Supporting Information

Visualization and Quantitative Analysis of G Protein-Coupled Receptor–β-Arrestin Interaction in Single Cells and Specific Organs of Living Mice Using Split Luciferase Complementation

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Material and Methods

Materials

DNA polymerase, restriction enzymes, and the cDNA library from human brain were obtained from Takara Bio Inc. (Japan). The cDNA of firefly luciferase (Fluc) and CBR luciferase (CBRluc), and luciferase assay kit, Bright-Glo Assay System, were obtained from Promega Corp. (Madison, WI). Mouse anti-Myc-Tag monoclonal antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA). Mouse anti-V5 antibody and donkey antimouse IgG antibody tagged with AlexaFluoro 488, and expression vectors of pcDNA3.1/myc-His (B) and pcDNA4/V5-His (B), Zeocin, and Hoechst 33342 were purchased from Invitrogen Corp. (Carlsbad, CA). pBlueScript was obtained from Stratagene Corp. (La Jolla, CA). Isoproterenol, propranolol, pindolol, D-luciferin potassium salt, and Dulbecco's modified eagle's medium (DMEM) were purchased from Wako Pure Chemical Industries Ltd. (Japan). Metaproterenol, ritodrine, terbutaline, dobutamine, butoxamine, and poly L-lysine (PLL) were purchased from Sigma-Aldrich Japan K.K. Fetal bovine serum (FBS), 0.05% (v/v) trypsin-ethylenediaminetetraacetic acid (EDTA), Hank's balanced buffered saline (HBSS), penicillin and streptomycin solution, G418, and Lipofectamine 2000 were obtained from Gibco BRL (Rockville, MD). TransIT Transfection was purchased from Mirus Bio Corp. (Madison, WI).

Construction of Mammalian Expression Vectors

The cDNAs used for the plasmid construction were generated using the standard polymerase chain reaction (PCR) with gene specific primers and Pyrobest DNA polymerase. The cDNAs of β2-adrenergic receptor (ADRB2) and β-arrestin type 2 (ARRB2) were amplified from the human brain cDNA library. An *E. coli* strain, DH5α, was used as a bacterial host for construction of all plasmids. The PCR fragments were cloned into restricted enzyme sites of mammalian expression vectors or pBlueScript, and sequenced using a genetic analyzer (ABI310; Applied Biosystems). The cDNA fragments used for mammalian expression were subcloned into expression vectors (pcDNA3.1/myc-His (B) or pcDNA4/V5-His (B)). For transfection of the cells, the expression vectors were purified using a DNA purification system (Promega Corp.).

Mutations at S284 and V348 were created by the QuikChange Site-Directed Mutagenesis protocol on the plasmid containing the sequence of FN-ARRB2 which inserted into *Bam*HI/*Hind*III restriction site. The following mutagenic primers were designed and used in PCR: S284T, 5'-TAC AAG ATT CAA A**C**T GCG CTG CTG GTG-3'; A348V, 5'-GAG ACT ACA TCA G**T**T ATT CTG ATT ACA-3' (mutated sites were indicated

in bold).

The plasmid containing FN-ARRB2 was used as a template. The PCR was carried out by Prime Star under the following condition: the initial denaturation at 95 °C for 5 min, 18 cycles of 95 °C for 30 sec, 55 °C for 10 sec and 72 °C for 7 min. Subsequently, the PCR products were treated with DpnI at 37 °C for 1 hr in order to digest the original template plasmids. Then, 2 μl of the reaction mixture was used for heat-shock transformation of the competent cells of *E. coli* (DH5α). After obtaining plasmids of the clone, we confirmed completely the sequence using genetic analyzer. The mutagenesis plasmids were digested with *Bam*HI/*Hind*III, and the fragments were inserted into the restriction site of digested pcDNA3.1 or pCold expression vector. The plasmids were transformed into the competent cells of *E. coli* (BL21) by heat shock for protein expression.

Protein expression and purification

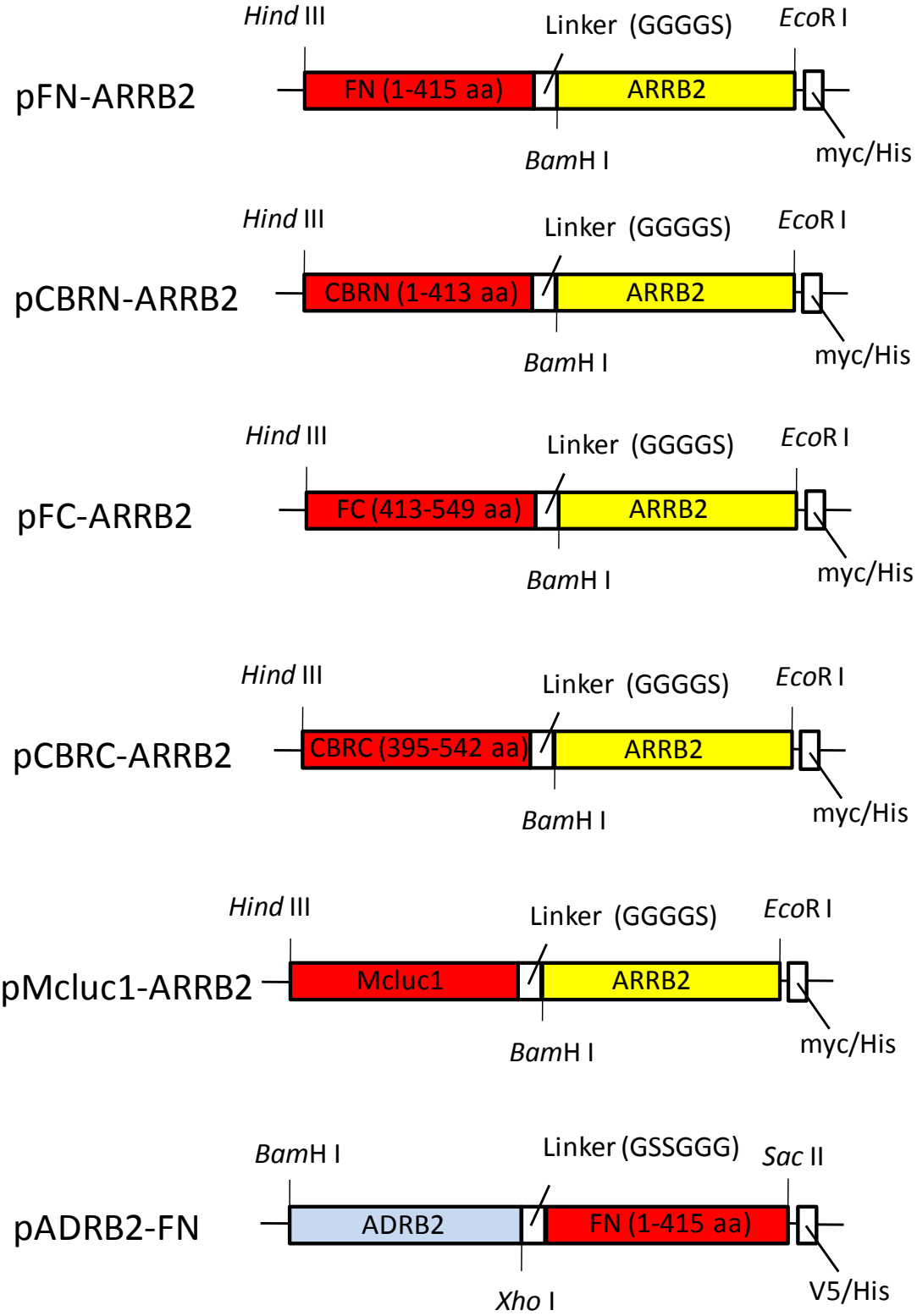
Transformed *E. coli* cells were added to 20~100 mL of LB medium in the presence of 100 μg/ml ampicillin. Cells were grown at 37 °C until the OD600 reached about 0.4, and after addition of IPTG (final concentration 0.5 mM) to the solution, the medium was incubated at 16 °C for 36~72 hr. The cells were harvested by centrifugation at 9000 rpm for 10 min at 4 °C, and the pellet was resuspended in 3~4 mL of PBS containing 1% Triton-100. After sonication for 10 min on ice, the lysate was centrifuged at 12000 rpm for 10 min at 4 °C, and the supernatant was collected. Purification of His6-tagged fusion protein was carried out using prepacked Ni-NTA column (GE Healthecare) as described in the manufacture. After equilibration, the supernatant was applied to the column, washed with PBS containing 30 mM imidazole, and eluted with PBS containing 300 mM imidazole. To remove excess imidazole, the eluate was passed through a PD-10 column (GE Healthcare).

Bioluminescence spectrum

The stable cell line, HEK293_FN-ARRB2_ADRB2-FC, was incubated on 35 mm culture dishes coated with PLL in DMEM with 10% FBS for 36−48 hr at 37 °C in 5% CO_2. Culture medium was exchanged to HBSS containing 10% FBS, and after addition of 1×10^{-6} M of Iso (final) incubated at 37 °C for 15 min. For *in vitro* assay, after removal of the medium, Bright Glo was added to the dish, and then collected into a tube. Bioluminescence spectra were measured using a luminescence spectrometer (LumiFlspectrometer, ATTO corp.). For living cells, bioluminescence spectra of the dish were measured using a luminescence spectrometer. In the case of purified protein, Fluc

protein and 1 mM of ATP (final) were added to a tube containing Bright Glo, and bioluminescence spectra of tube were measured using a luminescence spectrometer.

Figures


pFN-ARRB2
Hind III
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EcoR I
FN (1-415 aa)
ARRB2
BamH I
myc/His
pCBRN-ARRB2
Hind III
Linker (GGGGS)
EcoR I
CBRN (1-413 aa)
ARRB2
BamH I
myc/His
pFC-ARRB2
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Linker (GGGGS)
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CBRC (395-542 aa)
ARRB2
BamH I
myc/His
pMcluc1-ARRB2
Hind III
Linker (GGGGS)
EcoR I
Mcluc1
ARRB2
BamH I
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Xho I
V5/His

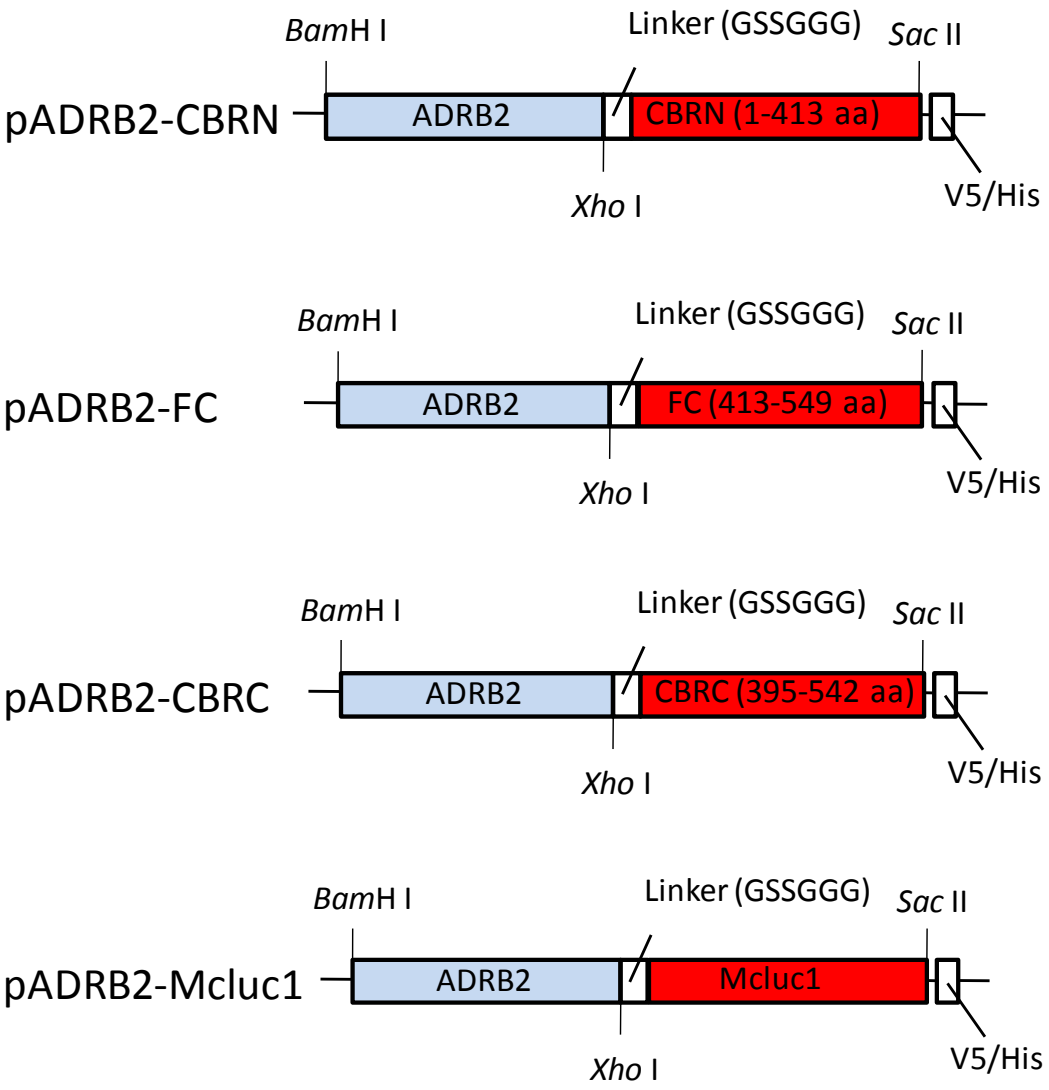

Figure S1. Schematic structures of cDNA constructs. FN; N-terminal fragment of firefly luciferase (Fluc), FC; C-terminal fragment of Fluc, CBRN; N-terminal fragment of click beetle luciferase in red (CBRluc), CBRC; C-terminal fragment of CBRluc, Mcluc1; the multi-complement fragment for all N-terminal fragments of split luciferase, ARRB2; β-arrestin2, ADRB2; β2-adrenergic receptor. ARRB2 and ADRB2 are attached to FN, FC, CBRN, CBRC and Mcluc1 through GS linker peptides, i.e. GGGGS or GSSGGG. Myc/His and V5/His indicate epitope sequences for antibody recognition.

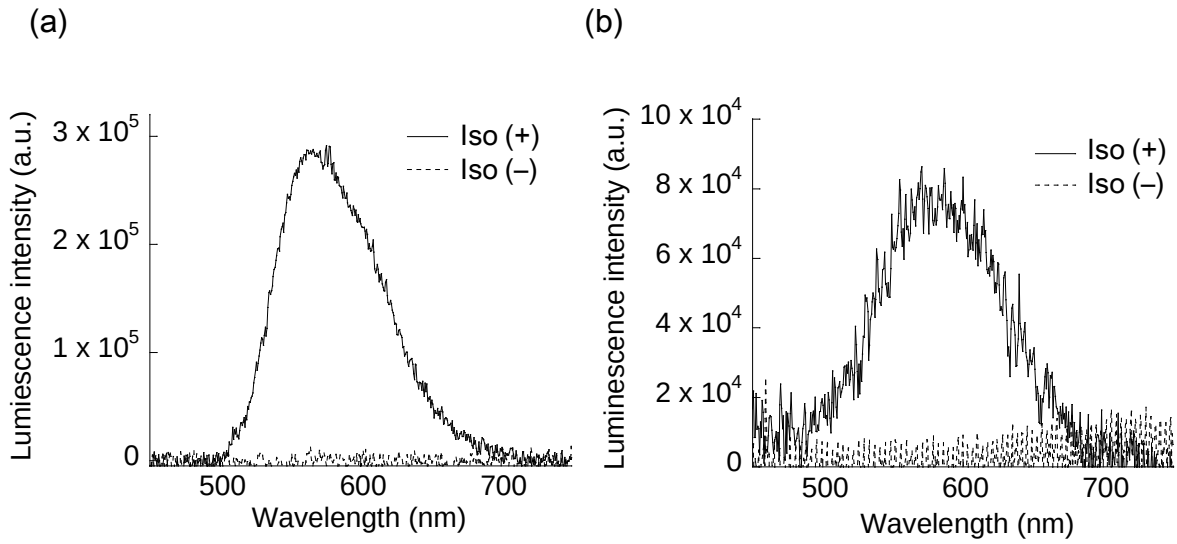

Figure S2. Bioluminescence spectra of complement Fluc fragments obtained from the cel line of HEK293_FN-ARRB2_ADRB2-FC in the presence and absence of Iso. (a) The cells were cultured in a 35 mm dish, and stimulated with 1×10^{-6} M of Iso (final) for 15 min. After the medium was removed, lysis buffer was added to the dish and the cells were collected to a tube. Bioluminescence spectra were measured by a luminescence spectrometer. (b) The cells were cultured in a 35 mm dish, and stimulated with 1×10^{-6} M of Iso (final) for 15 min. Bioluminescence spectra of the cells cultured on the dish were directly measured.

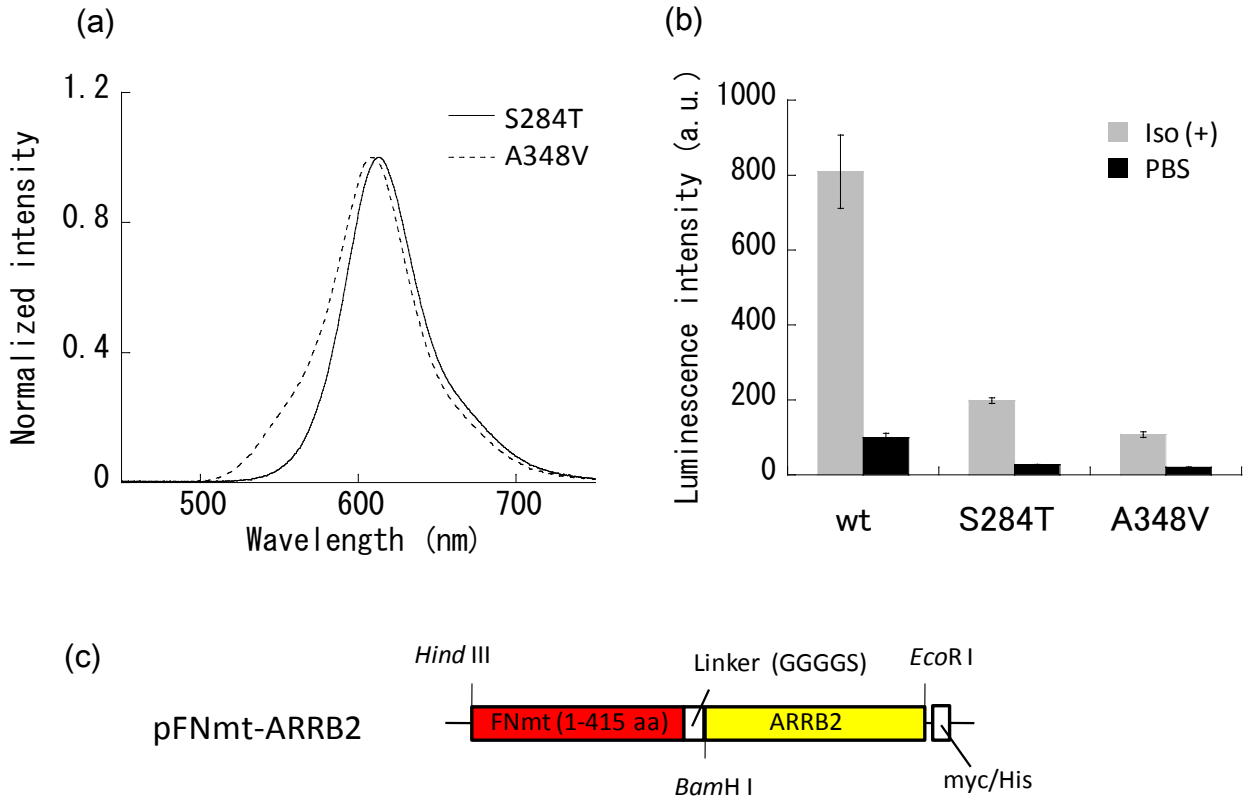

Figure S3. Bioluminescence assays using Fluc mutants (S284T and A348V) emitting red light. (a) Bioluminescence spectra of full-length Fluc mutants. The mutants from *E. coli* were measured by a luminescence spectrometer. (b) Bioluminescence of HEK293 cells transiently expressing mutated FN-ARRB2 (FNmt-ARRB2) and ADRB2-FC upon stimulation with Iso. Measurements were performed for 2 sec/well with a microplate reader ($n = 4$). (c) Schematic structures of the cDNA constructs.

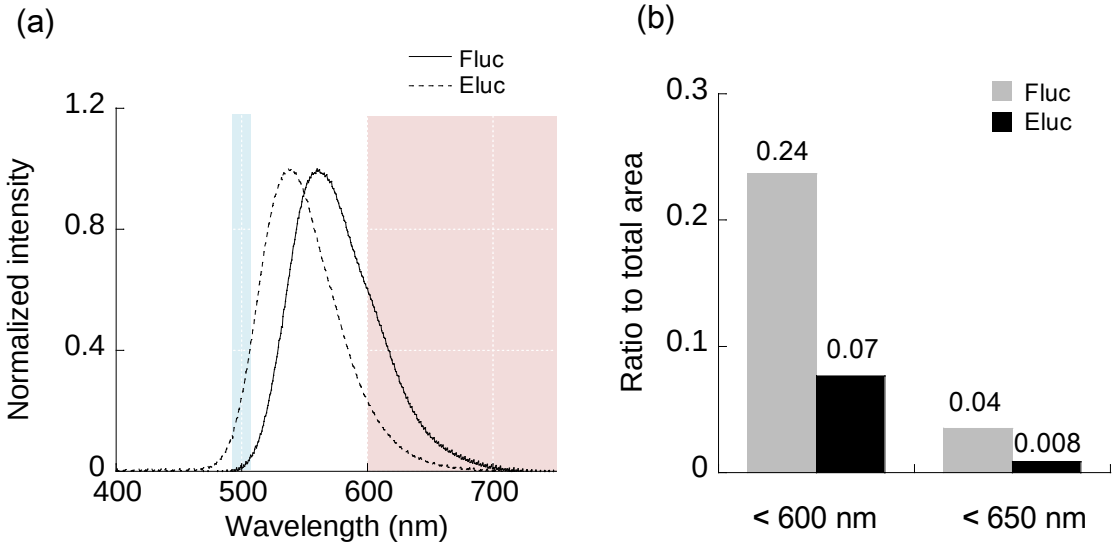

Figure S4. Spectral analysis of Fluc and Eluc. (a) Bioluminescence spectra of full length Fluc and Eluc. The range of the filter passed through green light (500 ± 7.5 nm) is indicated in blue, and that over 600 nm is indicated in red. Bioluminescence spectra of Fluc and Eluc purified from *E. coli* were measured by a luminescence spectrometer. (b) Ratio in the range over 600 nm or 650 nm to the total area of Fluc and Eluc.

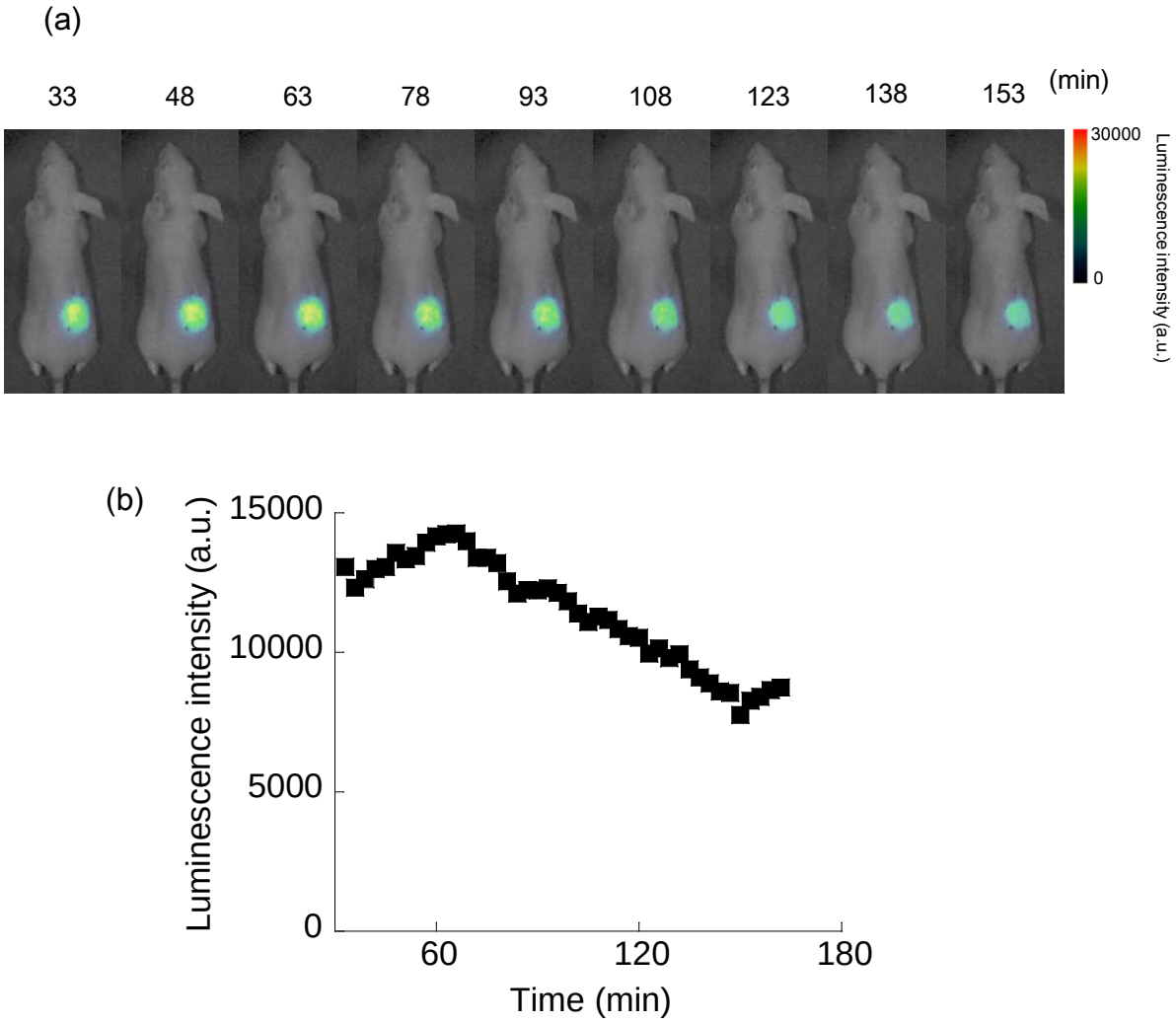

Figure S5. Bioluminescence imaging (BLI) of mouse implanted with HEK293 cells stably expressing Fluc. (a) Cells were cultured in a 10 cm dish, and harvested using a rubber scraper. The collected cells were suspended with PBS with D-luciferin (90 mg/kg body weight), and implanted under the skin of mouse. The time for injection of the cells was defined at 0 min. 30 min after i.p. injection, BLI was taken every 3 min using a CCD camera under isoflurane anesthesia. (b) Time-dependent bioluminescence changes in the area of implanted cells.

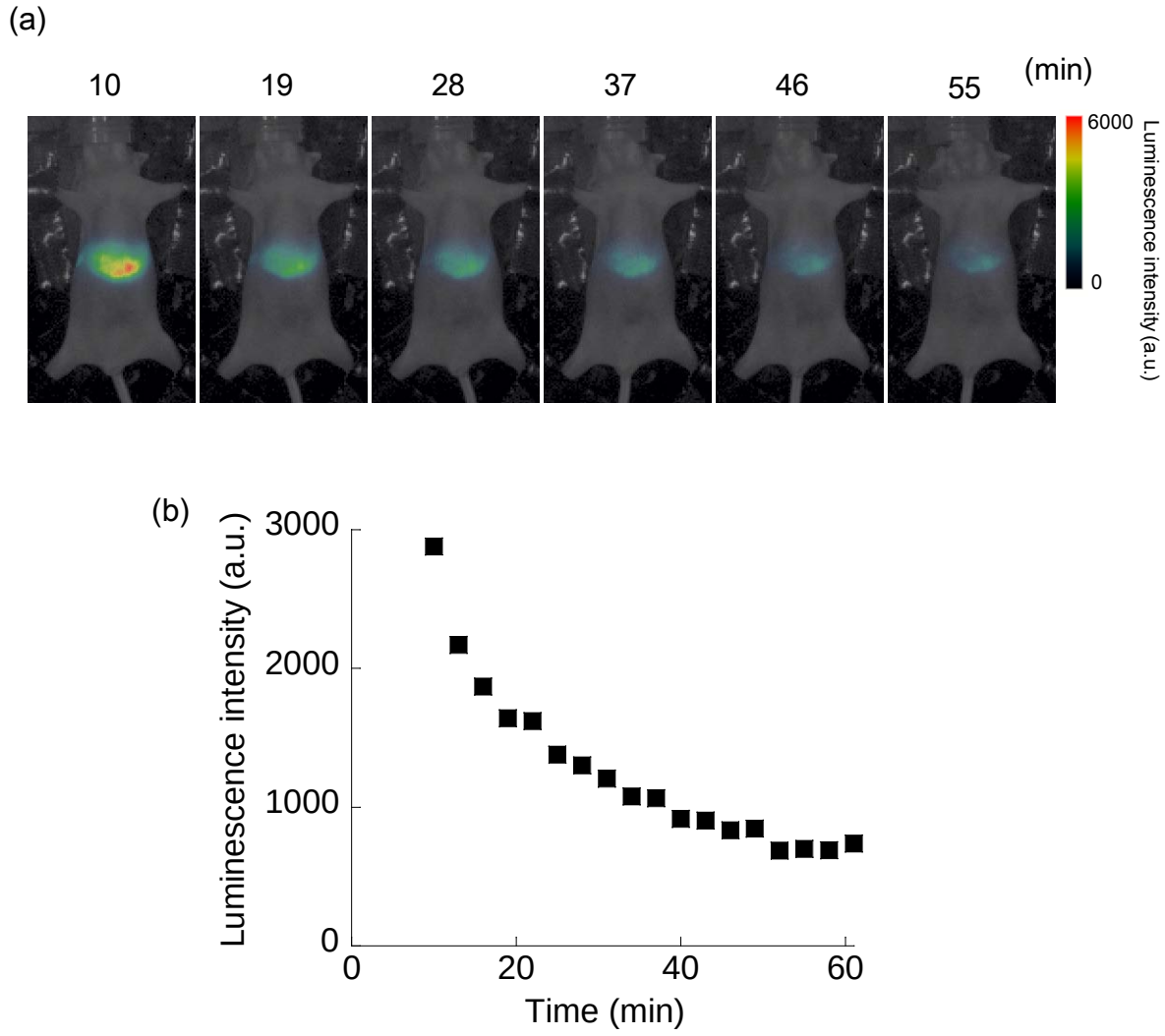

Figure S6. Bioluminescence imaging of Fluc expressed by the HTV method. (a) Mice were treated with HTV using the plasmid of full length Fluc. The mice were kept in dark for one day, and then D-luciferin (600 mg/kg body weight) was i.p. injected; the injection time was defined at 0 min. After i.p. injection, BLI was taken every 3 min using a CCD camera under isoflurane anesthesia. (b) Time-dependent bioluminescence changes in the area of the liver.

Movie S1. Real-time bioluminescence images of ADRB2-ARRB2 interactions. HEK293_FN-ARRB2_ADRB2-FC cells were cultured on a dish and were stimulated with 1.0 μM of isoproterenol.

Movie S2 Real-time fluorescence images of influx of Ca^{2+} inside the cells. HEK293_FN-ARRB2_ADRB2-FC cells were incubated with Fluo4, and were stimulated with 1.0 μM of isoproterenol.

Movie S3 Real-time fluorescence images of influx of Ca^{2+} the cells. HEK293_FN-ARRB2_ADRB2-FC cells were incubated with Fluo4 and 10 μM of propranolol, and were stimulated with 1.0 μM of isoproterenol.