Five colour variants of bright luminescent protein for real-time multicolour bioimaging

Kazushi Suzuki1, Taichi Kimura2, Hajime Shinoda1, Guirong Bai3, Matthew J. Daniels4, Yoshiyuki Arai1,2,3, Masahiro Nakano1,2,3 & Takeharu Nagai1,2,3

Luminescence imaging has gained attention as a promising bio-imaging modality in situations where fluorescence imaging cannot be applied. However, wider application to multicolour and dynamic imaging is limited by the lack of bright luminescent proteins with emissions across the visible spectrum. Here we report five new spectral variants of the bright luminescent protein, enhanced Nano-lantern (eNL), made by concatenation of the brightest luciferase, NanoLuc, with various colour hues of fluorescent proteins. eNLs allow five-colour live-cell imaging, as well as detection of single protein complexes and even single molecules. We also develop an eNL-based Ca$^{2+}$ indicator with a 500% signal change, which can image spontaneous Ca$^{2+}$ dynamics in cardiomyocyte and neural cell models. These eNL probes facilitate not only multicolour imaging in living cells but also sensitive imaging of a wide repertoire of proteins, even at very low expression levels.
live-cell imaging with fluorescent proteins (FPs) has become a gold standard of biological imaging with the aid of an expanded FP colour palette. However, the requirement of excitation light for fluorescence detection sometimes causes serious problems such as phototoxicity, perturbation of photo-dependent biological phenomena and autofluorescence from the specimen.

Instead of FPs, using luminescent proteins (LPs), such as luciferase, can circumvent these problems. LPs generate an emission signal by catalysing a confined chemical reaction with a luminescent compound, so they are totally independent from an external light source. However, the output photons from conventional LPs are generally insufficient to provide spatiotemporal resolution equivalent to fluorescence.

To overcome the dim signal limitation of LPs, researchers have used directed evolution to improve the intrinsic properties of luciferase such as its luminescent quantum yield, catalytic turnover and stability\(^1\). Recently, the brightest luciferase, NanoLuc (Nluc), was developed through genetic engineering of *Ophioscurus luciferase* (OLuc), which is derived from the deep-sea shrimp *Ophioscurus gracilicostiris*, along with its optimal substrate, furimazine\(^3,4\). Although the intense luminescence of Nluc is very useful for visualizing fast physiological events and proteins with lower expression levels, Nluc application is restricted to single subcellular structures, and gene expression\(^10\).

Colour variants of luciferase are typically developed by rationally altering residues in the active pocket, which alters the chemical form of the excited luminescent substrate, thereby allowing a colour-shifted emission spectrum\(^7\). This approach has not proven particularly effective for Nluc owing to the modified chemical structure of furimazine.

An alternative way to develop colour variants of luciferase is to harness Förster resonance energy transfer (FRET) to a FP that emits the colour of interest. Tight concatenation of luciferase with a FP results in luminescence from the acceptor FP owing to efficient FRET\(^8-10\). For example, colour hues of *Renilla* luciferase variants (Rluc8_S257G and Rluc8.6) were effectively expanded towards yellow and orange by concatenation with Venus (Yellow FP) and mKusabiraOrange2 (Orange FP), yielding yellow (YNL) and orange Nano-lantern (ONL), respectively\(^9,10\). These facilitate the use of eNLs as fusion tags for live-cell imaging, in a similar manner to the ‘universal point accumulation target molecules with Ni-NTA might enable sub-diffraction potential of eNLs for single-molecule-based superresolution imaging.'

Inspired by the strategy for Nano-lantern development, here, we create colour variants of Nluc with the aid of FRET. The efficiency of FRET generally increases as the linker shortens, whereas large truncation of the linker in FPs substantially affects folding. Thus, we systematically identify the optimal linker between luciferase and FP that confers high FRET efficiency. These colour variants allow five-colour live-cell imaging, as well as detection of single protein complexes and even single molecules. We also develop an enhanced Nano-lantern (eNL)-based \(\text{Ca}^{2+}\) indicator with a 500% signal change, which can image \(\text{Ca}^{2+}\) dynamics in cardiomyocytes.

**Results**

**Development of multicolour bright LPs.** To develop colour variants of Nluc, we fused Nluc with mTurquoise2 (mTQ2; ref. 11; cyan FP), mNeonGreen\(^12\) (green FP), Venus\(^3\) (yellow FP), mKOK\(^14\) (orange FP) and tdTomato\(^3\) (red FP) as FRET acceptors. We speculated that the unstructured residues of Nluc and FPs could work as linkers and be deleted without disrupting the function of either protein. First, we made a library of Nluc and FPs with different linker lengths by systematically truncating the N terminus of Nluc and C terminus of FPs, as well as randomizing two residues at the junction, derived from a KpnI site (Supplementary Figs 1–6). By screening for high FRET efficiency and absolute brightness, we identified colour variants that exhibited the highest FRET efficiency. We named them cyan eNL (CeNL), green eNL (GeNL), yellow eNL (YeNL) and red eNL (ReNL) (Fig. 1a).

However, the FRET efficiency of mKOK–Nluc pairs turned out to be moderate. Thus, we attempted to insert mKOK into the loop region of Nluc (Supplementary Fig. 7 and Supplementary Note 1). During screening, we found that a variant with the highest FRET efficiency had an accidental 22-residue insertion in addition to a GSSGGS linker at the N termini of the mKOK domain, whereas there was no linker at the C termini. We called it ‘orange eNL’ (OeNL; Fig. 1a and Supplementary Fig. 8).

Next, the luminescent spectrum of eNLs: CeNL, GeNL, YeNL, OeNL and ReNL, was measured with distinct emission spectra, peaking at 475, 520, 530, 565, and 585 nm, respectively (Fig. 1b). To investigate the brightness of eNLs, we performed side-by-side comparisons of the luminescent intensity of the eNLs, Nluc, Nano-lanterns (yellow, YNL; cyan, CNL; orange, ONL) and Rluc8. Interestingly, the luminescence intensity of CeNL and GeNL was greater than that of Nluc by 2.0- and 1.8-fold, respectively (Fig. 1c). Although the brightness of YeNL, OeNL and ReNL was moderate compared with that of Nluc, they were still brighter than Nano-lanterns with corresponding colour by 4- to 1.8-fold. (Fig. 1c).

To investigate how the luminescence intensities of CeNL and GeNL became brighter than Nluc, we compared luminescence quantum yield (LQY) and the enzymatic parameters (\(K_m\) and \(k_{\text{cat}}\)) of the eNLs and Nluc (Supplementary Fig. 9 and Supplementary Table 1). \(k_{\text{cat}}\) of all eNLs were almost identical to that of Nluc, suggesting that FP fusion did not perturb Nluc enzymatic activity. In contrast, the LQY of CeNL and GeNL became larger than that of Nluc, while LQY of others were comparable or less (Supplementary Table 1). These results indicate that the enhancement of luminescence intensities in CeNL or GeNL are due to the enhancement of LQY by means of efficient FRET from Nluc to FPs with high fluorescence quantum yield.

To our surprise, we could detect the luminescence from a single GeNL molecule immobilized on the glass surface (Fig. 2a). The average total photon numbers from individual spots was consistent with the value estimated from the kinetic parameters associated with bulk solution analysis (Fig. 2b and Supplementary Note 2). Moreover, luminescence intensity at each region of interest (ROI) exhibited a stepwise transition between ‘bright states’ with \(75 \pm 30\) photon emission and ‘dark states’ with emission similar to the background (Fig. 2c). We reasoned that those two states correspond to association and dissociation between Ni-NTA and single GeNL molecules fused with a his-tag, which might occur during the observation times (the reported dissociation half-times of his-tag and Ni-NTA are \(t_{\text{fast}} = 110\) s and \(t_{\text{slow}} = 386\) s, respectively\(^16\)). These results suggested not only the possibility of single-molecule observation but also the potential of eNLs for single-molecule-based superresolution luminescence imaging. The weak binding between GeNL and target molecules with Ni-NTA might enable sub-diffraction imaging, in a similar manner to the ‘universal point accumulation imaging in the nanoscale topography’ method.

**Application as a fusion tag for subcellular structures.** To demonstrate the use of eNLs as fusion tags for live-cell imaging, we constructed 14 eNL fusion proteins. These fusion proteins were localized appropriately in living cells, including to...
microtubules, histones and intermediate filaments that require a monomeric character (Fig. 3 and Supplementary Figs 10–13).

Notably, CeNL with a clathrin fusion tag demonstrated the monomeric character (Fig. 3 and Supplementary Figs 10–13).

According to a crystallographic study, only 180 molecules of the clathrin light chain are incorporated into clathrin-coated pits, meaning that eNLs are capable of serving as a fusion tag for visualizing supramolecular complexes that consist of a small number of proteins.

To demonstrate the utility of the eNL colour palette, we co-expressed Nluc and the eNLs in the mitochondria lumen (mito-Nluc), endoplasmic reticulum (ER) lumen (CeNL-ER), nucleus (GeNL-fibrillarin), inner plasma membrane (Lyn-OeNL) and nucleus (ReNL-H2B). As a result, five different colour luminescence signals were clearly visualized by optical filtering and spectral unmixing (Fig. 4 and Supplementary Fig. 14).

To track the dynamics of multiple subcellular structures, we expressed GeNL and ReNL in lysosome and nucleus by fusion with lysosome-associated membrane protein (LAMP, GeNL is located on the outer surface of lysosome) and histone H2B, respectively, and we tracked their trajectories for 20 min (Supplementary Movie 1). The results demonstrate that eNLs are sufficient for examining the dynamics of multiple subcellular structures simultaneously.

Development of Ca$^{2+}$ indicators based on GeNL. To expand the application of eNLs to biosensing, we sought to develop a Ca$^{2+}$ indicator based on GeNL. We inserted a fusion protein of calmodulin and M13 (CaM–M13) into Nluc, in which the conformational change of CaM–M13 by Ca$^{2+}$-binding induces the reconstitution of the split Nluc moiety (Fig. 5a,b). We found that insertion of CaM–M13 between Gly66 and Leu67 of the Nluc moiety could work as Ca$^{2+}$ indicator (Supplementary Note 3 and Supplementary Fig. 15). After two rounds of directed evolution by error-prone PCR, we obtained a construct with three mutations (K30R, E114V and V142E) in the CaM domain, and a signal change on Ca$^{2+}$ binding of 500%. The $K_d$ value for Ca$^{2+}$ of this construct was 480 nM. Thus, we named it GeNL(Ca$^{2+}$)480 (Fig. 5a,c,d). The Ca$^{2+}$ affinities of GeNL(Ca$^{2+}$) could be tuned from 60 to 520 nM by changing the linker length between CaM mutants and M13, as reported previously (Supplementary Table 2).

To demonstrate the performance of GeNL(Ca$^{2+}$), we observed Ca$^{2+}$ dynamics induced by histamine in HeLa cells. On addition of 10 µM histamine, an acute Ca$^{2+}$ spike followed by Ca$^{2+}$ oscillations with smaller amplitudes was detected at 30 Hz (Fig. 6a,b and Supplementary Movie 2). Furthermore, we were able to propagate the oscillation of cytoplasmic Ca$^{2+}$ wave from one end of the cell to the other (Fig. 6c).

We also made side-by-side comparisons of its performance with the benchmark Ca$^{2+}$ indicators, GCaMP3 (ref. 22) and Fura-2 (ref. 23), in GH3 cells (rat pituitary tumour), which show spontaneous Ca$^{2+}$ spikes (Supplementary Fig. 16). All indicators produced a dynamic signal trace. The signal to noise ratio of GeNL(Ca$^{2+}$)480 (SNR 120 ± 12 at 1.3 Hz frame rate and 74 ± 30 at 33 Hz frame rate; data are presented as mean ± s.d.; $n = 6$ cells) was superior to that of Fura-2 (SNR 9.6 ± 0.71 at 1.3 Hz frame rate; $n = 6$ cells), but inferior to that of GCaMP3 (SNR 590 ± 25 at 33 Hz frame rate; $n = 6$ cells). We also detected spontaneous Ca$^{2+}$ spikes in GH3 cells expressing GeNL(Ca$^{2+}$)480 over 20 min. In contrast, the signals from Fura-2 were severely diminished 10 min after starting of observation due to phototoxic and photobleaching effects.

Recently, cardiomyocytes derived from human-induced pluripotent stem cells (hiPSC-CMs) provide a platform for personalized drug screening in vitro. Several fluorescent indicators have been used to detect drug effects on cardiomyocyte beating behaviour. To test the applicability of GeNL(Ca$^{2+}$), we expressed GeNL(Ca$^{2+}$)520 in cardiomyocytes by means...
of an adeno-associated virus (AAV) infection system. GeNL(Ca^{2+})_{520} was imaged at 60 Hz revealing a periodic luminescence change synchronized with cardiomyocyte contraction for 35 min (Fig. 7a and Supplementary Movie 3). When cardiomyocytes were treated with astemizole, which perturbs beat behaviour by off-target human Ether-à-go-go...

Figure 2 | Detection of luminescence from single GeNL molecule. (a) Luminescence images of single GeNL molecule (left). The exposure time was 180 s. The magnified image of the square inset (right). Scale bars, 10 μm. (b) A histogram of the average photon numbers emitted from single spots (N = 919). (c) A time series of single GeNL molecule (above). Time course in the total photon numbers within the ROIs (below).

Figure 3 | Luminescence imaging of HeLa cells expressing eNLs targeted to the various cellular compartments. HeLa cells expressing GeNL in cytosol (a), nucleus (b), mitochondria (c), ER (d), inner plasma membrane (e), peroxisome (f), lysosome (g), nucleoli (h), actin (i), microtubule (j), vimentin (k), vinculin (l), zyxin (m), paxillin (n) and CeNL in a clathrin-coated pit (o). Scale bars, 10 μm.
Related Gene (hERG) inhibition, we detected irregular beating, like an arrhythmia (Fig. 7b). These results indicate that our luminescence-based Ca$^{2+}$ indicator can be used not only for imaging fast physiological events but also for analysing drug effects on cardiomyocyte function.

**Discussion**

Through systematic protein engineering, we identified five colour variants of bright LPs across the visible spectrum. The use of eNLs allowed us to perform five-colour luminescence imaging of subcellular structures. A remarkable feature of eNL is signal intensity sufficient to visualize luminescence from single molecules in vitro and single clathrin-coated pit in living cells. We also developed an eNL-based Ca$^{2+}$ indicator with a 500% signal change. This indicator enabled long-term and fast Ca$^{2+}$ imaging in cardiomyocytes.

Luminescence imaging in general has other limitations that the eNLs do not overcome. First, luminescence signal decays over time by consumption of the luminescent substrate. This issue may be possible to overcome by continuous perfusion with fresh luminescent substrate. Second, the luminescent substrates may affect cell behaviour. Coelenterazine is reported to possess high antioxidant activity against reactive oxygen species. Since furimazine is an analogue of coelenterazine, it might perturb cellular physiology by disruption of signal cascades involving reactive oxygen species. To minimize the potential for this effect, most of our experiments use 0.2 mM furimazine, which does not affect cell viability and morphology.

![Figure 4](image_url)  
**Figure 4 | Multicolour luminescence image of subcellular structures.** Mitochondria (mito-Nluc); ER (CeNL-ER); nucleoli (GeNL-fibrillarin); plasma membrane (Lyn-OeNL); and nucleus (ReNL-H2B). Each luminescence signal was separated by linear unmixing. Scale bar, 10 μm.

![Figure 5](image_url)  
**Figure 5 | Luminescent Ca$^{2+}$ indicators based on GeNL.** (a) Domain structure of four affinity variants of GeNL(Ca$^{2+}$). (b) Schematic explanation of Ca$^{2+}$-sensing mechanism in GeNL(Ca$^{2+}$). (c) Relative brightness of recombinant GeNL, GeNL(Ca$^{2+}$), with or without Ca$^{2+}$. (d) Ca$^{2+}$ titration curves for four affinity variants of GeNL(Ca$^{2+}$). Measurements in c, d were performed at least in triplicate, and the averaged data and s.d.'s are shown.
compartments, furimazine membrane permeability within the cells does not appear limiting.

The design of GeNL-based functional indicators could be expanded to other colour variants and biomolecules such as cyclic AMP and ATP. Furthermore, we believe that eNLs are promising reporters for endogenous proteins. It is now feasible to create endogenously tagged proteins using CRISPR/Cas9-mediated genome editing. The enhanced luminescence signal of eNL allows detection of low-copy-number proteins, which is difficult by fluorescence imaging, especially where the cellular substrate has autofluorescence.

The red-shifted emission of ReNL is also important for in vivo imaging because it can be distinguished from the background signal caused by self-oxidation of substrate in tissue. It can also overcome the optical scattering of luminescence signal that occurs in deep tissue. Thus, we expect that eNLs could facilitate detection of rare, but significant cell populations (cancer stem cells and so on) in living mice in addition to functional imaging in freely moving animals.

Methods

General. DNA oligonucleotides used for cloning and construction of gene libraries were purchased from Hokkaido System Science. The sequences of all the

Figure 6 | High-speed Ca\(^{2+}\) imaging with GeNL(Ca\(^{2+}\))\(_{480}\) in HeLa cells. (a) A series of intensity modulated display pseudo-coloured ratio images showing Ca\(^{2+}\) concentration change by histamine stimulation. Scale bars, 20 μm. (b) Long-range time course of the \(L/L_0\) (\(L\): luminescence intensity at arbitrary time; \(L_0\): luminescence intensity before stimulation). Arrow indicates the time point of 10 μM histamine addition in the ROI (white rectangle). (c) Short-range time courses of the \(L/L_0\) ratio change in ROIs (green, magenta and yellow boxes in a).

Figure 7 | High-speed Ca\(^{2+}\) imaging with GeNL(Ca\(^{2+}\)) in hiPSC-CMs. (a) Time course of the luminescence signal of GeNL(Ca\(^{2+}\))\(_{520}\) at around 0 and 35 min. (b) Time course of luminescence signal of GeNL(Ca\(^{2+}\))\(_{520}\) before and after treatment with 1 μM astemizole.
Modelling conclusions made on the basis of our results.

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Covalent labelling of Nluc with eosin maleimide dye

Construction of five colour eNL variants. cdNA of mNeonGreen was provided by Allele Biotechnology. The deletion mutant libraries of mNeonGreen–Nluc and Venus–Nluc were generated as described previously. C-terminally deleted mNeonGreen mutants (mNGCa0–10) and Venus mutants (VenusCa0–12) were amplified by PCR. They were digested with BamH1 and Kpn1. The cDNAs of N-terminally deleted Nluc mutants (NlucAn1–5 for mNG and NlucAn1–6 for Venus) were amplified by PCR and digested with EcoRI and Kpn1. The digested fragments were gel-purified, mixed together and cloned in-frame into the BamH1/EcoRI sites of pRESEf1 (Invitrogen) for bacterial expression (Supplementary Fig. 1). After screening, the linker amino acids (encoded by Kpn1, Gly–Thr–) of deletion mutants were randomized by inverse PCR techniques (nucleotide sequence NNKNNK, where N = A, G, C and T; and K = G and T) to generate 400 amino-acid combinations (1,024 nucleotide combinations; Supplementary Fig. 3).

Covalent labelling of Nluc with eosin maleimide dye

Eosin-5-maleimide was purchased from Molecular Probes (no. E-118). To achieve the labelling at specific positions, we substituted the endogenous cysteine of Nluc (166th residue) with cysteine, respectively. The mutated Nluc was expressed in E. coli, with such substitution would not affect the conclusions made on the basis of our results.

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Protein expression and purification. LP with an N-terminal polyhistidine tag was expressed in E. coli (JM109 (DE3)) at 23 °C for 6 h in LB bacterial growth medium supplemented with 0.1 mg ml⁻¹ carbenicillin. Cells were collected and ruptured with a French press (Thermo Fisher Scientific), and recombinant proteins were purified from the supernatant using Ni-NTA agarose affinity columns (Qiagen) followed by buffer-exchange (20 mM HEPES, pH 7.4) gel filtration (PD-10 column, GE Healthcare). The whole purification process after rupture was conducted on ice to avoid protein degradation. The protein concentration was determined by Bradford method (Protein assay kit, Bio-Rad).

LP characterization. Recombinant proteins and furimazine were diluted with 20 mM HEPES buffer (pH 7.4), and emission spectra were measured with a photonic multichannel analyser PMA-12 (Hamamatsu Photonics) at room temperature. The final concentration were screened and selected in two steps. Initially, the E. coli colonies were poured with a phosphate-buffered saline (PBS) solution supplemented with 5 μM coelenterazine-h and examined with an LAS-1000 luminescence imaging system (GE Healthcare). Bright colonies were picked, and those mutants were inoculated in the 96-well plate supplemented with 10 μM coelenterazine-h. The plate was incubated at 37 °C for 5 days before luminescence spectra were measured using a micro-plate reader (SH-9000; Corona Electric). A final concentration of 5 μM coelenterazine-h was used as the luminescent substrate for this measurement. Luminescent spectra were normalized at the 450 nm luminescence intensity and mutants with a high-FRET ratio (ratio of peak 530 nm/450 nm) were chosen. Finally, the protein samples were diluted to 50 μM for mKoc and 585/450 nm for tdTomato) picked and subjected to DNA sequencing and protein characterization. Because the emission peaks of Nluc (donor, ~460 nm) and mTQ2 (acceptor, 480 nm) were too close to discern, the cyan variants were directly purified and screened in vitro on the basis of brightness and FRET efficiency.

Construction of mammalian expression vectors. To ensure the robust expression of GeNL in mammalian cells, we replaced the wild-type codon with synonymous cdNA encoding the mNeonGreen with mammalian favourable codons obtained from Life Technologies (GeneArt Strings DNA Fragments). PCR-amplified eNLs were inserted into a pCDNA3 mammalian expression vector using BamHI and EcoRI RE sites. We localized eNLs to mitochondria, plasma membrane and nucleus, respectively, by replacing the Nano-lantern sequence with the eNL sequence in pCDNA3-CoxVIIENano-lantern (a duplicated mitochondrial localization sequence derived from the subunit-VIII precursor of human cytochrome c oxidase (Cox-VIII) at the N termtnus); pcDNA3-Nano-lantern-H2B (a DNA-binding protein histone 2B (H2B) at C terminus); and pcDNA3-lyn-Nano-lantern (a myristoylation and palmitoylation sequence from lyn at the N terminus) using two rounds of phenol/chloroform extraction, PEG 8000 precipitation and two rounds of phenol/chloroform extraction. cDNA sequences for all constructs were read by dye terminator cycle sequencing using the BigDye Terminator v1.1 Cycle Sequencing kit (Life Technologies). The luminescent substrate coelenterazine-h was purchased from Wako Chemicals, and furimazine was synthesized according to a previous report4. Cultured cells were not tested for the presence of plasmid, as such contamination would not affect the conclusions made on the basis of our results.

Modelling. The primary amino-acid sequence of Nluc4 was used as input to the I-TASSER structure prediction server30 using default parameters. The generation of I-TASSER structure prediction server30 using default parameters. The generation of I-TASSER structure prediction server30 using default parameters. The generation of I-TASSER structure prediction server30 using default parameters. The generation of I-TASSER structure prediction server30 using default parameters. The generation of I-TASSER structure prediction server30 using default parameters.

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Luminescent quantum yield and kinetic parameters. The luminescent quantum yields were estimated from the total light output by the complete consumption of 0.05 pm of furimazine. The luminescent quantum yields were measured in triplicate with a micro-plate reader (SH-9000, Corona Electric). The photon sensitivity of the detector was calibrated with luminal chemiluminescence under following reaction mixture: 200 nM horseradish peroxidase; 200 nM luminol; and 2 mM H2O2 and 100 mM KCl. The concentration of luminol (Wako, Osaka, Japan) was characterized by the absorbance at 347 nm with using the extinction coefficient of 7.640 M⁻¹ cm⁻¹ as reported previously. The luminescence characteristics of the detecting method was adjusted in relation to the photonic multichannel analyzer PMA-12 (Hamamatsu Photonics). The final concentrations of LP and furimazine were 1 nM and 500 nM, respectively. The protein and substrate solutions were diluted with HEPES buffer (50 mM HEPES, pH 7.5) supplemented with <0.1% casein.

Kinetic parameters were measured from the reactions of 10 pmol of GeNL(Ca²⁺) and 500 nM of furimazine. The initial reaction velocities were measured as the integrated luminescence intensities for the initial 12 s. Michaelis–Menten constants (KM) and maximum reaction velocities (Vmax) were estimated from the nonlinear fit to the Michaelis–Menten equation using Origin7 software (OriginLab).

Detection of luminescence from single-molecule GeNL. To immobilize the GeNL protein on the glass surface, we prepared the Ni-NTA agarose glass coverslips following a previous study. The fluorescent beads were used as the microbeads bound on the glass surface. First, fluorescent beads (FluoSpheres sulfate microspheres, 0.2 μm diameter, yellow-green fluorescent, no. F8848, Invitrogen) in MOPS/KCl buffer were incubated for 1 h at 37°C. The next day, HeLa cells (expressing each fusion construct) were grown to 70% confluency in 5% CO₂. HeLa cells were washed with phenol red-free DMEM/F12 and imaged in phenol red-free DMEM/F12. For Ca²⁺ imaging, Fura-2 or GeNL(Ca²⁺) was added to the imaging medium. When GeNL(Ca²⁺) was imaged in the absence of Ca²⁺, we used a Ca²⁺-dependent change in luminescence was assessed using the protein extracted from periplasmic fraction of E. coli. The brightest colonies were picked and cultured in liquid LB medium for 12 h. They were subjected to plasmid DNA isolation and subsequent DNA sequencing as described above.

To determine the insertion site of Nluc in plasmid DNA that yield a large signal increase with Ca²⁺, we used the TorA protein export plasmid (pTorPE) in which a Ca²⁺-indicator localized to the E. coli periplasm can be placed on Ca²⁺-bound states and easily extracted by cold osmotic shock. We subcloned Nluc into pTorPE using the TorA protein export plasmid (pTorPE) in which a Ca²⁺-indicator localized to the E. coli periplasm can be placed on Ca²⁺-bound states and easily extracted by cold osmotic shock. We subcloned Nluc into pTorPE using
400 µl of ice-cold 5 mM MgSO4 and gently agitated for 10 min on ice. Following centrifugation to pellet the intact bacteria (9,000g for 5 min at 4°C), the supernatant (the osmotic shock fluid containing the periplasmic protein fraction) was collected. The Ca2+-dependent change in luminescence was measured by a micro-plate reader as described in “In vitro characterization of GeNL(Ca2+).”

**Construction of GeNL(Ca2+)**

NcoI and SacI restriction sites were introduced at the 66/67 and 69/70 insertion sites of the Nuc moiety in pSRETa_GenL. This fragment was then ligated with a CaM-M13 fragment to yield pSRETa_GeNL_CaM-M13. The fragment was transformed into E. coli (DH5α) and grown in 35 mm dish with 5% O2. The recombinant plasmid was then purified and sequenced. The mutant with the largest brightness was selected and cultured in liquid LB medium for 12 h. The bacteria were then subjected to further DNA purification and sequencing. The mutant with the largest brightness and signal increase was designated GeNL(Ca2+), 480 (latter number indicates Kd value to Ca2+).

To generate affinity variants of GeNL(Ca2+), we introduced various length linkers between CaM and M13 by QuikChange site-directed random mutagenesis. For mammalian expression experiments, GeNL(Ca2+) cDNA digested with BamHI and EcoRI was cloned into the BamHI/EcoRI site of the pcDNA3 vector. The nucleotide sequences of GeNL(Ca2+) (60), GeNL(Ca2+) (250), GeNL(Ca2+) (480) and GeNL(Ca2+) (520) are given in Supplementary Note 4.

For the AAV expression system, pHelper and pAAV-DJ were obtained from Cell Biolabs, Inc. The cDNA for GeNL(Ca2+) (520) replaced the ArchT-GFP sequence in pAAV-CAG-ArchT-GFP. pAAV-CAG-ArchT-GFP was a gift from Edward Boyden (Addgene plasmid #29777).

**In vitro characterization of GeNL(Ca2+).** Emission intensity of the purified proteins was measured using a micro-plate reader (SH-9000, Corona Electric). A final concentration of 5 µM coelenterazine-h was used as the luminescent substrate for these measurements. Experiments were performed at least in triplicate, and the averaged data were used for further analysis. Ca2+ titrations were performed by the reciprocal dilution of Ca2+-saturated and Ca2+-free buffers containing 10 mM MOPS, 100 mM KCl and 10 mM EGTA with or without 10 mM Ca2+ added as CaCO3, at pH 7.2. 25°C. The free Ca2+ concentrations were calculated using 0.15 µM for the apparent Kd value of EGTA for Ca2+. The Ca2+ titration curve was used to calculate the apparent Kd value by nonlinear regression analysis. The averaged data were fitted to a single Hill equation using Origin® software (OriginLab).

**AAV production and infection.** HEK293T (RIKEN BRC Cell Bank RCB2202) cells were grown in DMEM (Sigma) containing heat-inactivated 10% FBS at 37°C in 5% CO2. Equal amounts of pHelper, pAAV-DJ and pAAV_CAG_GeNL(Ca2+) (520) were transfected by FuGene6 transfection reagent (Roche). The transfection efficiency was 70%. Three days after transfection, the cultures were collected. The solution containing AAV was distributed into small aliquots and stored at –80°C.

**hiPSC-CM culture and imaging.** Human iPSC cells (hiPS, 201B7, RIKEN BRC) were cultured with a priate ES medium (Reprocell Inc.) and 4 ng ml-1 human basic fibroblast growth factor (Wako Pure Chemical Industries, Ltd.) in an incubator with 5% CO2 at 37°C on a mouse feeder cell layer (SNL, CBA-316, C. Two days after incubation, the medium was replaced with the RPMI + Media 1640 (human FGF2 (both from R&D systems) and 10 mM Ca2+ in an incubator with 5% O2 and CO2 at 37°C. Two days after incubation, the medium was replaced with the RPMI + Media 1640 (human FGF2 (both from R&D systems) and 10% FBS medium). After 3 days of incubation in an incubator with 5% CO2 at 37°C. Four days after incubation, 8–10 embryo bodies were transferred to gelatinized 0.1% coverslips in six-well plates. The medium was replaced with fresh RPMI + FBS medium every 3 days afterwards. The embryo bodies were visually assessed for contraction on 9 days after incubation. Then they were treated with AAV for 4 days before observation. The hiPSC-CM was washed with Tyrode solution (Sigma) and imaged in Tyrode solution. Just before observation, 40 µM furimazine was added to the Tyrode solution. An inverted microscope (LV-200, Olympus) equipped with a ×10 objective (Olympus, UPlanSapo, numerical aperture 1.4) and ×0.5 relay lens was used. Emission signals were detected by an EM-CCD camera (ImageEM, Hamamatsu Photonics) with ×4 (for GeNL(Ca2+) 4) binning settings. During the entire imaging period, the temperature was kept at 28°C by a stage-top incubator. The background drift was manually subtracted using Origin® software (OriginLab).

**Data availability.** The data that support the findings of this study are available from the corresponding author on request. The nucleotide sequences of CaM, GeNL, YeNL, RNL, RoNL, GeNL(Ca2+) (520), GeNL(Ca2+) (310), GeNL(Ca2+) (480) and GeNL(Ca2+) (520) have been deposited to GenBank/EMBL/DDBJ database under the following entry IDs: LC128714; LC128715; LC128716; LC128717; LC128718; LC128719; LC128720; LC128721; and LC128722, respectively. The nucleotide sequences of all constructs are also in Supplementary Note 4.

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Author contributions

T.N. conceived and coordinated the project; K.S., T.K., H.S., G.B., M.J.D., Y.A., M.N. and T.N. designed the experiments; K.S. and T.K. constructed and characterized eNLs with the support of M.N. and Y.A.; K.S. performed imaging; H.S. constructed and characterized the eosin-conjugated Nluc; G.B. and M.J.D. established the iPS cell culture and performed the differentiation of iPSCs into cardiomyocytes; K.S., M.N., Y.A. and T.N. wrote the paper with contributions from all authors.

Additional information

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