Enhanced brightness of bacterial luciferase by bioluminescence resonance energy transfer

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Using the lux operon (luxCDABE) of bacterial bioluminescence system as an autonomous luminous reporter has been demonstrated in bacteria, plant and mammalian cells. However, applications of bacterial bioluminescence-based imaging have been limited because of its low brightness. Here, we engineered the bacterial luciferase (heterodimer of luxA and luxB) by fusion with Venus, a bright variant of yellow fluorescent protein, to induce bioluminescence resonance energy transfer (BRET). By using decanal as an externally added substrate, color change and ten-times enhancement of brightness was achieved in Escherichia coli when circularly permuted Venus was fused to the C-terminus of luxB. Expression of the Venus-fused luciferase in human embryonic kidney cell lines (HEK293T) or in Nicotiana benthamiana leaves together with the substrate biosynthesis-related genes (luxC, luxD and luxE) enhanced the autonomous bioluminescence. We believe the improved luciferase will forge the way towards the potential development of autobioluminescent reporter system allowing spatiotemporal imaging in live cells.

Light production in luminous bacteria results from an enzymatic reaction of a substrate catalyzed by a bacterial luciferase. Bacterial luciferase oxidizes reduced flavin mononucleotide (FMNH₂) and a long-chain fatty aldehyde (RCHO) to yield flavin mononucleotide (FMN) and the corresponding long-chain fatty acid (RCOOH). This reaction concomitantly generates blue-green light with a peak wavelength around 490 nm.

The fundamental enzymes required for bacterial luminescence are encoded by a single operon, luxCDABE, which is found in all species of luminous bacteria. The luxA and luxB genes encode for the α and β subunits of a heterodimeric protein of bacterial luciferase, respectively. The luxC, luxD and luxE genes encode for the complex components that serves to synthesize and recirculate fatty aldehyde, which is the substrate for luciferase. Co-expression of the five lux genes in non-luminous bacteria or yeast cells shows a light-emitting phenomenon with no external supply of the substrate. In mammalian or plant cells, the additional gene expression of FMN oxidoreductase (luxG), which provides a sufficient amount of FMNH₂, together with the five lux genes enables stable autobioluminescence.

Introduction of genetically encoded reporters into living cells has been widely used to observe biological phenomena. Compared to fluorescence imaging, bioluminescence imaging does not need external excitation illumination that can cause problems such as phototoxicity, photobleaching, and autofluorescence from the specimen. Bacterial luciferase-based reporter is a valuable tool because of its high signal-to-noise ratio and ease of operation. Additionally, a wider spectral information can be obtained from bioluminescence compared to fluorescence because the excitation illumination does not interfere with the spectral analysis. However, the application of bacterial bioluminescence imaging has been limited because of the low brightness. It has been shown that seven-times increment of bacterial bioluminescence allows imaging of single E. coli cells with improved spatiotemporal resolution. However, image acquisition using this enhanced luminescence still requires about 10 min of exposure time, which would be difficult to capture biological phenomena that change rapidly. Therefore, a higher luminescence intensity is expected to allow observation of biological phenomena that change within minutes or even less.

To further improve on the signal of the bacterial bioluminescence, we attempted to increase the luminescence of bacterial luciferase by fusion to a fluorescent protein. This approach is based on the phenomenon of bioluminescence resonance energy transfer (BRET) between the luciferase (as a donor) and fluorescent protein (as an acceptor). BRET results in luminescence emission from the acceptor without external excitation illumination. BRET efficiency depends on the spectral overlap between the donor emission and acceptor absorbance, and on the spatial arrangement of the donor and acceptor. High-efficient BRET has been shown to substantially increase...
luminescent intensity from the acceptor \cite{14-16}. In this study, we engineered the luciferase from the luminous bacterium *Photorhabdus luminescens* and showed that the optimal fusion of a yellow fluorescent protein Venus \cite{17} with the luciferase significantly enhanced its brightness.

**Results**

**Comparison of the gene constructs for expression of luxA and luxB in Escherichia coli.** To compare the genetic constructs for expression of two subunits of the luciferase in *Escherichia coli*, we designed three constructs: bicistronic expression of luxAB from the original operon, dual promoter-driven expression of luxA and luxB, and fusion protein of luxA and luxB by a 15 amino acids linker (GGGGS)_3 (Fig. 1a). The constructs were introduced into *E. coli* strain JM109(DE3), and the whole cell suspensions overexpressing the
recombinant proteins were used for the measurement of bioluminescence. Luminescence reaction was induced by the addition of decanal [CH₃(CH₂)₈CHO] as a luciferin. The luminescence intensity of luxA + luxB expressed by the dual promoters was not statistically significant compared to that of the bicistronic luxAB (Fig. 1b). On the other hand, the luminescence of luxA(GGGGS)₃luxB was substantially lower (about 5% of luxAB). We decided to use the dual promoter-driven expression vector to modify the luciferase, because the luminescence intensity was equally high compared to the bicistronic luxAB but this construct had more convenient restriction enzyme sites for manipulation.

**Validation of BRET for bacterial luciferase.** We designed a chimeric protein containing Venus as a BRET acceptor. We fused Venus to N- or C-terminus of luxA or luxB (Fig. 2a). The luminescence spectrum exhibited a high BRET efficiency when Venus was fused to the C-terminus of luxB (Fig. 2b). An additional peak in the emission spectrum at around 528 nm, corresponding to the fluorescence emission maximum of Venus was identified. When Venus was expressed separately from the luciferase (luxAB + Venus), the luminescence was not significantly affected. The resulting luminescence intensity of luxB-Venus + luxA was about five times higher than luxA + luxB (Fig. 2c). Therefore, to achieve brighter luminescence, the construct design of fusing Venus on the C-terminus of luxB was used for further modifications.

We confirmed the expression levels of Venus-fused proteins by Western blot (Supplemental Fig. 1). The fusion proteins constructed in this study were expressed with an N-terminal His-tag, and detected by a His-tag antibody.
The expression levels of luxA was substantially low compared to luxB in all constructs. The fusion of N- and C-terminal Venus to luxB did not affect the expression levels of their fused proteins. Therefore, the increased luminescence intensity by luxB:Venus is likely due to BRET.

Optimization of BRET by circularly permuted Venus. In order to further enhance the luminescence intensity, we attempted to optimize the spatial arrangement of the donor and acceptor by using circularly permuted Venus variants (cp50Venus, cp157Venus, cp173Venus, cp195Venus and cp229Venus)\textsuperscript{18,19}. The highest BRET efficiency was observed when cp157Venus was fused to the C-terminus of luxB (Fig. 3a). The brightness of luxB:cp157Venus + luxA was about ten times higher than that of luxA + luxB (Fig. 3b). The protein expression levels of circularly permuted Venus-fused luxB, analyzed by Western blot, were unaffected by their variations (Supplemental Fig. 2). The Venus-mediated BRET had led to the color shift of light emission from blue-green to green (Fig. 3c).
Transient expression of the engineered lux genes in human cells. The pCMV<sub>Lux</sub> vector which harbors a codon-humanized viral 2A-linked luxCDEBAG genes has been shown to evoke autonomous bioluminescence in human cells. To investigate the effect of Venus-fused luciferase on autobioluminescence intensity, we inserted cp157Venus into the C-terminus of codon-humanized luxB (hluxB) of pCMV<sub>Lux</sub> (Fig. 4a), and introduced it into human embryonic kidney cell lines (HEK293T). The luminescence intensity of cells containing cp157Venus-inserted pCMV<sub>Lux</sub> was about 3.5 times higher compared to conventional pCMV<sub>Lux</sub> (Fig. 4b). No significant difference in gene expression levels of hluxB and hluxB:cp157Venus was detected by real-time quantitative RT-PCR analysis (Fig. 4c).

Transient expression of the engineered lux genes in Nicotiana benthamiana leaves. To expand the application of the Venus-fused luciferase to autobioluminescent plants, we constructed plant vectors expressing lux genes (Fig. 5a). The luxCDE complex requires fatty acid as a substrate. Because fatty acid synthesis in plant is known to occur almost exclusively in the chloroplast, we fused a transit peptide of Arabidopsis thaliana Rubisco small subunit 1A (TPats1A) in front of each lux gene for localization of the proteins in the chloroplast. The genes were placed under the control of CaMV 35S promoter for constitutive expression. The resulting genes were integrated into two separate vectors: luxA + luxB and luxC + luxD + luxE units. Agrobacterium tumefaciens was transformed with each vector, and cotransfected in equal amounts into Nicotiana benthamiana leaves using a needle-less syringe. Autonomous bioluminescence was observed in the Agrobacterium-infiltrated regions (Fig. 5b). The luminescence intensity of the leaf disc expressing luxB:cp157Venus was about seven times higher compared to that expressing the non-fused luxB (Fig. 5c). No significant difference in gene expression levels of luxB and luxB:cp157Venus was observed (Fig. 5d).
Figure 5. Autobioluminescence of *N. benthamiana* leaves expressing *lux* genes. (a) Schematic representation of expression constructs containing *lux* genes in pRI201-AN vector. (b) Luminescence image of the leaf cotransfected with the indicated constructs by *Agrobacterium* infiltration. The exposure time for the luminescence image was 10 s. Scale bar, 2 cm. (c) Luminescence intensities of leaf discs cut from the *Agrobacterium*-infiltrated regions. Data are means ± SD of three different leaves. (d) Relative quantitation of *luxB* and *luxB:cp157Venus* expression. The transcript level was probed with *luxB* primers. Results were normalized by *PP2A* gene expression. Values are calculated by ΔΔCT from three biological replicates.
Discussion

Our results showed that the optimization of the BRET between the bacterial luciferase and yellow fluorescent protein Venus can enhance bioluminescence. The luminescence intensity relative to the protein expression level suggests that the enhanced brightness of Venus-fused luciferase was not because of the increased expression level of the luciferase, but is more likely due to BRET. BRET efficiency differed depending on the construct design, and the change in emission ratio from luciferase (490 nm) and Venus (528 nm) lead to the change in color. Among the assessed construct designs, the brightness was most enhanced when cp157Venus was fused to the C-terminus of luxB. It can be speculated that high BRET efficiency was achieved by optimizing the distance (by peptide linker) and relative dipole orientation (by circular permutation) between luciferase and cp157Venus.

The quantum yield of bacterial luciferase is about 0.1–0.1623,24 and Venus is about 0.618. Considering that cp157Venus has a similar brightness to the wild-type Venus18 (therefore, similar quantum yield), maximum of six times enhancement can be expected by BRET. However, we achieved ten times enhancement by fusing luciferase to cp157Venus, which was higher than what was expected. This may have resulted from the change in functional properties of luciferase caused by the fusion of cp157Venus. For example, the firefly luciferase fused to Venus had enhanced brightness not as a result of BRET, but from other reasons that is not understood25. Further investigations such as 3D structure and biophysicochemical properties (e.g. \( K_m \)) of the luxB fused with Venus may provide additional information for the cause of this additional enhancement and for further improvement of brightness.

We demonstrated that the brightness of this enhanced BRET-based luciferase was functional in autonomous luminous mammalian and plant cells generated by the coexpression of lux genes. Recently, autoboluminescence imaging of plant by using the fungal bioluminescence system was reported30. The fungal luciferase is a promising tool for autonomous luminous bioimaging, but the enzyme exhibits several drawbacks. The fungal luciferase is a temperature-sensitive enzyme, so the activity is almost lost at above 30 °C27. In addition, the transmembrane domain of the fungal luciferase can lead to low solubility27, making it difficult for high cytosolic or organelle targeted expression. The bacterial bioluminescence system would function in widely cell types including both animals and plants compared to the fungal bioluminescence system.

Naturally occurring energy transfer between bacterial luciferase and fluorescent protein has been found in some species. The Vibrio fischeri strain Y-1 expresses a yellow fluorescent protein YFP, and emits yellow light (around 545 nm) by energy transfer between the luciferase and YFP28,29. Furthermore, the blue fluorescent protein termed lumazine protein (LumP) was isolated from Photobacterium leiognathi, Photobacterium phosphoreum and Photobacterium kishitanii, and energy transfer between the luciferase and LumP causes blue light emission (around 475 nm)30–32. These YFP and LumP also enhanced the intensity of luminescence greater than three to four times29,30, however, the genes of YFP or LumP in these bacteria are not genetically fused to the lux genes and may not be optimal for high luminescence. Our results showed that the optimized fusion of the fluorescent protein to bacterial luciferase led to a more effective energy transfer.

Several attempts have been made to enhance the signal of bacterial bioluminescence, including codon optimization6,33 and random mutagenesis11,34. The improvement of bacterial luciferase by BRET is a useful strategy to enhance the bioluminescent signal. So far, BRET-based bacterial luciferase has been applied for the analysis of protein–protein interactions35, or biosensors36. Further optimization of BRET pair and linker in between them may improve the brightness of bacterial bioluminescence.

Luminescence using the bacterial lux system has been demonstrated in different species including plants and animals, and further development of enhanced brightness by fusing luxB to other fluorescent proteins may also be useful. For example, orange/red-light emitting luminescent proteins such as Antares37 and ReNL38 have been developed using other luciferases, which allows deep-tissue imaging by the effective light penetration of the longer wavelength. The BRET-based bacterial luciferase holds promise as a valuable autobioluminescent tool for long-term continuous imaging in live cells from bacteria to plants and animals.

Methods

Construction of E. coli expression vectors. Primers used in this study were listed in Supplementary Table 1. The luxCDABE genes of Photorhabdus luminescens was cloned from pAKlux139, which was provided by Attila Karsi (Addgene plasmid # 14073). For bicistronic expression of luxAB operon in E. coli, PCR-amplified luxAB fragment was digested with BamHI and EcoRI, and the fragment was inserted in-frame into the corresponding site of pRSET B (Thermo Fisher Scientific). For the fusion protein expression, a 15-amino-acid linker (GGGGS)3 was inserted between luxA and luxB by overlap extension PCR as previously described14. The resulting PCR product was inserted in-frame into the BamHI-EcoRI site of pRSET B.

For co-expression of two luciferase in E. coli by dual promoter from a single plasmid, the region including two multiple cloning sites (MCS) of pETDuet-1 (Merck) excised with BamHI and KpnI was inserted into the corresponding site of pRSET B. Venus and circularly permuted Venus were cloned from BRAC derivatives49. Venus or circularly permuted Venus variant was fused to a subunit of the luciferase by an EL (glutamic acid-leucine) linker encoded by Sacl recognition sequence. The fusion constructs were inserted in-frame into the BamHI-Nool site of the MCS1. The PCR-amplified fragment of another subunit of luciferase was inserted in-frame into the NdeI-KpnI site of MCS2.

Measurement of bioluminescence in E. coli. The colonies of transformed E. coli JM109(DE3) were grown at 23 °C for 60 h in 2 ml Luria–Bertani (LB) medium to express the recombinant protein, as previously described46. The OD600 of the cultures were adjusted to 1.0 by adding LB medium. Emission spectra of the whole cell suspensions overexpressing the recombinant proteins were measured using a photonic multi-channel analyzer (PMA-12, Hamamatsu Photonics) at room temperature. Decanal is known to cross membranes48 and a
final concentration of 1% (v/v) decanal (Wako Pure Chemical) was added just before the measurement, which is expected to be at or near saturating concentration41.

**Western blot.** The transformed *E. coli* cells were collected and resuspended in PBS buffer with 0.2 mg/ml lysozyme. The cells were lysed by sonication, and the supernatant was collected. The samples were mixed with SDS sample buffer and boiled for 5 min. The proteins were separated by SDS-PAGE. The proteins were transferred to Immobilon-P PVDF membrane (Merck). For detection of His-tagged proteins, Anti-His-tag pAb (MBL) at a dilution of 1:5000, and the secondary anti-rabbit IgG, HRP conjugate (Promega) at a dilution of 1:5000 were used. The chemiluminescence was imaged by ECL Prime Western Blotting Detection Reagent (GE Healthcare).

**Modification of pCMV<sub>Lux</sub> vector.** The pCMV<sub>Lux</sub> vector was purchased from 490 Biotech. A unique EcoRV site is located in the 3’ end of luxB of pCMV<sub>Lux</sub> and cp157Venus was inserted into the EcoRV site of pCMV<sub>Lux</sub> by In-Fusion HD cloning (Takara Bio).

**Transient expression assay in HEK293T cells.** HEK293T cells (RIKEN BRC, RCB2202) were cultured to 60% confluence. Then cells were transformed with an expression vector using polyethylene imine (PEI MAX, MW 40,000, Polysciences) according to the manufacturer’s instructions, and grown for 24 h. Before measurements, transfected cells on a 4 cm culture dish were detached and suspended by 1 ml of Gibco Dulbecco’s Modified Eagle’s Medium (DMEM/F-12, 15 mM HEPES, no phenol red, Thermo Fisher Scientific). Luminescence of the cells were measured by a microplate reader (SH-9000, Corona Electric).

**Construction of plant expression vectors.** We prepared two types of modified pRI201-AN (Takara Bio) as follows; One pRI201-AN was digested with BamHI, and digested ends were filled in with KOD -Plus-DNA polymerase (Toyobo). The subjected DNA fragment was circularized by DNA ligase (Promega) for yielding pRI201-AN(ΔBamHI). Another pRI201-AN was digested with KpnI and EcoRI to remove the MCS2 from the plasmid (MBL). Bio) as follows; One pRI201-AN was digested with BamHI, and digested ends were filled in with KOD -Plus-DNA polymerase (Toyobo). The subjected DNA fragment was circularized by DNA ligase (Promega) for yielding pRI201-AN(ΔBamHI). Another pRI201-AN was digested with KpnI and EcoRI to remove the MCS2 from the plasmid (MBL). Bio).

**Transient expression assay in *N. benthamiana* by Agrobacterium infiltration.** For plant material, wild-type *N. benthamiana* were germinated on soil and grown for one month at 24 °C under 24 h light. The third leaf from the top was used for *Agrobacterium* infiltration. All plant materials were handled and disposed of according to Osaka University guidelines. Transformed *A. tumefaciens* (GV3101) was collected and resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6, 100 μM acetoxyringone) to OD<sub>590</sub> = 0.5. The suspension was kept at room temperature for 4 h. For cotransfection of luxA + luxB and luxC + luxD + luxE, two transformed plasmids were mixed in equal amounts. A final concentration of 0.005% (v/v) silwet L-77 was added just before the infiltration. The suspension was infiltrated using a needleless syringe to the abaxial side of leaves of *N. benthamiana*. Three days after infiltration, bioluminescence was observed by a multi-functional in vivo imaging system (MIIS, Molecular Devices) equipped with EMCCD camera (iXon Ultra897, Andor Technology). For quantitative analysis of signal intensity, the infiltrated regions were cut into 5 mm diameter discs, and luminescence intensity of leaf discs were measured by a microplate reader (SH-9000).
Quantitative analysis of gene expression. Total RNA was extracted using TRizol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Before cDNA synthesis, genomic DNA contamination was removed using TURBO DNase (Thermo Fisher Scientific). One microgram of total RNA was reverse transcribed to first-strand cDNA using SuperScript III first-strand synthesis system (Thermo Fisher Scientific) with oligo(dT)$_{20}$ primer. Real-time quantitative RT-PCR was performed on StepOne Real-Time PCR system (Thermo Fisher Scientific) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The baseline and threshold cycles (C$_{T}$) for the amplification curves were calculated using the StepOne software v2.3. Results from HEK293 and N. benthamiana were normalized by GAPDH and P2PA$_{45}$ gene expression, respectively. Relative level of gene expression was analyzed by the comparative C$_{T}$ ($\Delta\Delta$C$_{T}$) method (StepOne software v2.3).

Statistical analysis. Statistical significance was accepted at P<0.05 assessed by Student’s t-test.

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References
1. Hastings, J. W. & Nealon, K. H. Bacterial bioluminescence. Ann. Rev. Microbiol. 31, 549–595 (1977).
2. Meighen, E. A. Molecular biology of bacterial bioluminescence. Microbiol. Rev. 55, 123–142 (1991).
3. Dunlap, P. Biochemistry and genetics of bacterial bioluminescence. Adv. Biochem. Eng. Biotechnol. 144, 37–64 (2014).
4. Frackman, S., Anhalt, M. & Nealon, K. H. Cloning, organization, and expression of the bioluminescence genes of Xenorhabdus luminescens. J. Bacteriol. 172, 5767–5773 (1990).
5. Gupta, R. P., Patterson, S. S., Ripp, S., Simpson, M. L. & Sayler, G. S. Expression of the Photobahdus luminescens lux genes (luxA, B, C, D, and E) in Saccharomyces cerevisiae. FEMS Yeast Res. 4, 305–313 (2003).
6. Close, D. M., Patterson, S. S., Ripp, S., Baek, S. J., Sanseverino, J. & Sayler, G. S. Autonomous bioluminescent expression of the bacterial luciferase gene cassette (lux) in a mammalian cell line. PLoS One 5, e12441 (2010).
7. Krichevsky, A., Meyers, B., Vainstein, A., Maliga, P. & Citovskv, Y. Autoluminescent plants. PLoS One 5, e15461 (2010).
8. Xu, T., Ripp, S., Sayler, G. S. & Close, D. M. Expression of a humanized viral 2A-mediated lux operon efficiently generates auto-

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natureportfolio
34. Cui, B., Zhang, L., Song, Y., Wei, J., Li, C., Wang, T., Wang Y., Zhao T. & Shen, X. Engineering an enhanced, thermostable, monomeric bacterial luciferase gene as a reporter in plant protoplasts. *PLoS One* **9**, e107885 (2014).
35. Cui, B., Wang, Y., Song, Y., Wang, T., Li, C., Wei, Y., Luo, Z. Q. & Shen, X. Bioluminescence resonance energy transfer system for measuring dynamic protein–protein interactions in bacteria. *mBio* **5**, e01050–14 (2014).
36. Gammon, S. T., Villalobos, V. M., Roshal, M., Samarakandi, M. & Pwinka-Worms, D. Rational design of novel red-shifted BRET pairs: Platforms for real-time single chain protease biosensors. *Biotechnol. Prog.* **25**, 559–569 (2009).
37. Chu, J. et al. A bright cyan-excitable orange fluorescent protein facilitates dual-emission microscopy and enhances bioluminescence imaging in vivo. *Nat. Biotechnol.* **34**, 760–767 (2016).
38. Suzuki, K. et al. Five colour variants of bright luminescent protein for real-time multicolour bioimaging. *Nat. Commun.* **7**, 13718 (2016).
39. Karsi, A., Menanteau-Ledouble, S. & Lawrence, M. L. Development of bioluminescent *Edwardsiella ictaluri* for noninvasive disease monitoring. *FEMS Microbiol. Lett.* **260**, 216–223 (2006).
40. Legocki, R. P., Legocki, M., Baldwin, T. O. & Szalay, A. A. Bioluminescence in soybean root nodules: Demonstration of a general approach to assay gene expression in vivo by using bacterial luciferase. *Proc. Natl. Acad. Sci. USA* **83**, 9080–9084 (1986).
41. Blouin, K., Walker, S. G., Smit, J. & Turner, R. Characterization of in vivo reporter systems for gene expression and biosensor applications based on luxAB luciferase genes. *Appl. Environ. Microbiol.* **62**, 2013–2021 (1996).
42. Liu, D., Shi, L., Han, C., Yu, J., Li, D. & Zhang, Y. Validation of reference genes for gene expression studies in virus-infected *Nicotiana benthamiana* using quantitative real-time PCR. *PLoS One* **7**, e46451 (2012).

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Author contributions

T.N. conceived and supervised the project. T.K., K.S., T.E., K.O. and T.N. designed the experiments. T.K. performed the gene construction, and the data acquisition. K.S. performed the transformation of HEK293T cells. T.E. constructed the plant expression vectors. T.K. wrote the original draft, and all authors reviewed and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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