnI, or KpnI/XhoI, at the 5' and 3' ends of each cDNA fragment as shown in Figure 4(A). The fabricated cDNA fragments were ligated and finally subcloned into pcDNA3.1(+) vector backbone.

The cDNA construct encoding A16-ERLBD-mNep-MLS was fabricated by ligating the cDNA construct encoding the human ER LBD (281–549 aa) between the cDNA fragments of ALuc16 and mNeptune of the original template A16-mNep-MLS.

The fidelity of the cDNA constructs in the vectors was ensured with a genetic sequence analyzer by order (Eurofin Genomics, Tokyo, Japan).

**3. Determination of the BL intensities of various marine luciferases in live cells according to their substrate specificities**

The substrate-driven absolute BL intensities in living COS-7 cells transiently expressing marine luciferases were determined according to the following protocol (Figure 1).

The cells were grown in 6-well plates to 80% confluence for transfection. The cells were transiently transfected using pcDNA3.1(+) vector encoding GLuc, RLuc, RLuc8, R86SG, ALuc16, ALuc23, ALuc47, or ALuc49, and incubated overnight in a 5%(v/v) $CO_2$ incubator at 37 °C. The cells were then subcultured into a 96-well clear-bottom black-frame microplate by further incubating overnight in the $CO_2$ incubator at 37 °C before using them for luciferase imaging.

All the culture media in the microplates were carefully aspirated, and the microplate wells were conceptionally divided into three sections. The wells in the three sections were simultaneously injected with 40 μL of PBS buffer containing nCTZ, BBlue2.3, and Cy5-CTZ, respectively using a 12-channel micropipette. The corresponding BL intensities of the microplate were immediately

determined with an IVIS Spectrum imaging system (PerkinElmer, USA) and analyzed with the Living Image version 4.7 software.

**4. Characterization of the BL spectra of selected marine luciferases with various BL signal-unmixing conditions**

The BL spectra of selected marine luciferases were determined with various BL signal-unmixing conditions, namely, the substrate type, the lysis condition, and the optical filters (Figure 2).

COS-7 cells grown in 6-well microplates were transiently transfected with pcDNA3.1(+) vector encoding each marine luciferase using a lipofection reagent (TransIT-LT1, Mirus). The cells were incubated overnight and subcultured into 96-well optical-bottom black-frame microplates. The cells in the microplates were further incubated overnight for stabilization. The wells were conceptionally sectioned into two regions, where one is prepared for live cell imaging whereas the other is for imaging after lysis. All the culture media in the microplates were completely removed by aspiration. Each well in the first section (for lysate imaging) was injected with 40 μL of a lysis buffer (Promega) and incubated for 20 minutes. On the other hand, the wells in the second section (for live cell imaging) were sealed and remained before substrate injection and imaging.

The wells were then simultaneously injected by 40 μL of PBS buffer dissolving nCTZ or BBlue2.3 (final concentration: 100 μM) using a multichannel micropipette (Gilson). The BL intensities were immediately determined with a microplate reader (Spark 10M, TECAN) equipped with a series of bandpass filters ranging from 495 to 655 nm in 15 nm increments.

**5. Determination of the intracellular localization of the BRET probes with FL and BL images**

The intracellular compartments in live COS-7 cells were imaged with FL for the determination of the localization of the BRET probes according to the following protocols (Figure 3).

COS-7 cells were grown in a 6-well microplate and transiently transfected with pcDNA3.1(+) vector encoding A16-mNep-MLS, iRFP-R86SG-NLS, or iRFP-R86SG using a lipofection reagent (TransIT-LT1, Mirus). The cells were then incubated overnight at 37 °C in a 5%(v/v) $CO_2$ incubator. The cells were then subcultured into both 35-mm glass-bottom microdishes and 6-channnel microslides (μ-Slide VI 0.4, ibidi, Germany). The cells were further incubated until they reach 90% confluency on the microdish or microslide surface. The culture media were rinsed once using PBS buffer.

The FL images of the cells containing A16-mNep-MLS or iRFP-R86SG-NLS in the microdishes were acquired with 580-nm Ex/ 640-nm Em filter set or 480-nm Ex/ 510-nm Em filter set

of a fluorescent microscope (EVOS M5000 Imaging System, Thermo Fisher Scientific, USA; Figure 3[A]).

In parallel, the BL images of the cells containing iRFP-R86SG-NLS or iRFP-R86SG were determined after replacement of the culture medium with the PBS buffer containing nCTZ (Figure 3[B]). The microslide was set in the dark chamber of the IVIS Spectrum imaging system and the corresponding BL images were immediately determined. The corresponding BL images were analyzed by Living Image version 4.7 software.

**6. Spectral confirmation of BRET signals and substrate-driven unmixing of the multiple BL spectra**

First, we determined if typical BRET spectrum is observed with the BRET probe, iRFP-R86SG (Figure 3[C]).

COS-7 cells grown in a 6-well plate was transiently transfected with pcDNA3.1(+) vector encoding iRFP-R86SG or NLuc and incubated overnight at 37 °C in a $CO_2$ incubator. The cell medium was then replaced with a fresh one containing 25 μM of biliverdin and incubated for one day. The cells were harvested by trypsinization and centrifuge. The cell numbers were counted with an automatic cell counter (Countess 3, Thermo Fisher Scientific). All the cells were finally adjusted with PBS to be $10^6$ cells/mL. Forty μL of the cell suspension were transferred to 200-μL PCR tubes. The corresponding BRET spectra were determined with a precision spectrophotometer (AB-1850, ATTO), which simultaneously measures all the wavelengths in the visible and NIR region, after injection of the same amount of a BBlue2.3 substrate solution dissolved in PBS buffer.

Secondly, multiplex BL spectra of various live COS-7 cell cocktails were determined and unmixed with a specific CTZ analogue BBLue2.3 (Figure 3[D]).

COS-7 cells were grown in 6-well plates. When the cells reach 70% confluency, the cells were transiently transfected with ALuc16, ALuc23, NLuc, RLuc86SG, or iRFP-R86SG using a lipofection reagent (TransIT-LT1, Mirus). The cells were incubated at 37 °C in a $CO_2$ incubator for two days. The cells were collected by trypsinization and resuspended in PBS. The cell numbers were counted with an automatic cell counter (Countess 3, Thermo Fisher Scientific). All the cells were finally adjusted with PBS to be $10^6$ cells/mL.

Among the harvested live cells, the cells containing ALuc16, NLuc, and iRFP-R86SG were mixed in the ratios of 5:5:50, 5:10:50, 5:10:40, or 5:20:20 respectively, as indicated in the figure legend, each of which was transferred to 200-μL PCR tubes. After injection of 60 μL of PBS buffer dissolving nCTZ into each PCR tube, the corresponding BL spectra were determined with the precision spectrophotometer (AB-1850, ATTO) (Figure 3[D], left). The spectral data were analyzed with the specific software LumiFLSpectroCapture version 1.0.

In parallel, the cells containing ALuc16, NLuc, R86SG, and iRFP-R86SG were separately prepared in a 200-μL PCR tube (Figure 3[D], right). The corresponding BL spectra were determined with the same spectrophotometer after injection of 60 μL of PBS buffer dissolving BBlue2.3.

**7. Determination of the quantitative relationship between luciferases in multiplex reporter systems**

The quantitative relationship between the BL intensities of the live cells containing iRFP-R86SG, ALuc16, and NLuc were examined according to the following protocols (Figure S2[A] and 2[B]).

The cells containing iRFP-R86SG, NLuc, or ALuc16 were prepared with the same method as that of Figure 3(C) and 3(D) and adjusted the number of cells to be $10^6$ cells/mL using PBS. This cell suspension was entitled "100% stock solution". Firstly, the 100% stock solution of each cell line was consecutively diluted using PBS to be 80%, 60%, 40%, and 20%. PBS buffer alone was set as the negative control (0%).

Thirty μL of diluted cell suspension were deployed to each well in a fresh 96-well clear-bottom black-frame microplate. The wells were then simultaneously injected with 30 μL of DBC or BBlue2.3 substrates diluted in PBS using a multichannel micropipette. The corresponding BL images were acquired using the IVIS Spectrum imaging system equipped with 720-nm or 500-nm bandpass filter in order. As the control, the BL images were also taken in open channel, and the results were annotated as "open" in Figure S2(A).

**8. Locating molecular events in the subcellular compartments of live cells with the templates**

The BL signals generated from the subcellular compartments of live COS-7 cells were located with the images of the templates (Figure S3).

COS-7 cells were grown in a 6-well culture plate to a 70% confluency. The cells were then transiently transfected with pcDNA3.1(+) vector encoding iRFP-R86SG-NLS, A16-mNep-MLS, or NLuc using the lipofection reagent (TransIT-LT1, Mirus, USA). The cells were incubated overnight at 37 °C incubator in the 5%(v/v) $CO_2$ environment, and then harvested by trypsinization and centrifugation. The cells were finally resuspended in PBS to a concentration of 5 x $10^5$ cells/mL. One hundred mL of the cells were then seeded in each 3.5-cm glass-base dish (Iwaki, Japan) and further incubated overnight for stabilization.

The cells were then washed once with PBS and immersed with 200 mL of fresh PBS. The FL images of the cells in the dishes were firstly determined in advance to confirm the localization of the probes in the intended intracellular compartments using the fluorescent microscope (EVOS M5000 Imaging System) equipped with a Cy5.5 bandpass filter. Secondly, the dishes were transferred to the dark chamber of the IVIS Spectrum imaging system (PerkinElmer). The BL images of the cells were

then determined with 500-nm, 660-nm, and 720-nm BP filters after replacing the cell-immersing PBS in the dishes with DBlueC, nCTZ, or BBlue2.3 substrates dissolved in PBS. In the measurement of BP filters, 500-nm, 660-nm, and 720-nm filters were chosen for selectively collecting the BL signals from NLuc, A16-mNep-MLS, and iRFP-R86SG-NLS, respectively. Thirdly, the cells in the dishes were collected by trypsinization. The harvested cells were resuspended in PBS to a concentration of 5 x $10^6$ cells/mL. The cells containing A16-mNep-MLS and iRFP-R86SG-NLS were mixed in the ratios totaling 50 mL as indicated in the legend. The corresponding BL spectra were immediately determined using the precision spectrophotometer (AB-1850, ATTO) after injection of 50 mL of the BBLue2.3 substrate solution.

**9. Determination of subcellular steroid hormone activities in the PM and ER compartments of mammalian cells.**

The subcellular steroid hormone activities were determined with live COS-7 cells (Figure 4).

COS-7 cells were first grown in a 3.5-cm glass-base dish (Iwaki, Japan) and a 96-well black-frame microplate to a 70% confluency. The cells were transiently transfected with pcDNA3.1(+) vector encoding A16-ERLBD-mNep-MLS using the lipofection reagent (TransIT-LT1, Mirus, USA). The cells were incubated overnight at 37 °C incubator in the 5%(v/v) $CO_2$ environment.

The FL images from the 3.5-cm glass-base dish were obtained with the same protocol as that of Figure 3(A).

On the other hand, the cells in the 96-well black-frame microplate were stimulated for 4 hours with one of the following ligands dissolved in culture media: i.e., 17β-estradiol (E2), dihydrotestosterone (DHT), cortisol, 4-hydroxytamoxifen (OHT), forskolin, and the vehicle (0.1% DMSO). The culture media in the microplate were eliminated. The wells in the microplate were simultaneously injected by 40 μL of PBS dissolving nCTZ. The microplate was immediately deployed in the chamber of IVIS Spectrum imaging system and the corresponding BL images were determined and analyzed with the specific software Living Image 4.7.

# References


1. R. Nishihara, R. Paulmurugan, T. Nakajima, E. Yamamoto, A. Natarajan, R. Afjei, Y. Hiruta, N. Iwasawa, S. Nishiyama, D. Citterio, M. Sato, S. B. Kim and K. Suzuki, *Theranostics*, 2019, **9**, 2646-2661.
2. S. B. Kim, M. Torimura and H. Tao, *Bioconjugate Chem.*, 2013, **24**, 2067–2075.
3. S. B. Kim and H. Izumi, *Biochem. Bioph. Res. Co.*, 2014, **448**, 418-423.

4. S. B. Kim, R. Fujii, A. Natarajan, T. F. Massoud and R. Paulmurugan, *Chem. Commun.*, 2020, **56**, 281-284.
5. S. B. Kim, Y. Ito and M. Torimura, *Bioconjugate Chem.*, 2012, **23**, 2221-2228.
6. S. B. Kim, T. Ozawa, S. Watanabe and Y. Umezawa, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 11542-11547.