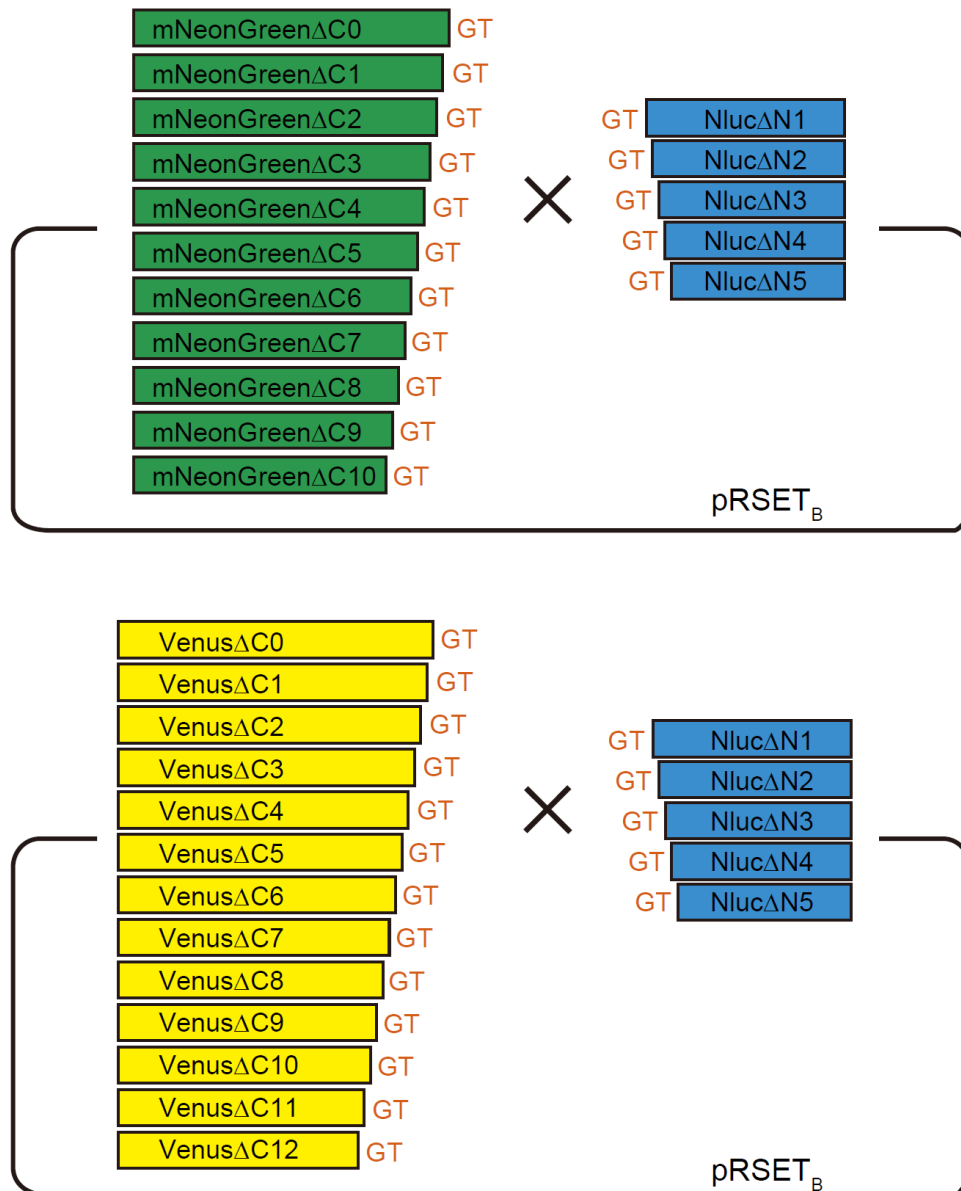
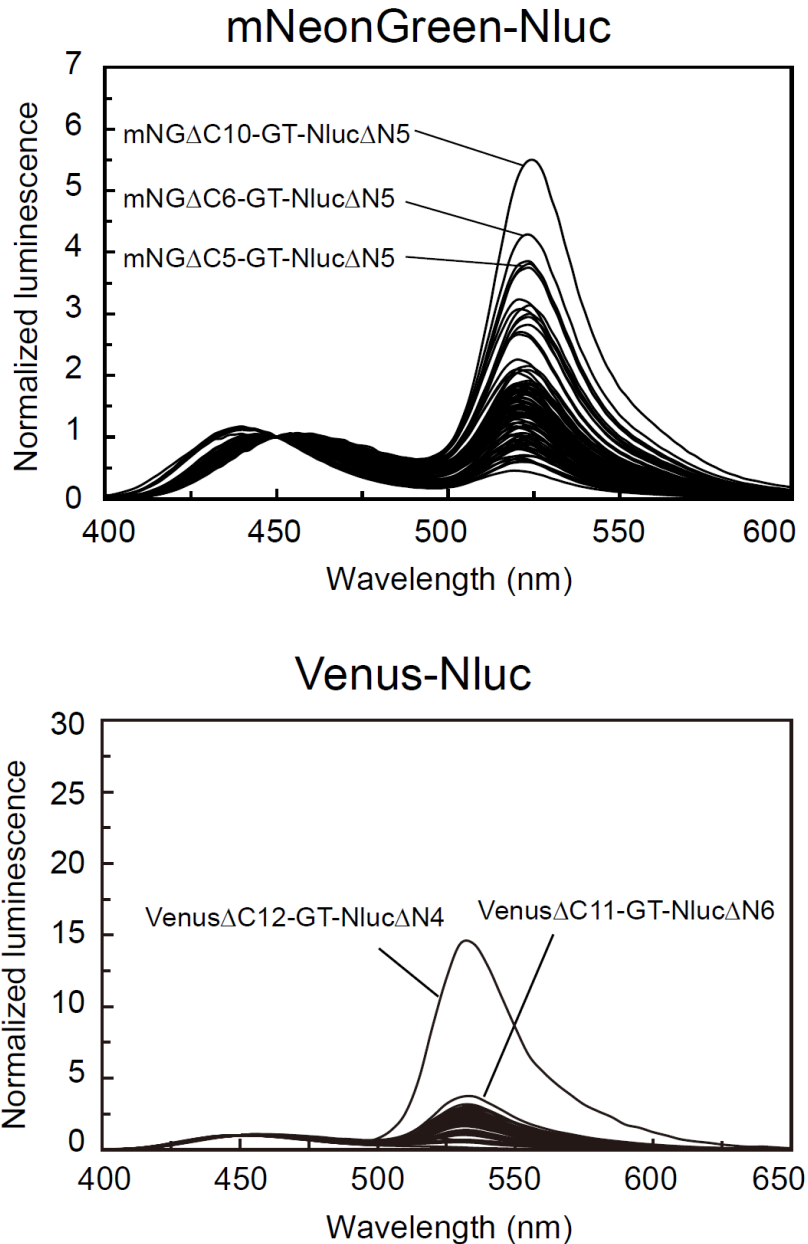


1 **Supplementary Figure 1. Design of linker truncation library between**  
 2 **Nluc and either Venus or mNeonGreen**  
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 5 The cDNA of C-terminally deleted FPs (mNeonGreen or Venus) mutants  
 6 and N-terminally deleted Nluc mutants with two residues GT (highlighted  
 7 in orange color), derived from the recognition sequence of *KpnI* (ggtacc),  
 8 were mixed together, and subcloned in-frame into the *BamHI/EcoRI* sites  
 9 of pRSET<sub>B</sub>. We generated and screened 55 and 65 linker combinations of  
 10 mNeonGreen-Nluc and Venus-Nluc pairs, respectively.

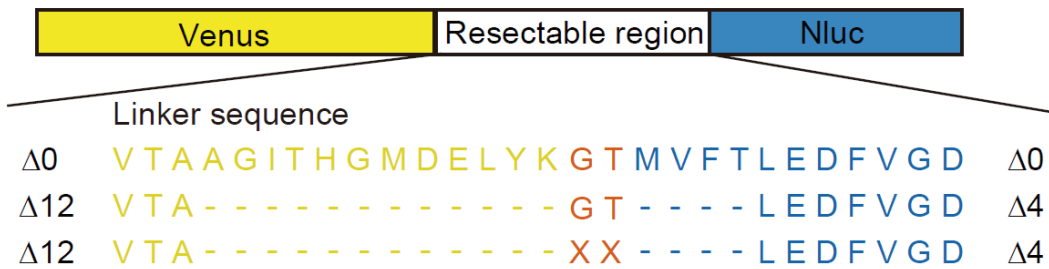
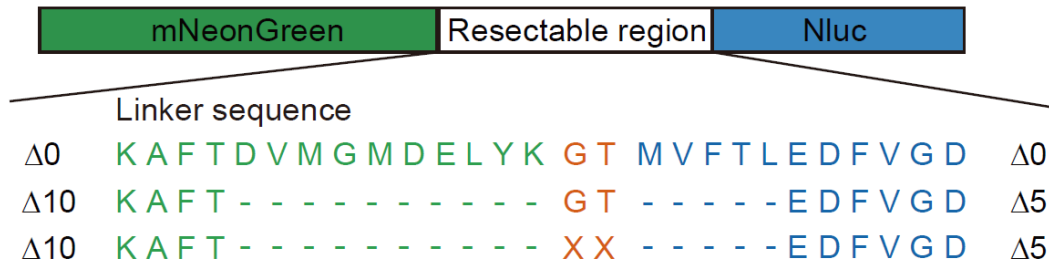
11 **Supplementary Figure 2. Emission spectra of linker truncation**  
12 **variants of mNeonGreen-Nluc and Venus-Nluc**



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14 Luminescence spectra of *E. coli* suspension colonies that showed bright  
15 luminescence signal on an agar plate at first screening. The spectra were  
16 normalized to the peak of Nluc emission intensity. Spectra were measured  
17 by micro-plate reader (SH-9000; Corona Electric). Among them,  
18 mNeonGreen $\Delta$ C10-GT-Nluc $\Delta$ N5 and Venus $\Delta$ C12-GT-Nluc $\Delta$ N4 exhibited  
19 high FRET efficiency.

20 **Supplementary Figure 3. Design of randomized linker library in**  
 21 **mNeonGreen-Nluc and Venus-Nluc fusion protein**

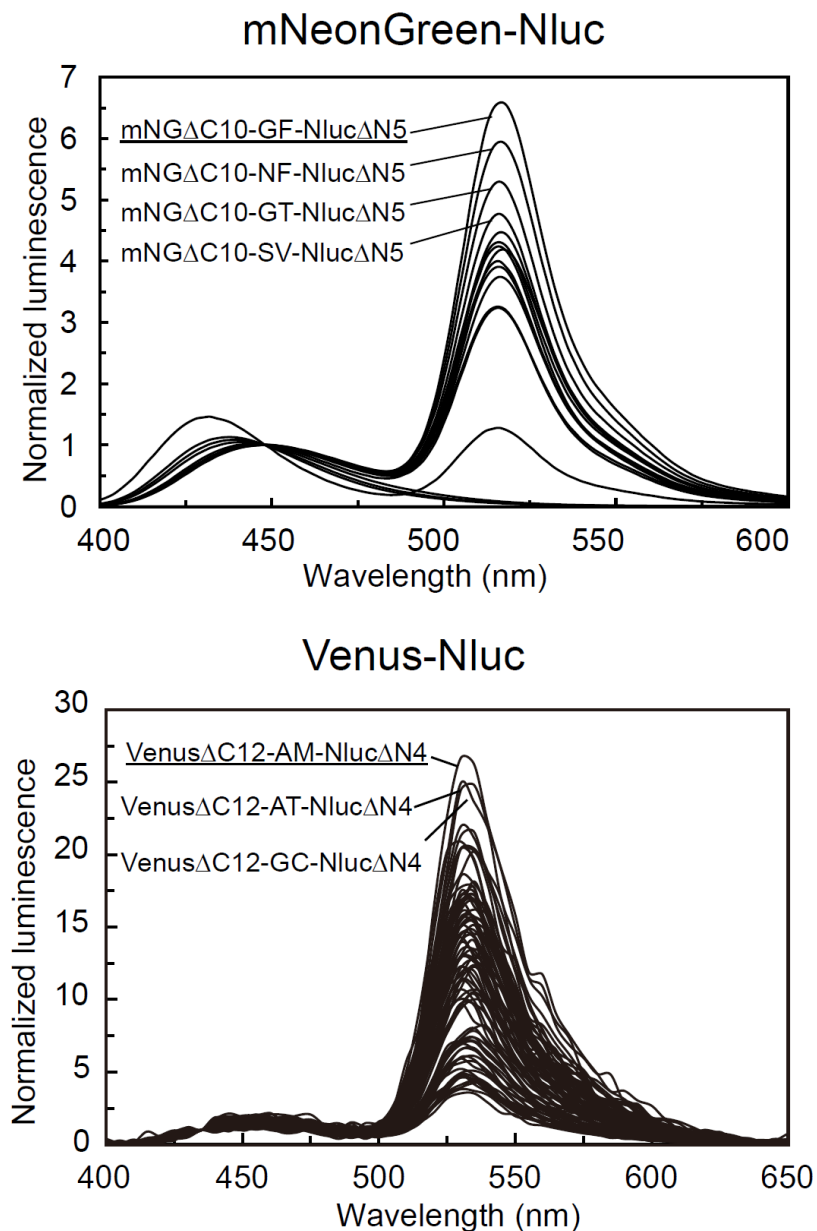
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Using mNeonGreen $\Delta$ C10-GT-Nluc $\Delta$ N5 and Venus $\Delta$ C12-GT-Nluc $\Delta$ N4 as templates, we performed site-directed random mutagenesis at the linker region, -Gly-Thr-.

36 **Supplementary Figure 4. Emission spectra of randomized linker**  
37 **variants in mNeonGreen-Nluc and Venus-Nluc fusion protein**

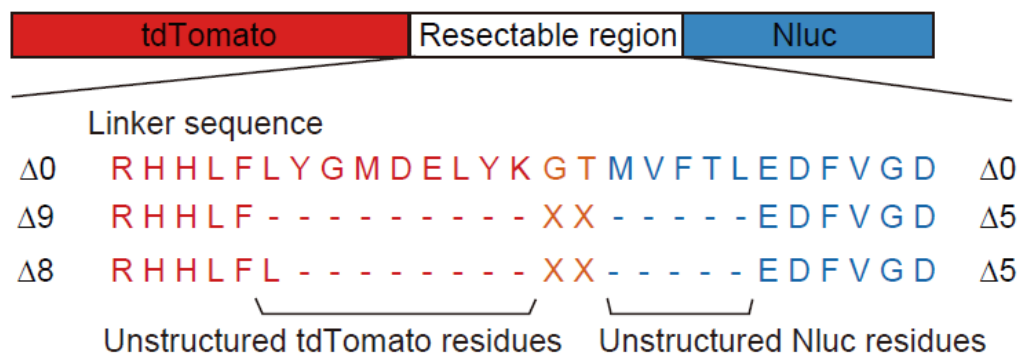
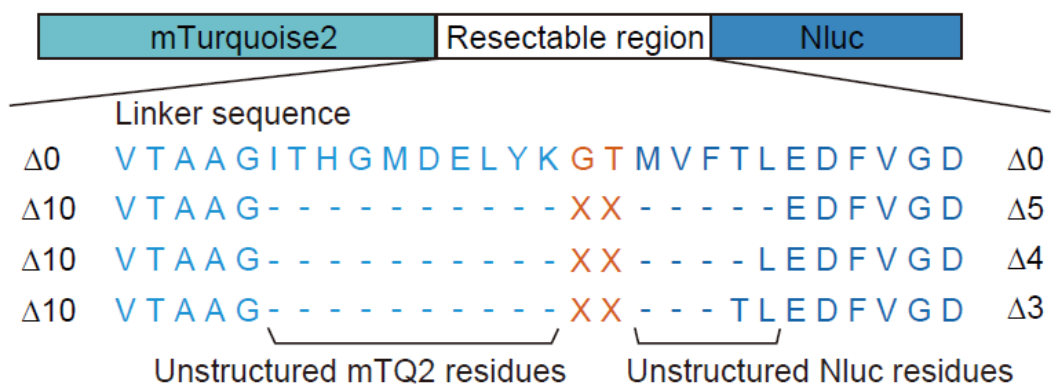


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39 Luminescence spectra of *E. coli* suspension of colonies that showed bright  
40 luminescence signal on an agar plate at first screening. The spectra were  
41 normalized to the peak of Nluc emission intensity. Spectra were measured  
42 by micro-plate reader (SH-9000; Corona Electric).  
43 mNG $\Delta$ C10-GF-Nluc $\Delta$ N5 (GeNL) and Venus $\Delta$ C12-AM-Nluc $\Delta$ N4 (YeNL)  
44 exhibited the highest FRET efficiency.

45 **Supplementary Figure 5. Design of linker library in mTurquoise2-Nluc**  
 46 **and tdTomato-Nluc fusion protein**

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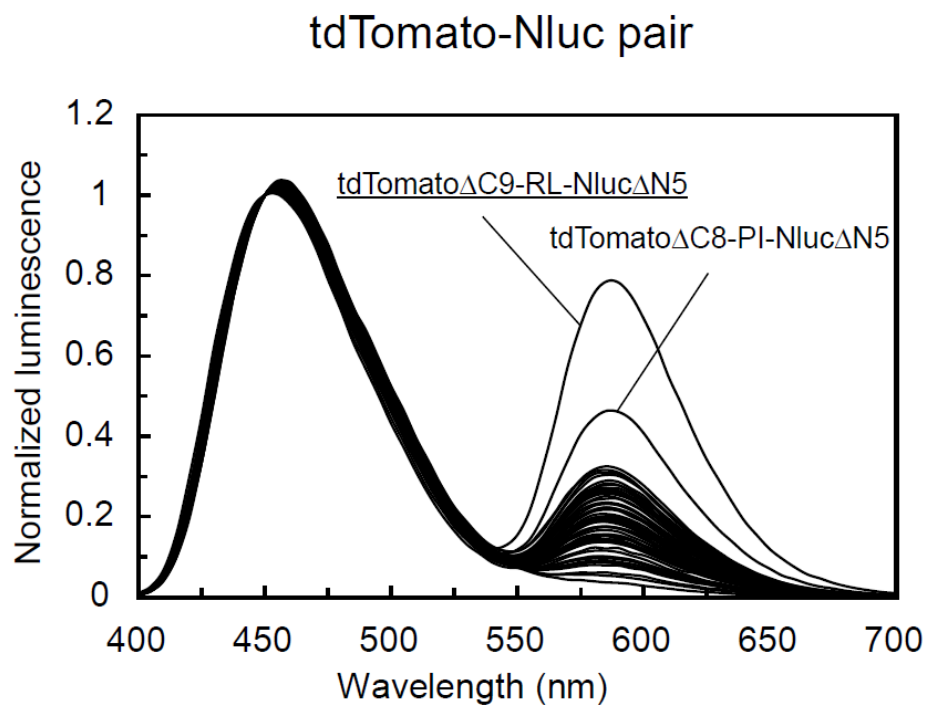


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In the mTurquoise2-Nluc and tdTomato-Nluc pair, we created a series of linker libraries by systematically truncating resectable regions and randomizing two residues at the junction simultaneously. Those libraries with different linker lengths were mixed together and screened.

58 **Supplementary Figure 6. Emission spectra of linker library in**  
59 **tdTomato-Nluc fusion protein**

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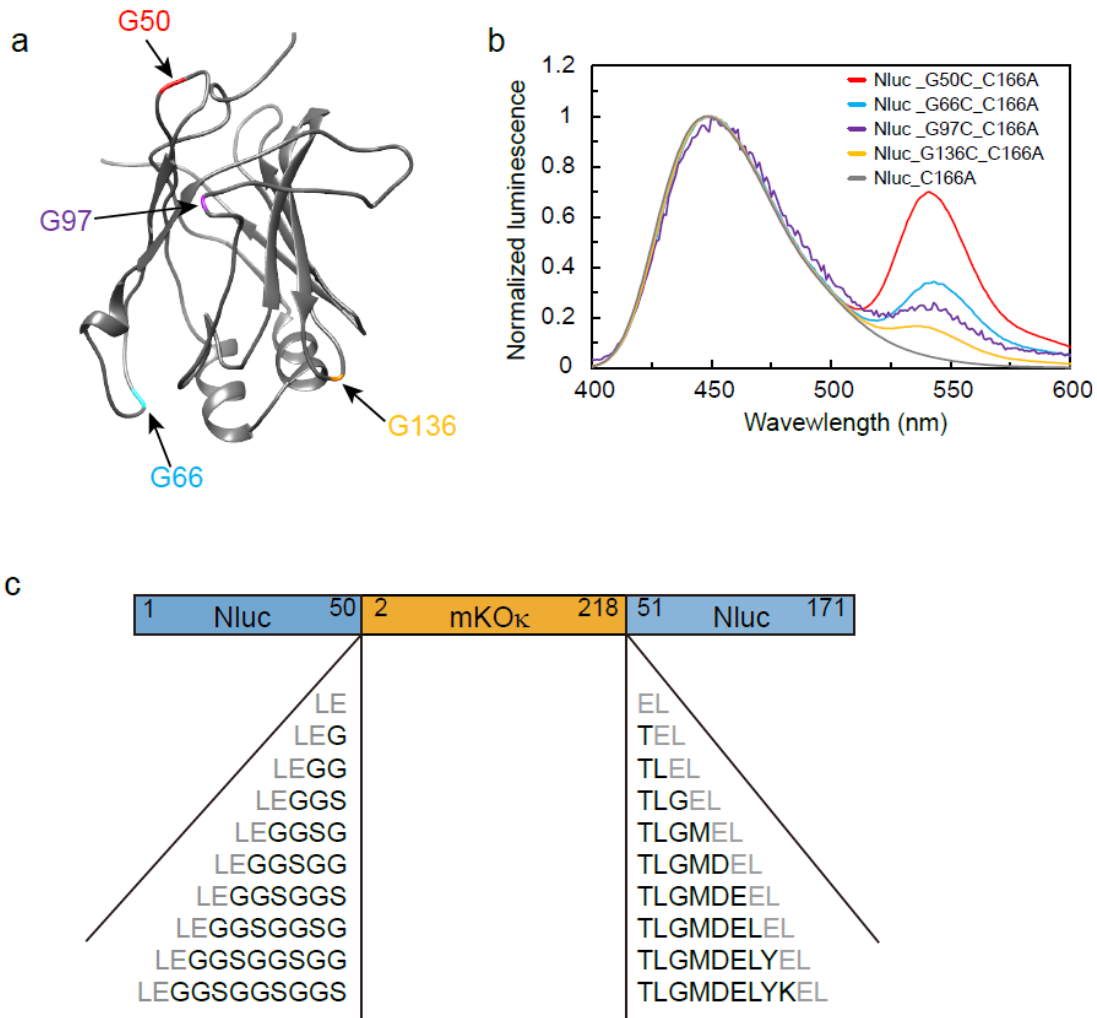


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Luminescence spectra of *E. coli* suspension of colonies that showed bright luminescence signal on an agar plate at first screening. The spectra were normalized to the peak of Nluc emission intensity. Spectra were measured by micro-plate reader (SH-9000; Corona Electric). Among them, tdTomato $\Delta$ C9-RL-Nluc $\Delta$ N5 (ReNL) exhibited the highest FRET efficiency.

75 **Supplementary Figure 7. Design of Orange eNano-lantern**

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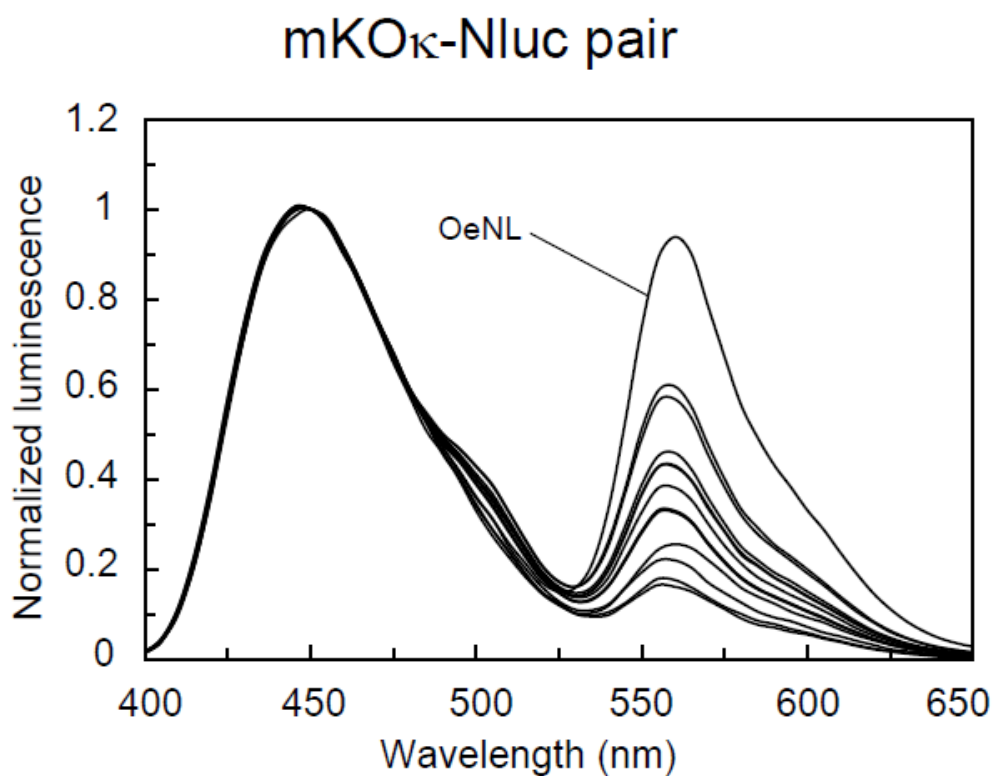
79 (a) 3D structure of Nluc predicted by I-TASSER with the positions of  
 80 cysteine insertion (50, 66, 97 and 136th). (b) Luminescence spectra of Nluc  
 81 conjugated with eosin-maleimide at designated cysteine. Each cysteine  
 82 substitution was introduced to mutated Nluc whose intrinsic cysteine  
 83 residue was substituted with alanine (C166A). (c) The flexible linker was  
 84 introduced to flank mKOκ. Typical Glycine-rich linkers and mimics of  
 85 GFP C-terminal 10 amino acids were employed as flexible linkers at the  
 86 N-terminus and C-terminus of mKOκ, respectively. All possible  
 87 combinations were screened.





89 **Supplementary Figure 8. Emission spectra of FRET proteins based on**  
90 **the design of OeNL**

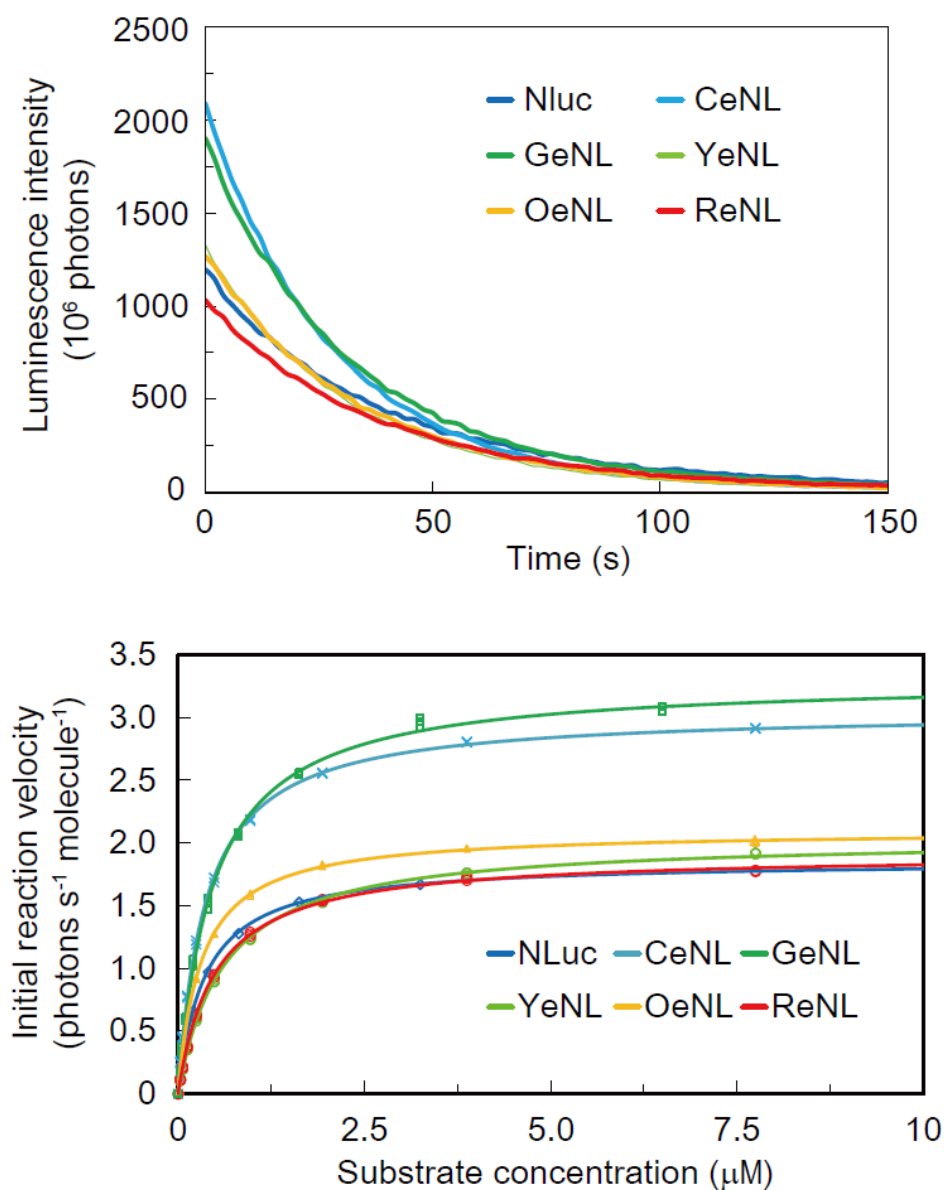
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Luminescence spectra of *E. coli* colony suspensions showing the brightest luminescence signal on initial agar plate screening. The spectra measured by micro-plate reader (SH-9000; Corona Electric) and were normalized to the peak of Nluc emission intensity.

104 **Supplementary Figure 9. Characterization of luminescent proteins**

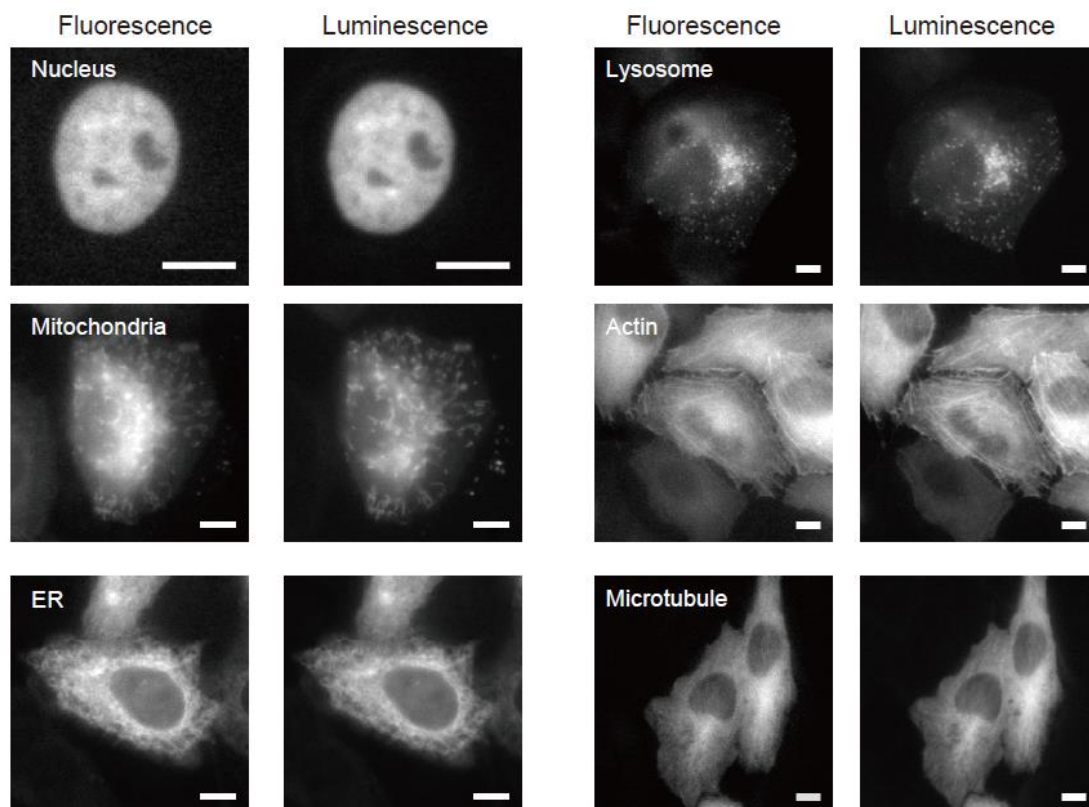


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107 (a) Quantum yield was estimated from the integrated light output for 200 s  
 108 until reaction of 500 pM of furimazine with 1 nM luminescent protein  
 109 approached completion. Intensities were measured in triplicate, and data  
 110 are presented as mean. (b) Kinetics parameters were estimated from the  
 111 plot of the initial light output (first 12 second integration) versus the  
 112 concentration of the furimazine. All intensities were measured in triplicate,  
 113 and the average data were fitted to Michaelis-Menten equation. The results  
 114 are summarized in Table S3.

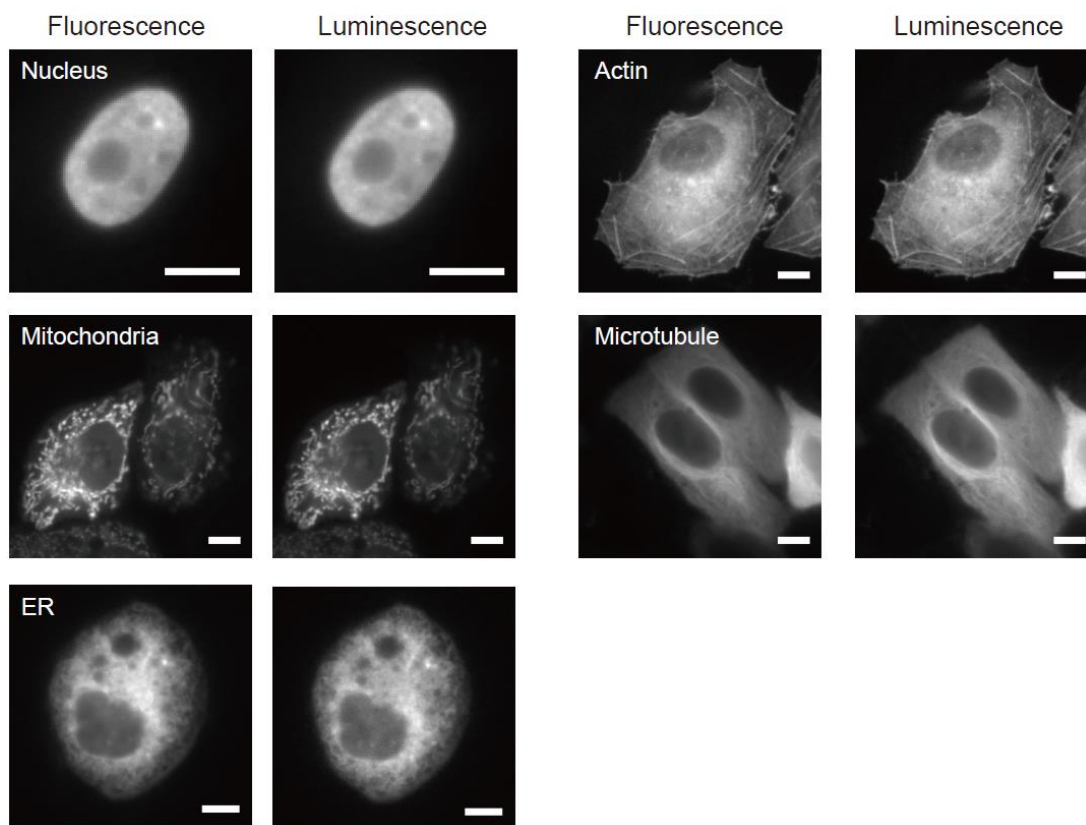
115 **Supplementary Figure 10. Single-cell imaging of HeLa cells expressing**  
116 **CeNL localized to various cellular compartments**  
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Fluorescence images (left) of mTurquoise2 moiety in CeNL and luminescence images (right) of CeNL localized to the nucleus, mitochondria, ER, lysosome, actin, and microtubule (exposure times for luminescence image acquisition times were 2 s, 20 s, 5 s, 10 s, 20 s, and 5 s, respectively). Scale bars, 10  $\mu$ m.

128 **Supplementary Figure 11. Single-cell imaging of HeLa cells expressing**  
129 **YeNL localized to various cellular compartments**  
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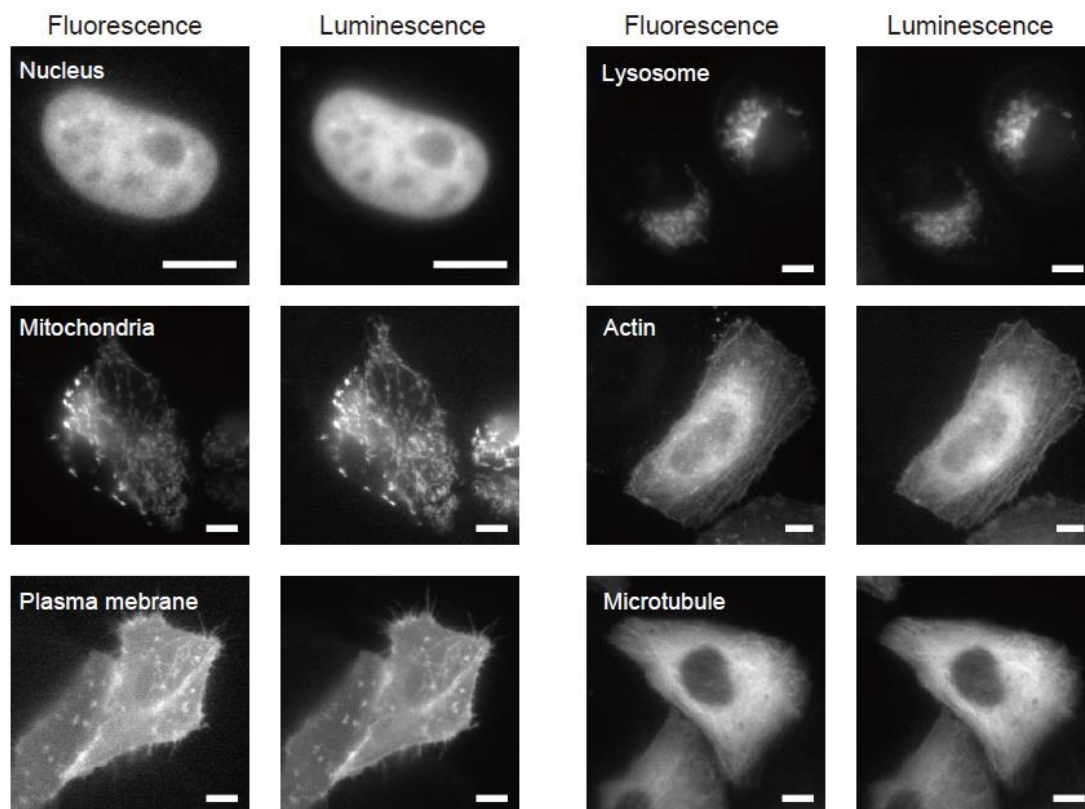


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136 Fluorescence images (left) of the Venus moiety in YeNL and luminescence  
137 images (right) of YeNL localized to the nucleus, mitochondria, ER, actin,  
138 and microtubule (exposure times for luminescence images were 5 s, 5 s, 5 s,  
139 5 s, and 10 s, respectively). Scale bars, 10  $\mu$ m.  
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142 **Supplementary Figure 12. Single-cell imaging of HeLa cells expressing**  
143 **OeNL localized to various cellular compartments**

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150 Fluorescence images (left) of mKOκ moiety in OeNL and luminescence  
151 images (right) of OeNL localized to the nucleus, mitochondria, plasma  
152 membrane, lysosome, actin, and microtubule (exposure time for  
153 luminescence images were 3 s, 10 s, 10 s, 5 s, 10 s, and 5 s, respectively).  
154 Scale bars, 10 μm.

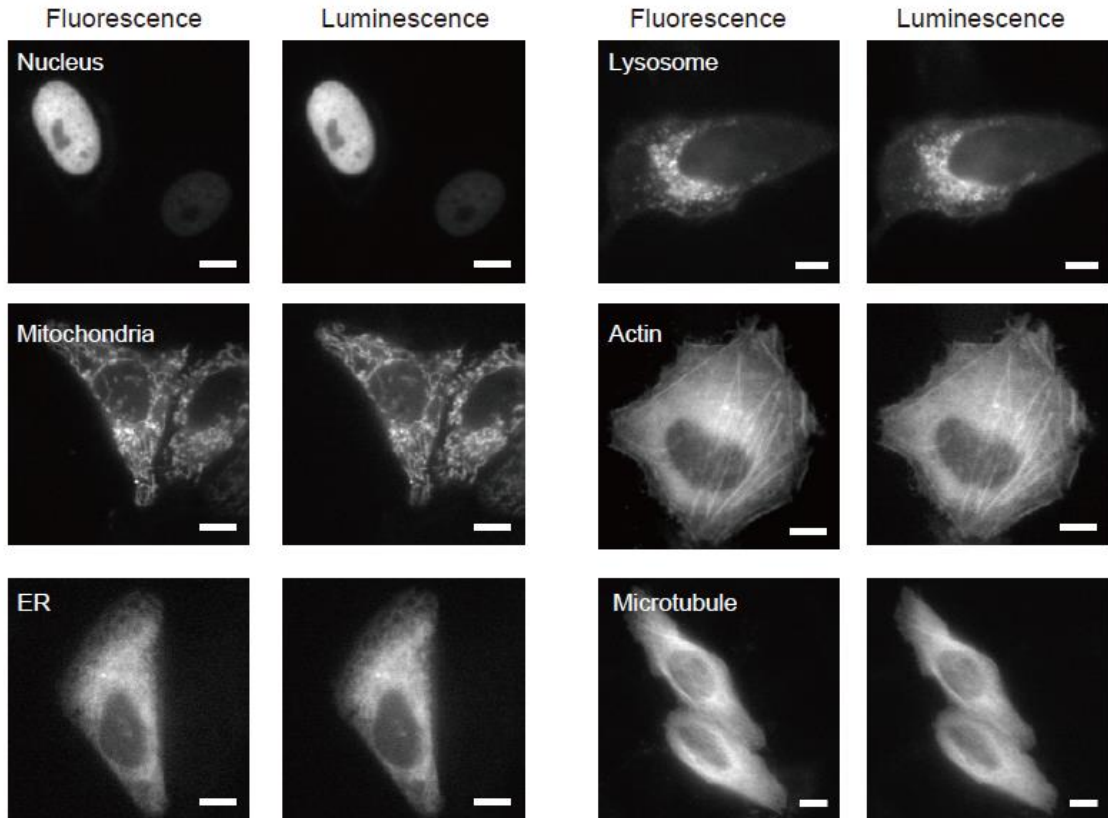
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157 **Supplementary Figure 13. Single-cell imaging of HeLa cells expressing**  
158 **ReNL localized to various cellular compartments**

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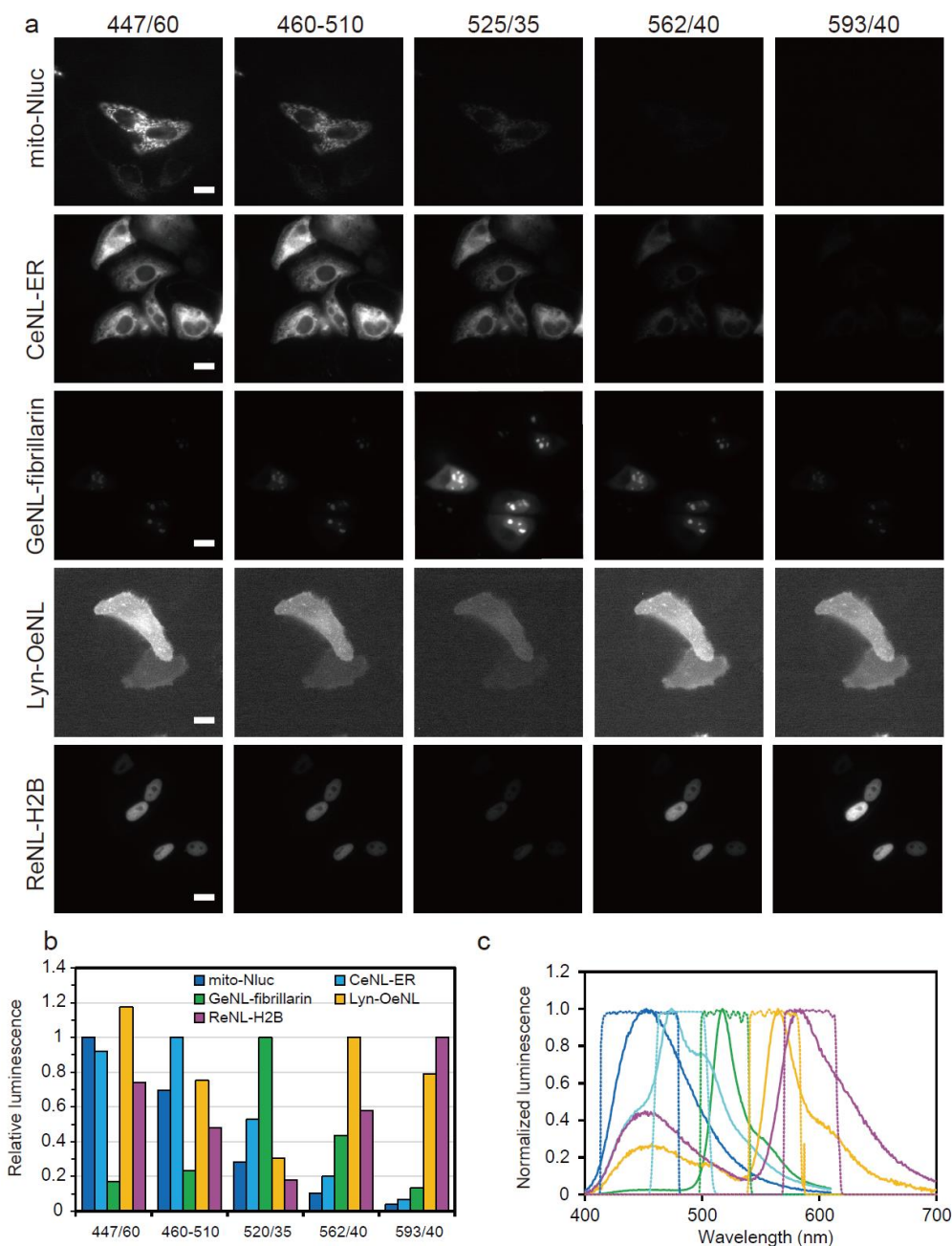
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165 Fluorescence images (left) of the tdTomato moiety in ReNL and  
166 luminescence images (right) of ReNL localized to the nucleus,  
167 mitochondria, ER, lysosome, actin, and microtubule (exposure time for  
168 luminescence images were 3 s, 10 s, 10 s, 5 s, 10 s, and 5 s, respectively).  
169 Scale bars, 10  $\mu\text{m}$ .

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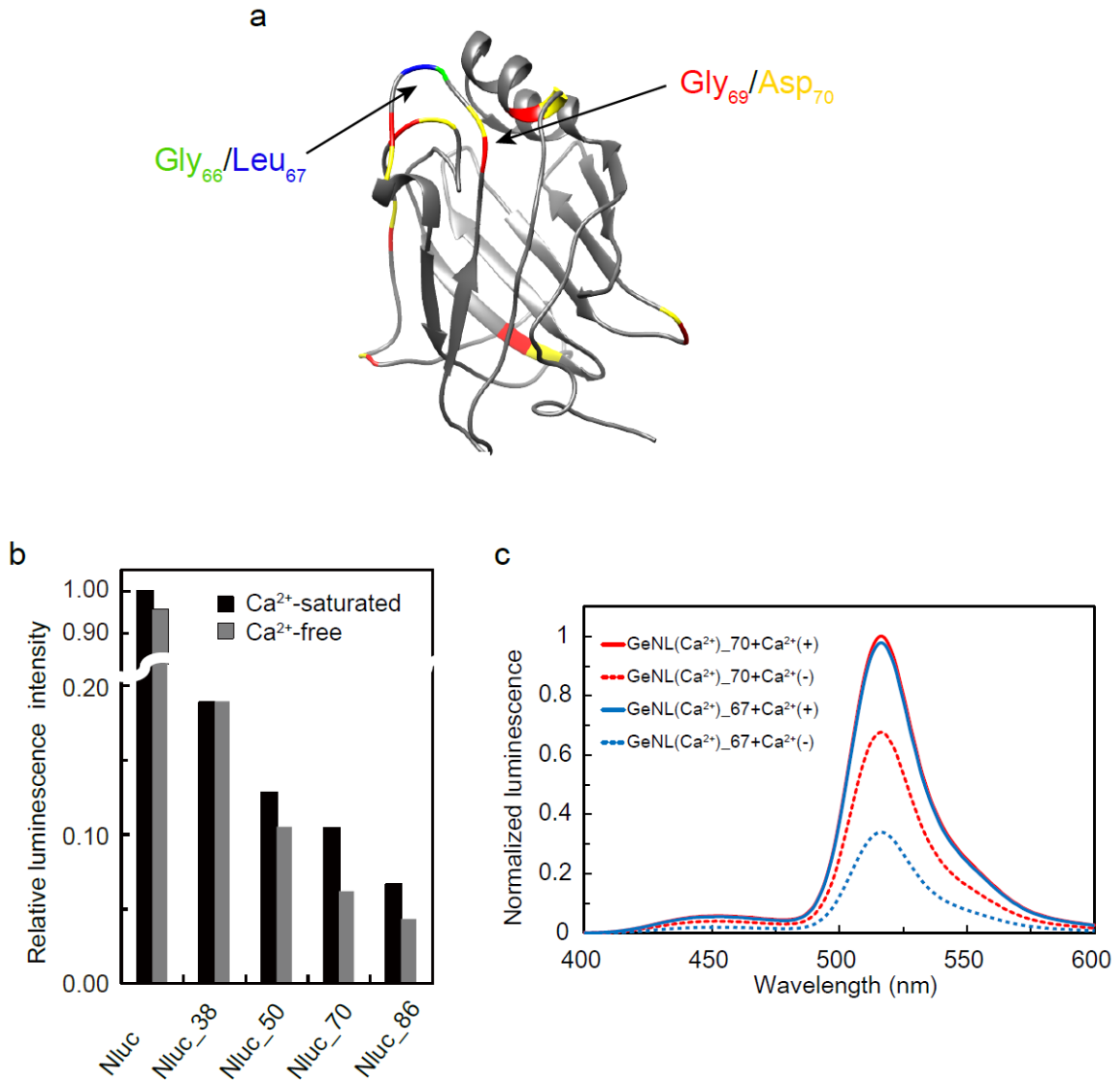
172 **Supplementary Figure 14. Multi-color luminescence imaging with**  
 173 **linear unmixing**



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 175 (a) HeLa cells expressing either mito-Nluc, CeNL-ER, GeNL-fibrillarin  
 176 Lyn-OeNL or ReNL-H2B were imaged with five filters. Scale bars, 20  $\mu$ m.  
 177 (b) Linear spectral unmixing coefficient was determined from the control  
 178 experiments as in **a**. (c) The emission spectra of Nluc, CeNL, GeNL, OeNL,  
 179 and ReNL. Dashed line represents the emission filters used to acquire each  
 180 luminescent protein signal.

181 **Supplementary Figure 15. Identification of optimal insertion site of**  
 182 **CaM-M13 into Nluc**

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185 (a) 3D structure of Nluc predicted by I-TASSER with the candidate

186 CaM-M13 insertion sites (37/38, 63/64, 69/70, 97/98, 103/104, 107/108,

187 121/122 and 148/149th with red and yellow color) and final optimal

188 insertion site at 66/67th of GeNL(Ca<sup>2+</sup>) with green and blue color. (b)

189 Relative brightness of Nluc\_CaM-M13 protein extracted from periplasmic

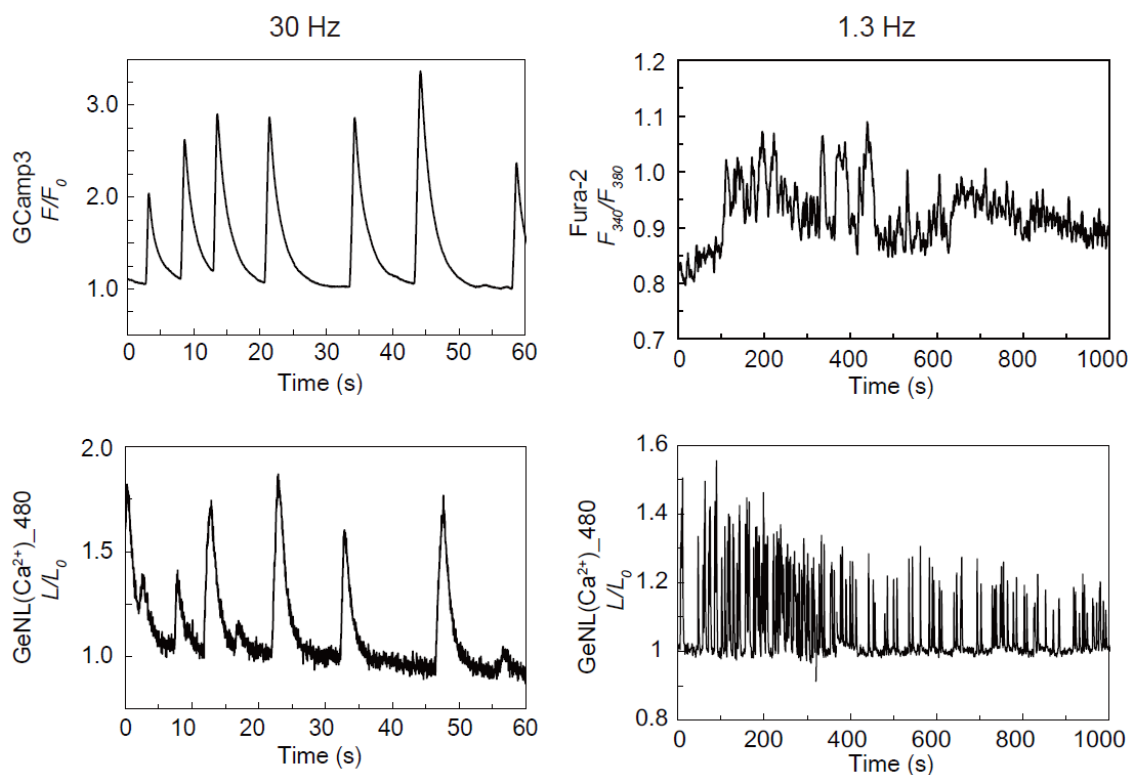
190 region with or without Ca<sup>2+</sup>. (c) Normalized luminescence spectra of

191 GeNL(Ca<sup>2+</sup>)\_67 and GeNL(Ca<sup>2+</sup>)\_70 with or without Ca<sup>2+</sup>.

192



193 **Supplementary Figure 16. Direct comparison of GeNL(Ca<sup>2+</sup>) and**  
194 **either GCaMP3 or Fura-2**  
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198 Spontaneous Ca<sup>2+</sup> spiking in GH3 cells were monitored with the indicators  
199 (vertical axis) at designated frame rate (Upper). Illumination power was  
200 130 mW cm<sup>-2</sup> for GCaMP3, 100 mW cm<sup>-2</sup> (384 nm) and 34 mW cm<sup>-2</sup> (340  
201 nm) for dual excitation ratio imaging of Fura-2. The background drift was  
202 manually subtracted using Origin7 software (OriginLab)

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**Supplementary Table 1. Enzymatic characteristics of luminescent proteins**

	<i>LQY</i> (%)	$K_m$ ( $\mu\text{M}$ )	$V_{\text{max}}$ (photon $\text{s}^{-1}$ molecule $^{-1}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )
Nluc	28 $\pm$ 3.0	0.36 $\pm$ 0.0038	1.9 $\pm$ 0.0061	6.6 $\pm$ 0.73
CeNL	42 $\pm$ 1.1	0.37 $\pm$ 0.0042	3.0 $\pm$ 0.010	7.1 $\pm$ 0.19
GeNL	45 $\pm$ 1.8	0.47 $\pm$ 0.010	3.3 $\pm$ 0.021	7.3 $\pm$ 0.30
YeNL	33 $\pm$ 1.1	0.61 $\pm$ 0.0088	2.0 $\pm$ 0.088	6.1 $\pm$ 0.34
OeNL	30 $\pm$ 0.48	0.31 $\pm$ 0.0025	2.1 $\pm$ 0.043	7.0 $\pm$ 0.18
ReNL	26 $\pm$ 1.0	0.49 $\pm$ 0.0064	1.9 $\pm$ 0.069	7.3 $\pm$ 0.20
Rluc8 <sup>a</sup>	5.8 $\pm$ 0.63	2.0 $\pm$ 0.057	0.22 $\pm$ 0.0018	3.8 $\pm$ 0.12

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207 LQY, luminescent quantum yield. Data are presented as mean  $\pm$  s.d., n = 3.208 <sup>a</sup> measured with 0.1% BSA

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**Supplementary Table 2. Affinity for Ca<sup>2+</sup> of GeNL(Ca<sup>2+</sup>) variants**

	Linker of CaM and M13	Dynamic range / %	Relative luminescence	K <sub>d</sub> / nM	Hill coefficient
GeNL(Ca <sup>2+</sup> )_520	104Q-2G	280	1	520	1.5
GeNL(Ca <sup>2+</sup> )_480	104Q-2GS	490	0.87	480	1.2
GeNL(Ca <sup>2+</sup> )_250	104Q-3GS	190	0.79	260	1.2
GeNL(Ca <sup>2+</sup> )_60	104E-4GS	270	0.76	56	1.1

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Supplementary Table 3. Oligonucleotides used in this study

Name of primer	Oligonucleotide sequence (5' to 3')
F-BH1-G-gfp_1	TTGGATCCGATGGTGAAGCAAGGGCGAGGAG
R-ER1-Nluc_171	ATGAATTCGCCAGAATGCGTTCGCACAG
F-Kpn1-Nluc_2	GCCGGTACCGTCTTCACACTCGAAGATTTTCG
F-Kpn1-Nluc_3	GCCGGTACCTTCACACTCGAAGATTTTCGTTG
F-Kpn1-Nluc_4	GCCGGTACCACACTCGAAGATTTTCGTTGGG
F-Kpn1-Nluc_5	GCCGGTACCCTCGAAGATTTTCGTTGGGGAC
F-Kpn1-Nluc_6	GCCGGTACCGAAGATTTTCGTTGGGGACTGGC
R-Kpn1-mNG_235	GCCGGTACCGTACAGCTCGTCCATGCCATC
R-Kpn1-mNG_234	GCCGGTACCAGCTCGTCCATGCCATCAC
R-Kpn1-mNG_233	GCCGGTACCCTCGTCCATGCCATCACATCG
R-Kpn1-mNG_232	GCCGGTACCGTCCATGCCATCACATCGG
R-Kpn1-mNG_231	GCCGGTACCCATGCCATCACATCGGTAAAG
R-Kpn1-mNG_230	GCCGGTACCGCCATCACATCGGTAAAGGCC
R-Kpn1-mNG_229	GCCGGTACCCATCACATCGGTAAAGGCCTTTTGC
R-Kpn1-mNG_228	GCCGGTACCACATCGGTAAAGGCCTTTTGC
R-Kpn1-mNG_227	GCCGGTACCATCGGTAAAGGCCTTTTGCAC
R-Kpn1-mNG_226	GCCGGTACCGGTAAAGGCCTTTTGCCTCC
F-XX-Nluc_6	NNKNNKGAAGATTTTCGTTGGGACTGG
R-mNG_226	GGTAAAGGCCTTTTGCCTCC
R-Kpn1-gfp_229	ATGGTACCCCCGGCGCGGTACGAAC
F-XX-Nluc_5	NNKNNKGAAGATTTTCGTTGGGACTG
F-XX-Nluc_4	NNKNNKCTCGAAGATTTTCGTTGGGG
F-XX-Nluc_3	NNKNNKACACTCGAAGATTTTCGTTG
R-gfp_229	CCCGGCGGCGGTACGAAC
F-XhoI-mKO_2	ATTCTCGAGGTGAGCGTGATCAA
F-XhoI-G-mKO_2	ATTCTCGAGGGCGGTGAGCGTGATCAAGC
F-XhoI-GG-mKO_2	ATTCTCGAGGGCGGTGAGCGTGATCAAGC
F-XhoI-GGS-mKO_2	ATTCTCGAGGGCGGTAGCGTGAGCGTGATCAAGC
F-XhoI-GGSG-mKO_2	ATTCTCGAGGGCGGTAGCGGTGGCGTGAGCGTGATCAAGCCCCGA
F-XhoI-GGSGGS-mKO-2	ATTCTCGAGGGCGGTAGCGGTGGCAGCGTGAGCGTGATCAAGCCCCGA
F-XhoI-GGSGGSG-mKO-2	ATTCTCGAGGGCGGTAGCGGTGGCAGCGGAGTGAGCGTGATCAAGCCCCGA
F-XhoI-GGSGGSGG-mKO-2	ATTCTCGAGGGCGGTAGCGGTGGCAGCGGAGGTGAGCGTGATCAAGCCCCGA
F-XhoI-GGSGGSGGS-mKO-2	ATTCTCGAGGGCGGTAGCGGTGGCAGCGGAGGTAGCGTGAGCGTGATCAAGCCCCGA
R-Sac1-mKO_218	ATTGAGCTCGGAGTGGGCCACGGCG
R-Sac1-T-mKO_218	ATTGAGCTCAGTGGAGTGGGCCACGGCG
R-Sac1-TL-mKO_218	ATTGAGCTCGAGAGTGGAGTGGGCCACGGCG
R-Sac1-TLG-mKO_218	ATTGAGCTCGCCGAGAGTGGAGTGGGCCACGGCG
R-Sac1-TLGM-mKO_218	ATTGAGCTCCATGCCGAGAGTGGAGTGGGCCACGGCG
R-Sac1-TLGMDE-mKO_218	ATTGAGCTCCTCGTCCATGCCGAGAGTGGAGTGGGCCACGGCG
R-Sac1-TLGMDEL-mKO_218	ATTGAGCTCCAGCTCGTCCATGCCGAGAGTGGAGTGGGCCACGGCG
R-Sac1-TLGMDELY-mKO_218	ATTGAGCTCGTACAGCTCGTCCATGCCGAGAGTGGAGTGGGCCACGGCG
R-Sac1-TLGMDELYK-mKO_218	ATTGAGCTCCTGTACAGCTCGTCCATGCCGAGAGTGGAGTGGGCCACGGCG
R-Kpn1-tdTA_467	GCCGGTACCCAGGAACAGGTGGTGGCGGCC
F-Nluc	GGGACTGGCGACAGACAGCCG
R-Nluc_6-XX-tdTA_467	CAACGAAATCTTCMNNMNNCAGGAACAGGTGGTG
R-Nluc_6-XX-tdTA_466	CAACGAAATCTTCMNNMNNGAACAGGTGGTG
R-Nluc_6-XX-tdTA_465	CAACGAAATCTTCMNNMNNCAGGTGGTGGCG
F-Sac1-Nluc_38	GCCGAGCTCGTGTCCGTAACCTCCGATCCAAAG
R-Nco1-Nluc_37	GAACCATGGCCCCGAGATTCTGAAACAACTG
F-Sac1-Nluc_64	GCCGAGCTCTATGAAGTCTGAGCGGCGAC
R-Nco1-Nluc_63	GAACCATGGCGGGATGATGACATGGATGTC
F-Sac1-Nluc_67	GCCGAGCTCCTGAGCGGCGACCAATGGGC
R-Nco1-Nluc_66	GAACCATGGACCTTCATACGGGATGATGAC

F-Sac1-Nluc_70	GCCGAGCTCGACCAAATGGGCCAGATC
R-Nco1-Nluc_69	GAACCATGGGCGCTCAGACCTTCATACGG
F-Sac1-Nluc_98	GCCGAGCTCACACTGGTAATCGACGGGG
R-Nco1-Nluc_97	GAACCATGGGCCATAGTGCAGGATCACC
F-Sac1-Nluc_104	GCCGAGCTCGTTACGCCGAACATGATC
R-Nco1-Nluc_103	GAACCATGGCCCCGTCGATTACCAGTGTG
F-Sac1-Nluc_108	GCCGAGCTCATGATCGACTATTTTCGGACGG
R-Nco1-Nluc_107	GAACCATGGGTTCGGCGTAACCCCGTC
F-Sac1-Nluc_122	GCCGAGCTCTTCGACGGCAAAAAGATCACTG
R-Nco1-Nluc_121	GAACCATGGCACGGCGATGCCTTCATAC
F-Sac1-Nluc_149	GCCGAGCTCGGCTCCCTGCTGTTCCGAG
R-Nco1-Nluc_148	GAACCATGGGTTCGGGGTTGATCAGGCGCTC
F-BH1-koz-hmNG	ATGGATCCCGCCACCATGGTGTCCAAGGGCGAAGAG
F-BH1-G-hmNG	ATGGATCCGATGGTGTCCAAGGGCGAAGAG
F-BH1-hmNG	ATGGATCCATGGTGTCCAAGGGCGAAGAG
F-Kpn1-hmNG_2	ATGGTACCGTGTCCAAGGGCGAAGAG
F-Hind3-koz-hmNG_1	ATAAGCTTCGCCACCATGGTGTCCAAGGGCGAAGAG
F-Sal1-link-hmNG_1	ATGTCGACGGTACCGCGGGCCGGGATCCAATGGTGTCCAAGGGCGAAGAG
F-Nhe1-koz-hmNG_1	ATGCTAGCCGCCACCATGGTGTCCAAGGGCGAAGAGG
R-Xho1-x-Nluc_171	ATCTCGAGTTACGCCAGAATGCGTTTCGCACAG
R-ER1-x-Nluc_171	ATGAATTCTTACGCCAGAATGCGTTTCGCACAG
R-Nluc_171-ER1	ATGAATTCGCCAGAATGCGTTTCGCACAG
R-Kpn1-Nluc_171	TATGGTACCCGCCAGAATGCGTTTCGCACAG
R-Kpn1-GSG-Nluc_171	ATGGTACCGCCTGATCCACCCGCCAGAATGCGTTTCGCACAG
R-Not1-x-Nluc_171	ATGCGGCCGCTTACGCCAGAATGCGTTTCGC
R-Bgl2-Nluc_171	ATTAGATCTCGCCAGAATGCGTTTCGCACAG
F-Sal1-GS10 linker	TCGACCGGATCTGGCGGCGGAGGAAGCGGAGGG
R-Sal1-GS10 linker	TCGACCTCCGCTTCTCCGCCGCCAGATCCGG
F- BH1-koz-gfp_1	ATGGATCCCCACCATGGTGTGAGCAAGGGCGAGGAG
F-Hind3-koz-gfp_1	ATAAGCTTCGCCACCATGGTGTGAGCAAGGGCGAGGAG
F- BH1-gfp_1	TTGGATCCATGGTGTGAGCAAGGGCGAGGAG
F-GS-VCL_2	GGAGGCGGAGGATCAGGCGGATCTGGGCCGCTTCCACACGCGCAC
F-Kpn1-GS	ATGGTACCGGCGGCGGAGGAAGCGGAGGCGGAGGATCAGGCGGATC
R-ER1-x-VCL_1066	ATGAATTCTTACTGATACCATGGGGTCTTTC
F-Hind3-koz-LAMP_1	ATAAGCTTCGCCACCATGGCGGCCCGGGCGCCCGG
R-Kpn1-LAMP_407	ATGGTACCGATGGTCTGATAGCCCGGTGAC
F-BH1-Nluc_1	A GGATCC G ATGGTCTTCACACTCGAAGATTTTCGTTGGGGACTGG
F-BH1-NLuc_1	ATGGATCCATGGTCTTCACACTCGAAG
F-Hind3-koz-Nluc_1	ATAAGCTTCGCCACCATGGTCTTCACACTCGAAG
F-ER1-Nluc_1	AGAATTCATGGTCTTCACACTCGAAGATTTTCGTTGGGGACTGG
Nluc_C166A	GGCTGGCGGCTGGCCGAACGCATTCTG
F-Nluc_G50C	TGCCAAAATGGGCTGAAGATC
R-Nluc_49	GCTCAGGACAATCCTTTG
F-Nluc_G66C	TGCCTGAGCGGCGACCAAATG
R-Nluc_65	TTC ATACGGGATGATGAC
F-Nluc_G97C	TGCACACTGGTAATCGACGGG
R-Nluc_96	ATAGTGCAGGATCACC
F-Nluc_G136C	TGCAACAAAATTATCGACGAGC
R-Nluc_135	GTTCCACAGGGTCCCCTG

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218 **Supplementary Note 1**

219 **Consideration of the insertion site of mKOκ**

220 To achieve efficient FRET from Nluc to mKOκ, we decided to insert mKOκ into the  
221 loop region of Nluc. To find the closest position of the luciferin-binding site, we  
222 substituted a glycine residue with a cysteine residue at four putative flexible loop  
223 regions of Nluc (50th, 66th, 97th, and 136th) and conjugated eosin, a yellowish orange  
224 fluorescent dye, with each cysteine residue via the maleimide functional group  
225 (**Supplementary Fig. 7a and 7b**). The Nluc conjugated with eosin at the 50th residue  
226 exhibited the highest FRET efficiency among tested constructs, suggesting that the  
227 luciferin binding site is close to the 50th residue of Nluc. Thus, we inserted mKOκ in  
228 between Gly<sub>50</sub> and Glu<sub>51</sub> of Nluc and generated a small library with flexible linker  
229 amino acids inserted at the N- and C-termini of the mKOκ domain (**Supplementary Fig.**  
230 **7c**).

231

232 **Supplementary Note 2**

233 **Estimation of photon number that single GeNL molecule emits**

234 Based on enzymatic parameters, the photon number that can be emitted by a single  
235 molecule of GeNL during camera exposure was  $590 \pm 3.8$  photons ( $3.3 \text{ photons s}^{-1} \times$   
236  $180 \text{ s}$ , mean  $\pm$  SD,  $n = 3$ ). The reported photon detection efficiency of an objective lens  
237 (NA 1.45, x100 magnification, oil immersion), similar to that used is 15% when the  
238 specimen is located at a height of 200 nm from the glass-water interface<sup>1</sup> (typical  
239 thickness of agarose used here<sup>2</sup>). Thus the number of photons reaching the camera is  
240 anticipated to be  $89 \pm 0.57$  photons (mean  $\pm$  SD). Separately, we calculated the total  
241 number of counts collected from single luminescence spots and then converted this  
242 number to the number of photons using 5.8 conversion efficiency, 1200 electron  
243 multiplication, an ADC gain setting of 5, and a quantum efficiency of 0.9 at 520 nm  
244 (ImagEM, Hamamatsu Photonics). The number of detected photons was  $75 \pm 30$   
245 photons (mean  $\pm$  SD,  $n = 919$ ).

246

247

## 248 **Supplementary Note3**

### 249 **Consideration of the insertion site for eNL-based indicators**

250 To develop a  $\text{Ca}^{2+}$  indicator based on GeNL, we adopted the intramolecular  
251 complementation of split luciferase, in which the sensor domain of a bioactive molecule  
252 is inserted into luciferase. The conformational change of the sensor domain by analyte  
253 binding induces the reconstitution of the split luciferase domains. We chose a fusion  
254 protein of calmodulin and M13 as a  $\text{Ca}^{2+}$  sensing domain. To create high-performance  
255 CSL-based indicators, it is important to design an appropriate insertion site that allows  
256 the split Luciferase to display a large dynamic range in signal change, an intensity  
257 bright enough for imaging. Thus we systematically screened and identified the  
258 appropriate sites of Nluc for CaM-M13 insertion. Through the use of transposon-based  
259 mutagenesis<sup>3</sup>, we constructed a library of Nluc gene variants that contained 15 base  
260 pairs of DNA inserted at a random location, followed by expression in *E. coli*<sup>3</sup>. We  
261 screened several thousand individual clones and picked 40 based on the luminescence  
262 signal intensity. Subsequent DNA sequencing revealed the insertion sites within the  
263 Nluc (37/38, 63/64, 69/70, 97/98, 103/104, 107/108, 121/122 and 148/149th residue) at  
264 which five amino acids were inserted without obliterating the intrinsic activity of Nluc  
265 (Supplementary Fig. 16a). Next we inserted the CaM-M13 domain into the identified  
266 sites within Nluc. As a result, CaM-M13 insertion in between Gly<sub>69</sub> and Asp<sub>70</sub> of Nluc  
267 yielded a 60% signal increase upon  $\text{Ca}^{2+}$  binding (Supplementary Fig. 16b). The  
268 construct, in which CaM-M13 was inserted in between Gly<sub>69</sub> and Asp<sub>70</sub> of the Nluc  
269 moiety in GeNL, showed almost the same signal change (60%). The spectrum profile  
270 was unchanged upon  $\text{Ca}^{2+}$  binding, indicating that the eNL-based  $\text{Ca}^{2+}$  indicator indeed  
271 used a  $\text{Ca}^{2+}$ -dependent CSL mechanism whose emission color was changed by FRET,  
272 but which was insensitive to  $\text{Ca}^{2+}$ . We searched for a more optimal insertion sites  
273 around Gly<sub>69</sub>/Asp<sub>70</sub>, and identified that the insertion in between Gly<sub>66</sub> and Leu<sub>67</sub> of the  
274 Nluc moiety in GeNL yielded a 180% signal increase upon  $\text{Ca}^{2+}$  binding  
275 (Supplementary Fig. 16c). Thus we named this construct GeNL( $\text{Ca}^{2+}$ ).

276

277

278 **Supplementary Note4**

279 Nucleotide sequences of eNL constructs used in this study are listed below. Acceptor  
280 fluorescent proteins is highlighted in yellow, Nluc highlighted in cyan, CaM-M13  
281 highlighted in green.

282

283 >CeNL

284 ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTC  
285 GAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGC  
286 GAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCG  
287 GCAAGCTGCCCGTGCCCTGGCCACCCTCGTGACCACCCTGTCCTGGGGCGT  
288 GCAGTGCTTCGCCCCGTACCCCGACCACATGAAGCAGCACGACTTCTTCAAG  
289 TCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACG  
290 ACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGG  
291 TGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCC  
292 TGGGGCACAAGCTGGAGTACA ACTACTTTAGCGACAACGTCTATATCACCGC  
293 CGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACAT  
294 CGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCAT  
295 CGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCCAGTC  
296 CAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGA  
297 GTTCGTGACCGCCGCGGGTTGCATACACTCGAAGATTTTCGTTGGGGACTGG  
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299 TCCAGTTTGTTCAGAATCTCGGGGTGTCCGTAACCTCCGATCCAAAGGATTGT  
300 CCTGAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTATG  
301 AAGGTCTGAGCGGCGACCAAAATGGGCCAGATCGAAAAATTTTAAGGTGG  
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303 GTAATCGACGGGGTTACGCCGAACATGATCGACTATTTTCGGACGGCCGTATGA  
304 AGGCATCGCCGTGTTTCGACGGCAAAAAGATCACTGTAACAGGGACCCTGTG  
305 GAACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTG  
306 CTGTTCCGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCA  
307 TTCTGGCGTAA

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309 >GeNL

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311 GAGCTGCACATCTTCGGCAGCATCAACGGCGTGGACTTCGACATGGTGGGAC  
312 AGGGCACCGGCAACCCCAACGACGGCTACGAGGAACTGAACCTGAAGTCCA  
313 CAAAGGGCGACCTGCAGTTCAGCCCCTGGATTCTGGTGCCCCACATCGGCTA  
314 CGGCTTCCACCAGTACCTGCCCTACCCCGACGGCATGAGCCCTTTCAGGCC  
315 GCTATGGTGGATGGCAGCGGCTACCAGGTGCACCGGACCATGCAGTTTGAGG  
316 ACGGCGCCAGCCTGACCGTGAACCTACCGGTACACATACGAGGGCAGCCACAT  
317 CAAGGGCGAGGCCCAAGTGAAGGGCACAGGCTTTCAGCCGACGGCCCCGT  
318 GATGACCAATAGCCTGACAGCCGCCGACTGGTGCAGAAGCAAGAAAACCTA  
319 CCCCAATGACAAGACCATCATCAGCACCTTCAAGTGGTCTACACCACCGGC  
320 AATGGCAAGCGGTACAGAAGCACCGCCCGGACCACCTACACCTTCGCCAAA  
321 CCTATGGCCGCCAACTACCTGAAGAACCAGCCTATGTACGTGTTCCGCAAGA  
322 CCGAGCTGAAGCACTCCAAGACAGA AACTGAACTTCAAAGAGTGGCAGAAA  
323 GCCTTCAACGGGTTTGAAGATTTTCGTTGGGGACTGGCGACAGACAGCCGGCT  
324 ACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTTTGTTCAGAA  
325 TCTCGGGGTGTCCGTAACCTCCGATCCAAAGGATTGTCTGAGCGGTGAAAAT  
326 GGGCTGAAGATCGACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGGCG



327 ACCAAATGGGCCAGATCGAAAAAATTTTAAAGGTGGTGTACCCTGTGGATGA  
328 TCATCACTTTAAGGTGATCCTGCACTATGGCACACTGGTAATCGACGGGGTTA  
329 CGCCGAACATGATCGACTATTTTCGGACGGCCGTATGAAGGCATCGCCGTGTT  
330 GACGGCAAAAAGATCACTGTAACAGGGACCCTGTGGAACGGCAACAAAATT  
331 ATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTCCGAGTAACCA  
332 TCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGTAA

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335 ATGGTGAGCAAGGGCGAGGAGCTGTTACCCGGGGTGGTGCCCATCCTGGTC  
336 GAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGC  
337 GAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACC  
338 GGCAAGCTGCCCCTGCCCTGGCCACCCTCGTGACCACCCTGGGCTACGGCC  
339 TGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAA  
340 GTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC  
341 GACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTG  
342 GTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATC  
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344 CCGACAAGCAGAAGAACGGCATCAAGGGCCAACCTTCAAGATCCGCCACAACA  
345 TCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCA  
346 TCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTC  
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353 GGATGATCACTTTAAGGTGATCCTGCACTATGGCACACTGGTAATCGACG  
354 GGGTTACGCCGAACATGATCGACTATTTTCGGACGGCCGTATGAAGGCATCGC  
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356 CAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTCCGA  
357 GTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCG  
358 AA

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360 >OeNL

361 ATGGTCTTCACACTCGAAGATTTTCGTTGGGGACTGGCGACAGACAGCCGGCT  
362 ACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTTTGTTCAGAA  
363 TCTCGGGGTGTCCGTAACCTCCGATCCAAGGATTGTCCTGAGCGGTCTCGAG  
364 GGCGGTAGCGGTGGCAGCGTGAGCGTGATCAAGCCCGAGATGAAGAAGGTG  
365 GAGGACGCCGTGGCCCACTCCACTCTCGAGGGCGTGAGCGTGATCAAGCCC  
366 GAGATGAAGATGAGGTAATACTACATGGACGGCTCCGTCAATGGGCATGAGTTCA  
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368 CACTGCGCGTCACAATGGCCGAGGGCGGGCCAATGCCTTTCGCCTTCGACCT  
369 GGTGTCCACGTGTTCTGTTACGGCCACAGAGTGTTTACTAAATATCCAGAAG  
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371 GTCCCTGGAGTTCGAGGACGGCGGCTCCGCCTCCGTGAGCGCCACATCAG  
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373 CCCGCCGACGGCCCCATCATGCAGAACCAGAGCGTGGACTGGGAGCCCTCC  
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383 GGGACCCTGTGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCC  
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385 TGTGCGAACGCATTCTGGCG TAA

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387 >ReNL

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401 CTGTTCCCTGGGGCATGGCACCCGGCAGCACCGGCAGCGGCAGCTCCGGCACC  
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425 AA

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427 >GeNL(Ca<sup>2+</sup>)\_520

428 ATGGTGTCCAAGGGCGAAGAGGACAACATGGCCAGCCTGCCTGCCACCCAC  
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463 >GeNL(Ca<sup>2+</sup>)\_480

464 ATGGTGTCCAAGGGCGAAGAGGACAACATGGCCAGCCTGCCTGCCACCCAC  
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499 >GeNL(Ca<sup>2+</sup>)\_250

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533 GGCTGTGCGAACGCATTCTGGCG TAA

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535 >GeNL(Ca<sup>2+</sup>)\_60

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555 ATGAGGTCGCTTGGACAAAACCCAACGGAAGCAGAATTGCAGGATATGATCA  
556 ATGAAGTCGATGCTGATGGCAATGGAACGATTACTTTCTTCTACTACTA  
557 TGATGGCTAGAAAAATGAAGGACACAGACAGCGAAGAGGAAATCCGAGAA  
558 GCATTCCGTGTTTTTGACAAGGATGGGAACGGCTACATCAGCGCTGCTGAAT  
559 TACGTCACGTCATGACAAACCTCGGGGTGAAGTTAACAGATGAAGAAGTTGA  
560 TGAAATGATAAGGGAAGCAGATATCGATGGTGTGATGGCCAAGTAAACTATGAA  
561 GAGTTTGAACAAATGATGACAGCAAAGGGGGGAGGTGGCTCCAAGAGGCGC  
562 TGGAAGAAAAACTTCATTGCCGTCAGCGCTGCCAACCGGTTCAAGAAGATCT  
563 CCAGCTCCGGGGCACTG GAGCTCCTGAGCGGCGACCAAATGGGCCAGATCG  
564 AAAAAATTTTAAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGTGATC  
565 CTGCACTATGGCACACTGGTAATCGACGGGGTTACGCCGAACATGATCGACTA  
566 TTTCGGACGGCCGTATGAAGGCATCGCCGTGTTTCGACGGCAAAAAGATCACT  
567 GTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGACGAGCGCCTGATC  
568 AACCCCGACGGCTCCCTGCTGTTCCGAGTAACCATCAACGGAGTGACCGGCT  
569 GGCGGCTGTGCGAACGCATTCTGGCGTAA

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