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Abstract. Luciferase, a bioluminescent protein, has been used as an analytical tool to visualize intracellular phenomena. Luciferase with red light emission is particularly useful for bioluminescence imaging because of its high transmittance in mammalian tissues. However, the luminescence intensity of existing luciferases with their emission over 600 nm is insufficient for imaging studies because of their weak intensities. We developed mutants of Emerald luciferase (Eluc) from Brazilian click beetle (*Pyrearinus termitilluminans*), which emits the strongest bioluminescence among beetle luciferases. We successively introduced four amino acid mutations into the luciferase based on a predicted structure of Eluc using homology modeling. Results showed that quadruple mutations R214K/H241K/S246H/H347A into the beetle luciferase emit luminescence with emission maximum at 626 nm, 88-nm red-shift from the wild-type luciferase. This mutant luciferase is anticipated for application in *in vivo* multicolor imaging in living samples. [DOI: 10.1117/1.JBO.20.10.101205]

Keywords: luciferase; red-shift; Pyrearinus termitilluminans; bioluminescence.

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1 Introduction

Luciferase is an enzyme that catalyzes the oxidation of D-luciferin to produce oxyluciferin in the electronically excited state, resulting in the emission of bioluminescence.[1] Luciferase has been widely used to detect and visualize intracellular events such as gene expression,[2] protein translocation,[3] protein–protein interactions,[4,5] enzymatic activities,[6] and pH changes.[7] The wavelength range of beetle luciferases expands from the 538 nm of the Brazilian click beetle (*Pyrearinus termitilluminans*) luciferase (PtGR)[8] to the 628 nm of the railroad worm (*Phrixothrix hirtus*) luciferase (PhRE).[9] Actually, D-luciferin is a common substrate for beetle luciferases. Therefore, the environment in the catalytic pocket is a major determinant of the bioluminescence wavelength.

Luciferases with red light emission are beneficial for *in vivo* imaging because of their high transmittance in mammalian tissues.[10] Moreover, red light emitting luciferases, in addition to green ones, are useful to investigate two or more biological phenomena at the same time. For example, a technique of multicolor luminescence imaging has been used to monitor antiphasic oscillations of two clock genes in living mice.[11] Also, two different colors of bioluminescence were applied for visualizing stage-specific spatial distributions of Smad1–Smad4 and Smad2-Smad4 interactions in single *Xenopus* embryos.[4] Therefore, red-shifted luciferase mutants have been extensively studied using a luciferase of *Photinus pyralis* (*Ppy*) (λ max = 552 nm). The color variation of *Ppy* luciferase mutants ranges from 549 to 623 nm, which is nearly comparable to that of natural beetle luciferases (538 to 628 nm).[12] However, the bioluminescence intensities of both natural and mutant luciferases with red light emission are much weaker than those of green light emitting luciferases.[13] To propose another choice for red light emitting luciferase, we generated Emerald luciferase (Eluc) mutants by site-directed mutagenesis. Eluc is one of the brightest beetle luciferases and has the same amino acid sequence as that of PtGR. The DNA sequence is optimized for expression in mammalian cells.[14] The Eluc structure was predicted using homology modeling. We identified four amino acid residues surrounding D-luciferin. By site-directed mutagenesis in the four residues, we obtained an Eluc mutant with its bioluminescence spectrum peak at 626 nm, which was 88 nm longer than that of original Eluc. This Eluc mutant constitutes a good candidate for use as a tool for multicolor *in vivo* bioluminescence imaging in living samples.

2 Materials and Methods

2.1 *Homology Modeling*

Homology modeling of Eluc was conducted using SWISS-MODEL.[15] The three-dimensional (3-D) structure of a substrate binding luciferase derived from *Luciola cruciata* (PDB: 2D1R) was used as a template. Amino acid sequences of click beetle green (CBG) (AY258595.1) and click beetle red (CBR) (AAP83303.1) were obtained from GenBank.

2.2 *Site-Directed Mutagenesis*

The cDNA for Eluc (Emerald Luc®) was purchased from TOYOBO (Shiga, Japan). Mutations at R214, H241, S246,

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Table 1 Mutagenic primers. DNA sequences of the mutagenic primers are listed. The mutations are shown as italics.

Primer name	DNA sequence
R214X	5'-ACC CAC AGG AAC ATC TGT GTG *NNN* CTC ACA CAC GCA TCT GAC CCC-3'
H241X	5'-C CTG CCA TTC TTC *NNN* GCC TTC GGC TTC AG-3'
S246H	5'-GCC TTC GGC TTC *CA*T ATC AAC CTG GGC-3'
H347Q	5'-CC TCC GCC AAC ATC CA*A* ACT CTG CAC AAC GAG TTC-3'
H347A	5'-CC TCC GCC AAC ATC *GCC* ACT CTG CAC AAC GAG TTC-3'

and H347 of Eluc were introduced by site-directed mutagenesis with the primers shown in Table 1 and their complementary primers. Template DNA (approx. 0.1 μmol) was added to a polymerase chain reaction (PCR) mixture containing 0.8 μM 5'-phosphorylated primer, 0.2 mM dNTP mix, 5× PrimeSTAR Buffer, and 2.5 U of PrimeSTAR HS DNA Polymerase (Takara Bio Inc.). The PCR cycling parameters were the following: the solution was heated at 98°C for 2 min, followed by 30 cycles of 10 s at 98°C, 5 s at 55°C, and 7 min at 72°C. The mixture was treated with *Dpn*I (10 U; Takara Bio Inc.) at 37°C for 4 h. The introductions of the mutations were verified using DNA sequencing (Eurofins Sequencing Service).

2.3 *Expression and Purification of Luciferases*

Wild-type and mutant luciferases were expressed in *E. coli* BL21. The *E. coli* harboring the expression plasmid was grown in 1 mL of LB medium at 37°C until the mid-log phase ($O.D._{600} = 0.4$–0.6). Then 1 mM IPTG was added and the cultures were incubated at 16°C for 24 h. The cells were collected by centrifugation at 4°C and were suspended in 30 μL of phosphate buffered saline (PBS) with subsequent sonication for 10 min at medium intensity using Bio Ruptor (Cosmo Bio Co. Ltd., Tokyo, Japan). The lysate was centrifuged to obtain the soluble fraction containing the luciferase. The solution was loaded onto equilibrated His SpinTrap (GE Healthcare) and was centrifuged at 4°C for 2 min. The column was washed several times with 300 μL of PBS containing 30 mM imidazole until the absorbance of the flowthrough solution at 280 nm became negligible. The protein was eluted with 200 μL of 300 mM imidazole in PBS and was stored on ice until measurements of bioluminescence were conducted.

2.4 *Luminescence Measurement*

First, 5 μL of the crude or purified luciferase solution was added to 45 μL of reaction mix containing 400 μM luciferin, 1 mM adenosine triphosphate (ATP), 5 mM $MgSO_4$, and 30 mM HEPES (pH = 7.6). Bioluminescence spectra were measured using a spectroluminometer (AB-1850 LumiFlSpectroCapture; Atto Corp., Tokyo, Japan) with a slit width of 0.5 mm. Background luminescence from the reaction mix without luciferases was subtracted from the obtained spectra. Luminescence was detected with the exposure time of 75 s and the intensities were evaluated by photon counts at the wavelength of the peak maximum. To obtain kinetics of luminescence, luminescence intensities were integrated every 15 s. The half-life of the luminescence was evaluated by the duration from the luminescence peak to the point when luminescence was decreased to the half of its peak intensity.

3 Results

3.1 *Eluc Structures Based on Homology Modeling*

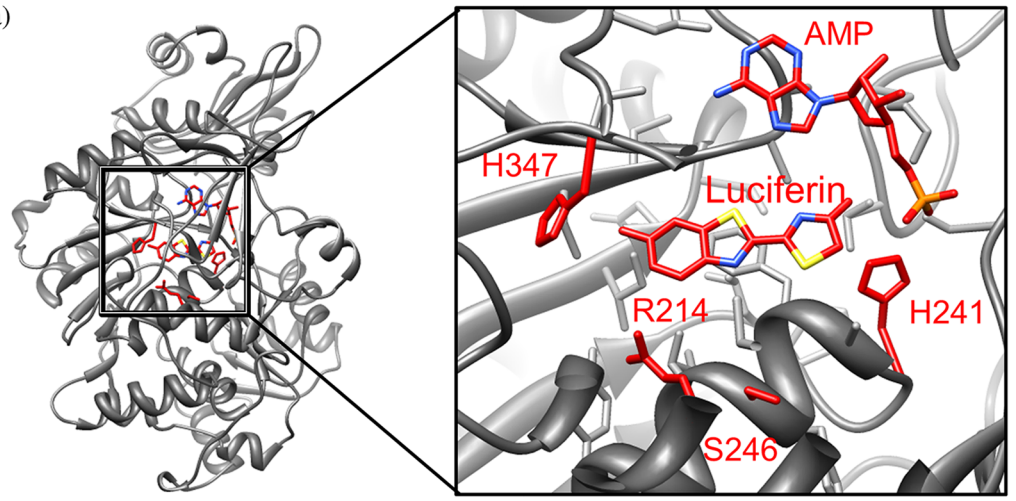
According to a previous research on *Ppy* luciferase mutants, residues near the luciferin binding pocket affect the bioluminescence wavelength.[16] We inferred that amino acid mutations around the D-luciferin binding site of Eluc induce a red-shift in the bioluminescence.[17] To identify the residues around the luciferin binding site of Eluc, we performed homology modeling of Eluc using SWISS-MODEL.[15] The amino acid sequence of Eluc was aligned with that of *Luciola cruciata* (*Lcr*) luciferase, of which a crystal structure incorporating a substrate analog 5'-*O*-[*N*-(dehydroluciferyl)sulfamoyl]adenosine (DLSA) has already been reported (PDB: 2D1R).[18] Based on the alignment results, we constructed a predicted structure of Eluc. The structure displayed that amino acid residues of R214, H347, H241, and S246 are located in the binding pocket, close to D-luciferin within a distance of 6 Å. R214 and H347 are near the benzothiazole ring of the substrate, whereas H241 is close to the thiazolone ring. The residue of S246 is directed perpendicularly to the center of D-luciferin [Fig. 1(a)].

To obtain additional clues to induce a red-shift to Eluc, we compared the amino acid sequence of Eluc with CBG and CBR luciferases, both of which are derived from *Pyrophorus plagiophthalamus*. The amino acid residues that are conserved in Eluc and CBG, but not in CBR, are candidates for the mutation sites to generate red-shifted Eluc mutants. We identified such candidates of four amino acid residues of Eluc: V223, S246, N345, and H347 [Figs. 1(b) and 1(c)]. Taken together with the result of the homology modeling, we selected S246 and H347 as amino acid residues for mutagenesis to produce red-shifted Eluc mutants.

3.2 *Spectral Properties of Eluc Mutants at S246 and H347*

Based on a comparison between the amino acid sequences of Eluc and CBR [Figs. 1(b) and 1(c)], we examined spectral properties of Eluc mutants, S246H and H347Q, in a physiological condition (pH = 7.6). The emission maxima of the bioluminescence spectra were red-shifted upon insertion of the single mutations (Fig. 2). The S246H mutation caused a red-shift of 21 nm, and the H347Q mutation showed a greater peak shift of 66 nm. The H347Q mutant showed a larger red-shift than any other single-mutated beetle luciferases already reported. The luminescence intensity of the H347Q mutant was, however, 20 times lower than that of the wild-type Eluc. To obtain a mutant with higher bioluminescence intensity, we introduced an alanine instead of a glutamine at the position of H347. The H347A mutant showed a 54-nm red-shift compared with wild-type Eluc (Fig. 2). The bioluminescence intensity was 10 times stronger than that of the H347Q mutant. We introduced a mutation of S246H into the mutant H347A, causing the emission maximum of 602 nm, which is a 64 nm longer wavelength than that of the wild type. This value was almost identical to

(a)



(b)

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                10        20        30        40        50        60        70
                |         |         |         |         |         |         |
PtGR  MEREKNVVYGPEPKHPLGNFTAGEMLYNALHKHSHIPQAILDVMGNESLSYQEFFDTTVKLGQSLQNCGY
CBG   MVKREKNVIYGPEPLHPLEDLTAGEMLFRALRKHSHLPQALVDVVGDESLSYKEFFEATVLLAQSLHNCGY
CBR   MVKREKNVIYGPEPLHPLEDLTAGEMLFRALRKHSHLPQALVDVVGDESLSYKEFFEATVLLAQSLHNCGY

                80        90        100       110       120       130       140
                |         |         |         |         |         |         |
PtGR  KMNDVVSICAENNKRFFIPIISAWYIGMVVAPVNEDYIPDELCKVTGISKPILVFTTRKILPKVLEVKDR
CBG   KMNDVVSICAENNTRFFIPVIAAWYIGMIVAPVNESYIPDELCKVMGISKPQIVFTTKNILNKVLEVQSR
CBR   KMNDVVSICAENNTRFFIPVIAAWYIGMIVAPVNESYIPDELCKVMGISKPQIVFTTKNILNKVLEVQSR

                150       160       170       180       190       200       210
                |         |         |         |         |         |         |
PtGR  TNYIKRIIILDSEENLLGCESLHNFMSRYSDNNLQTFKPLHYDPVDQVAAILCSSGTTGLPKGVMQTHRN
CBG   TNFIKRIIILDTVENIHGCESLPNFISRYSDGNIANFKPLHFDPVEQVAAILCSSGTTGLPKGVMQTHQN
CBR   TNFIKRIIILDTVENIHGCESLPNFISRYSDGNIANFKPLHFDPVEQVAAILCSSGTTGLPKGVMQTHQN

                220       230       240       250       260       270       280
                |         |         |         |         |         |         |
PtGR  ICVRLTHASDPRVGTQLIPGVSVLAYLPFFHAFGFSINLGYFMVGLRVVMLRRFNQEVFLKAIQDYEVRS
CBG   ICVRLIHALDPRVGTQLIPGVTVLVYLPFFHAFGFSITLGYFMVGLRVIMFRRFDQEAFLKAIQDYEVRS
CBR   ICVRLIHALDPRYGTQLIPGVTVLVYLPFFHAFGFHITLGYFMVGLRVIMFRRFDQEAFLKAIQDYEVRS

                290       300       310       320       330       340       350
                |         |         |         |         |         |         |
PtGR  VINVPSTILFLSKSPLVDKYDLSTLAELCCGAAPLAKEVAEIAVKRLNLPGIRCGYGLTESTSANIHTLH
CBG   VINVPSVILFLSKSPLVDKYDLSSLRELCCGAAPLAKEVAEVAAKRLNLPGIRCGFGLTESTSANIHSLR
CBR   VINVPSVILFLSKSPLVDKYDLSSLRELCCGAAPLAKEVAEVAAKRLNLPGIRCGFGLTESTSAIIQTLG

                360       370       380       390       400       410       420
                |         |         |         |         |         |         |
PtGR  NEFKSGSLGKVTPYMAAKIIDRNTGEALGPNQVGELCIWGPMVTKGYVNNPQATKEAIDDDGWLHSGDFG
CBG   DEFKSGSLGRVTPLMAAKIADRETGKALGPNQVGELCIKGPMVSKGYVNNVEATKEAIDDDGWLHSGDFG
CBR   DEFKSGSLGRVTPLMAAKIADRETGKALGPNQVGELCIKGPMVSKGYVNNVEATKEAIDDDGWLHSGDFG

                430       440       450       460       470       480       490
                |         |         |         |         |         |         |
PtGR  YYDEDEYFYIVDRYKELIKYKGYQVAPVELEEILLQHPGIRDVAVVGIPDIEAGELPAGFVVKQPGAQLT
CBG   YYDEDEHFYVVDRYKELIKYKGSQVAPAELEEILLKNPCIRDVAVVGIPDLEAGELPSAFVVKQPGKEIT
CBR   YYDEDEHFYVVDRYKELIKYKGSQVAPAELEEILLKNPCIRDVAVVGIPDLEAGELPSAFVVKQPGTEIT

                500       510       520       530       540
                |         |         |         |         |
PtGR  AKEVYDFLAQRVSHSKYLRGGVRFVDSIPRNVTGKISRKELREALMEKASKL
CBG   AKEVYDYLAERVSHTKYLRGGVRFVDSIPRNVTGKITRKELLKQLLEKAGG
CBR   AKEVYDYLAERVSHTKYLRGGVRFVDSIPRNVTGKITRKELLKQLLVKAGG
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(c)

Residues	223	246	345	347	348	487	537	λmax (nm)
PtGR	V	S	N	H	T	A	K	538 (green)
CBG	V	S	N	H	S	K	E	537 (green)
CBR	Y	H	I	Q	T	T	V	613 (red)

Fig. 1 (a) The luciferin binding site in the predicted structure of Emerald luciferase (Eluc). Luciferin is located in the center. Amino acid residues close to the substrate are shown in color. (b, c) Amino acid sequences of three click beetle luciferases, Eluc, CBG, and CBR. The seven residues which differ between CBG and CBR are indicated by arrows. Black arrows indicate residues conserved only in Eluc and CBG. Gray arrows indicate residues not conserved in click beetle luciferases (CBG and CBR) and in green luciferases (Eluc and CBG).

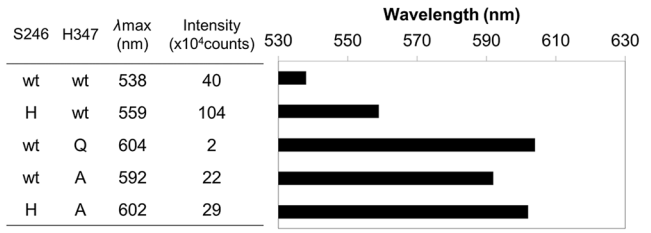

Fig. 2 Wavelengths and intensities of emission maxima for the wild-type Eluc and its mutants.

that of the H347Q mutant. The bioluminescence intensity was not decreased by the extra mutation of S246H (Fig. 2).

3.3 *Comprehensive Mutagenesis at R214 and H241 of Eluc*

We obtained an Eluc mutant with red-shifted luminescence only by insertion of S246H/H347A. However, the emission maximum of the mutant was still shorter than that of natural red luciferases such as PhRE (λ max = 628 nm). Then we successively

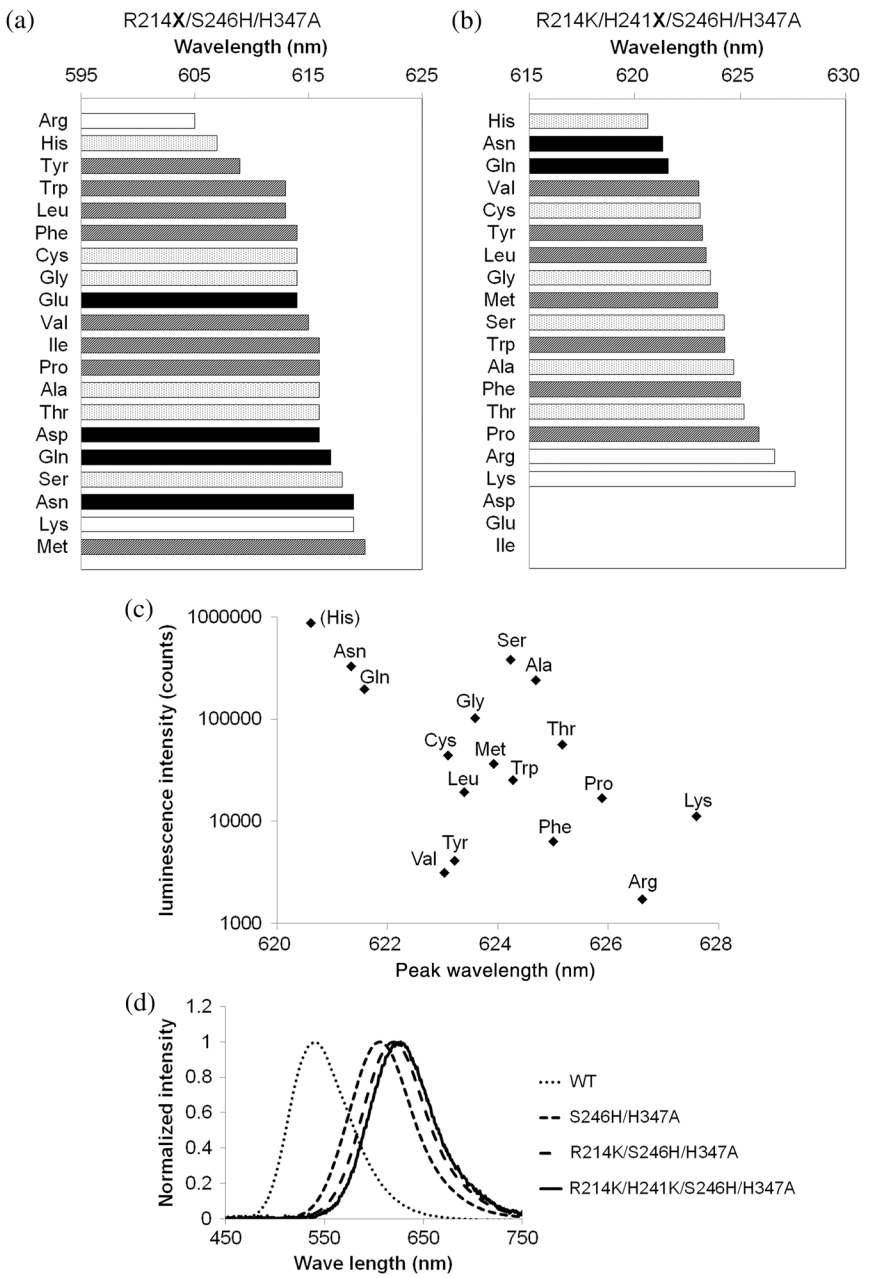

Fig. 3 (a,b) Emission maxima of the bioluminescence for R214X/S246H/H347A (a) and H241X/R214K/S246H/H347A (b) mutants. Each column indicates a single mutant with a mutation at R214 (a) or H241 (b). Negatively and positively charged residues are colored, respectively, in black and white. Hydrophobic residues are shown in gray. Asp, Glu, Ile mutants for R214K/H241X/S246H/H347A showed no detectable bioluminescence. (c) Wavelengths and intensities of emission maxima for the R214K/H241X/S246H/H347A mutants. The concentration of luciferases was six times higher than the condition of the experiment shown in Fig. 2. (d) Bioluminescence spectra of wild-type Eluc and its mutants are shown. Each spectrum was normalized at peak intensity.

Table 2 Luminescence properties of the wild type and mutant Emerald luciferases. Half-life was evaluated by the duration from the luminescence peak to the time point when the luminescence intensity decreased to the half of its peak intensity.

	λ max (nm)	Relative intensity	Half-life (min)
WT	538	1	11
S246H/H347A	602	0.47	9.5
R214K/S246H/H347A	619	0.082	6.5
R214K/H241K/S246H/H347A	626	0.001	6

inserted mutations at two other amino acid residues, R214 and H241. Previous studies of mutagenesis and modeling of *Ppy* and *Lcr* luciferases demonstrated that mutations at R214 and H241 affected bioluminescence spectra.[19–22] Based on such information, we performed systematic mutagenesis at the positions in the S246H/H347A Eluc mutant, and evaluated the properties of bioluminescence. First, the R214 residue in the S246H/H347A mutant was mutated to yield the triple mutants, and the bioluminescence spectra of each mutant were investigated. All the mutants caused red-shifted bioluminescence [Fig. 3(a)], of which the R214M/S246H/H347A mutant showed the most red-shifted emission (λ max was 620 nm). The intensity of this mutant was, however, low (data not shown). Therefore, we further systematically introduced mutations at H241 in the R214K/S246H/H347A mutant instead of the R214M/S246H/H347A mutant to prepare the 19 mutants. In these mutants, luminescence of aspartic acid, glutamic acid, and isoleucine mutants was not detectable [Figs. 3(b) and 3(c)]. Mutations of H241K in the R214K/S246H/H347A Eluc mutant showed the largest wavelength shift; the mutant emitted 7-nm longer wavelength light than that of the R214K/S246H/H347A mutant. The luminescence properties of the wild type and its mutant luciferases are summarized in Table 2 and Fig. 3(d). The luminescence of mutant luciferases showed transient increases in a few minutes with gradual decreases over 15 min thereafter, whose property is almost the same as that of the wild-type luciferase (Fig. 4). Consequently, we developed an 88-nm red-shift Eluc mutant by insertion of four amino acid mutations (R214K, H241K, S246H, and H347A) into the wild-type Eluc [Fig. 3(d)].

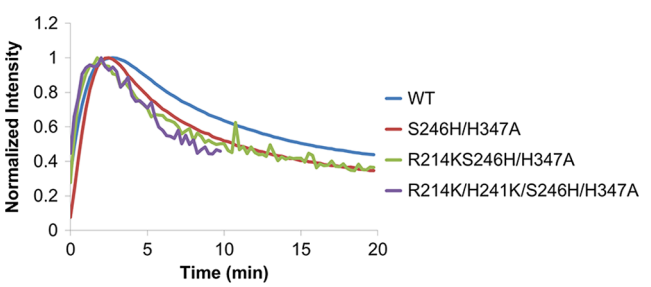

Fig. 4 Time dependence of luminescence intensities for wild type and three typical Eluc mutants. Luminescence intensities were normalized at their peak intensities.

4 Discussion

We conducted a quadruple mutation on Eluc and achieved a 88 nm red-shift, which is the greatest red-shift ever reported for beetle luciferases. The substituted four amino acid residues were selected based on the result of homology modeling of Eluc [Fig. 1(a)]. Two mutations, S246H and H347A, were performed first, causing a 64-nm red-shift. Additional mutations, R214K and H241K, were identified, respectively, through comprehensive amino acid substitutions, causing further 17 and 7 nm red-shifts. Consequently, we generated a new Eluc mutant by insertion of four amino acid mutations, which showed 88-nm red-shifted luminescence over that of WT Eluc.

Several residues are known to affect the bioluminescence wavelength from previous studies of random mutagenesis.[16,19–21] A recent study revealed the 3-D structure of a luciferase binding to a luciferin analog.[18] Based on these findings, the wavelength of bioluminescence is possibly affected by three factors; polarity of the pocket,[23,24] the size of the cavity,[18] and the electrostatic microenvironment.[22] In our study, the mutated positions seem close to the oxyluciferin [Fig. 1(a)]. Hence, the mutations in the Eluc mutants possibly affect the electrostatic microenvironment, resulting in the large red-shift of the luminescence. Regarding the microenvironment, previous studies demonstrated changes in the bioluminescence wavelength caused by mutations on the luciferases using mathematical calculations.[22,25] These calculations displayed a difference of electron distribution between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of oxyluciferin. In the HOMO, the benzothiazole ring is electronically more populated than the thiazolone ring. In contrast, in the LUMO, electrons distribute uniformly in the oxyluciferin[22] (Fig. 5). Negative and positive charges near the benzothiazole and the thiazolone rings, respectively, will increase the energy level of the HOMO, and reduce the energy gap separating the HOMO and the LUMO. This energy level alteration results in the red-shift of the bioluminescence spectra (Fig. 5). When one considers that the amino acid residue of H241 is close to the thiazolone ring, it is reasonable that the insertion of positively charged residues such as Lys and Arg at H241 caused a larger red-shift. The observations that the insertion of negatively charged residues into R214 caused a larger red-shift show good agreement with the fact that R214 is located near the benzothiazole ring. However, the most red-shifted mutants of R214X had methionine and lysine residues, which are neutral or even positively charged residues. This discrepancy is possibly caused by perturbation of the substrate-binding pocket conformation with induction of flexible side chains at the positions.

Many studies have been conducted to investigate the effect of the mutations on the spectral changes of bioluminescence using the firefly luciferase. It has been reported that two mutations in PtGR, C311A[26] and V224F,[27] caused a large red-shift (more than 40 nm), although other mutations had little effect on the spectrum.[27,28] The study suggested that the mutations at C311 and V224, whose side chains do not interact with the substrate, reduced the stability of the binding pocket and induced a large red-shift. Our study showed that the mutations in the residues that constitute the binding pocket also induced a large red-shift. Furthermore, by combining the red-shift mutations on Eluc, the present red-shifted quadruple mutant showed the largest wavelength shift in the Eluc mutants ever reported.

A pair of luciferases with sufficient wavelength separation has been used for simultaneous monitoring of multiple

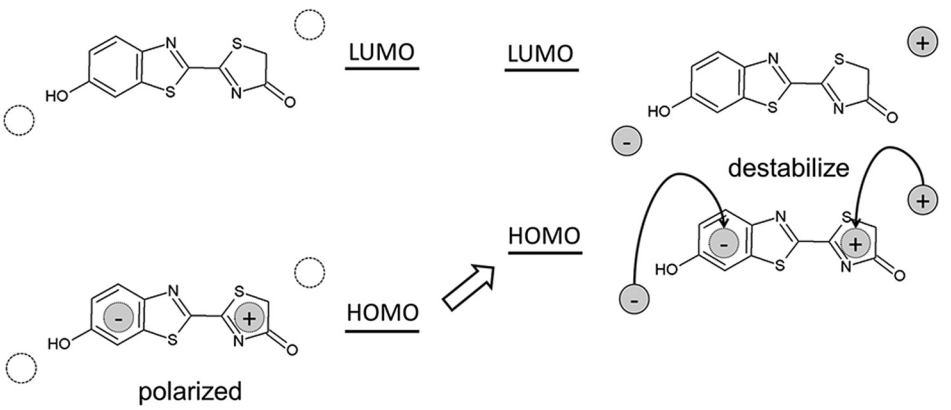

Fig. 5 Schematic diagram of the electron distribution and the energy levels of the highest occupied molecular orbital (HOMO) and the lowest occupied molecular orbital (LUMO). The HOMO energy level of oxyluciferin is highly polarized (left). When a negative charge is placed near the benzothiazole ring and a positive charge is placed near the thiazolone ring, the polarized HOMO energy level is destabilized (right). Accordingly, the energy gap separating the HOMO and the LUMO decreases and the wavelength of the luminescence is red-shifted.

biological molecules or phenomena in cultured cells. For example, a blue photoprotein, aequorin, and a red-emitting luciferase, PhRE, were used for simultaneous monitoring of oxygen and ATP levels in cultured cells.[29] Another pair of luciferases, Eluc and PhRE, was used as intracellular reporters of peroxisomes and nuclei in living cultured cells.[30] The Eluc mutant in the present study showed bioluminescence with an 88-nm longer emission maximum than that of wild-type Eluc. This large peak separation provides advantages for dual-color live cell imaging using the wild type and the mutated Eluc. On the other hand, emission with a long wavelength over 600 nm is suitable for bioluminescence imaging on living bodies or tissues because of the low optical absorbance. The maximum wavelength of the Eluc mutant was 626 nm, which is comparable to other red luciferases used in luminescence imaging on living subjects.[10,11] Thus, the present Eluc mutant, generated only by a four amino acid substitution to the wild type, will be applicable for dual-color live cell imaging and for whole body or tissue imaging.

In conclusion, we developed a red-shift mutant of Eluc with a peak wavelength at 626 nm merely by introducing four mutations. The color shift of 88 nm from the wild-type Eluc, which is the largest red-shift ever reported for mutants derived from a single luciferase, is sufficient to separate the bioluminescence spectra with optical filters. This property implies that the pair of wild-type Eluc and the mutant is suitable for dual-color imaging on living samples such as cultured cells and animals. The peak wavelength of luminescence spectrum suggests that the present Eluc mutant is also appropriate for whole body imaging with samples of living animal bodies or tissues. Thus, the present Eluc mutant will become a candidate of luciferases with a long wavelength for *in vivo* and *in vitro* visualization of multiple targets.

Acknowledgments

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Biographies for the authors are not available.