Expanded palette of Nano-lanterns for real-time multicolor luminescence imaging

Akira Takai1,2, Masahiro Nakano1,2, Kenta Saito1, Remi Harunob, Tomonobu M. Watanabe1,2, Tatsuya Ohyanagi1, Takashi Jin1, Yasushi Okada1,2, and Takeharu Nagai2

Laboratories for 1Cell Polarity Regulation, 2Comprehensive Bioimaging, 3Nano-Bio Probes, and 4Cell Dynamics Observation, Quantitative Biology Center, RIKEN, Osaka 565-0874, Japan; 5The Institute of Scientific and Industrial Research, Osaka University, Osaka 567-0047, Japan; and 6PRESTO, Japan Science and Technology Agency, Tokyo 102-0075, Japan

Edited by Jennifer Lippincott-Schwartz, National Institutes of Health, Bethesda, MD, and approved February 18, 2015 (received for review September 25, 2014)

Fluorescence live imaging has become an essential methodology in modern cell biology. However, fluorescence requires excitation light, which can sometimes cause potential problems, such as autofluorescence, phototoxicity, and photobleaching. Furthermore, combined with recent optogenetic tools, the light illumination can trigger their unintended activation. Because luminescence imaging does not require excitation light, it is a good candidate as an alternative imaging modality to circumvent these problems. The application of luminescence imaging, however, has been limited by the two drawbacks of existing luminescent protein probes, such as luciferases: namely, low brightness and poor color variants. Here, we report the development of bright cyan and orange luminescent proteins by extending our previous development of the bright yellowish-green luminescent protein Nano-lantern. The color change and the enhancement of brightness were both achieved by bio-luminescence resonance energy transfer (BRET) from enhanced Renilla luciferase to a fluorescent protein. The brightness of these cyan and orange Nano-lanterns was ~20 times brighter than wild-type Renilla luciferase, which allowed us to perform multicolor live imaging of intracellular submicron structures. The rapid dynamics of endosomes and peroxisomes were visualized at around 1-s temporal resolution, and the slow dynamics of focal adhesions were continuously imaged for longer than a few hours without photobleaching or photodamage. In addition, we extended the application of these multicolor Nano-lanterns to simultaneous monitoring of multiple gene expression or Ca2+ dynamics in different cellular compartments in a single cell.

Significance

The application of luminescence imaging has been limited mainly by the two drawbacks of luciferases: low brightness and poor color variants. Here, we report the development of cyan and orange luminescent proteins approximately 20 times brighter than the wild-type Renilla luciferase. The color change and enhancement of brightness were both achieved by exploring bioluminescence resonance energy transfer (BRET) from enhanced Renilla luciferase to a fluorescent protein, a technology that we previously reported for the development of the bright yellowish-green luminescent protein Nano-lantern. These cyan and orange Nano-lanterns along with the original yellow Nano-lantern enable monitoring of multiple cellular events, including dynamics of subcellular structures, gene expressions, and functional status, such as intracellular Ca2+ change.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB982217–AB982329 and AB983214–AB983377).

1A.T. and M.N. contributed equally to this work.
2To whom correspondence may be addressed. E-mail: ng1@sanken.osaka-u.ac.jp or y.okada@riken.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1418468112/-/DCSupplemental.

PNAS | April 7, 2015 | vol. 112 | no. 14 | 4352–4356

www.pnas.org/cgi/doi/10.1073/pnas.1418468112
First, we apply the LPs as fusion tags to visualize the dynamics of subcellular microstructures, such as cytoskeletons and vesicular organelles. These submicron structures were continuously visualized at ~1-s temporal resolution for longer than a few hours without photobleaching or photodamage. Second, an application as a multiple gene expression reporter is shown with photosensitive ES cells. Third, Ca\(^{2+}\) indicators were developed based on cyan Nano-lantern (CNL) and orange Nano-lantern (ONL), showing their usefulness as the basis for the development of luminescent indicators that are fully compatible with optogenetic tools and other optical manipulations.

Results

Development of CNL. We have first replaced Venus of the original Nano-lantern [hereafter, we call it yellow Nano-lantern (YNL)] with mTurquoise2, a cyan FP with the highest QY (QY = 0.93) among all known FPs (7) (Fig. 1A). The chimera of mTurquoise2-RLuc8-S257G emitted bright cyan light (Fig. 1B), and we named it CNL. CNL was about 20 times brighter than RLuc (Fig. 1C). The measured QY of CNL was similar to YNL (Table S1), which means that the higher QY of mTurquoise2 compensated for the lower spectral overlap between RLuc8 and mTurquoise2 than between RLuc8 and Venus. The nearly two times higher brightness of CNL over YNL, therefore, might reflect the higher enzymatic activity of CNL.

Development of ONL. We next attempted to develop a longer wavelength variant of Nano-lantern and tried various combinations of donor RLuc mutants, including long-wavelength mutants of RLuc (8), and acceptor FPs, including bright orange and red FPs (9, 10) or FPs with large Stokes shifts (11) (Figs. S2, S3, and S4). We have also tried deletions of the linker between RLuc and FP (Fig. S3). Among all of the tested constructs, the combination of RLuc8.6–535 (hereafter, RLuc8.6) and mKusabiraOrange2 (Fig. 1D) showed the highest BRET efficiency (Table S2). This variant emitted orange light brighter than YNL and more than 15 times brighter than RLuc (Fig. 1B and C), and we named it ONL. For the red color variants, the combination of RLuc8.6–545 with mCherry or TurboFP635 showed relatively high BRET efficiency (Fig. S4), but additional increase of the BRET efficiency is required to use it as a fourth color combined with other Nano-lanterns. Nonetheless, these red variants will have a potential benefit for deep-tissue imaging, because absorption by tissues, especially hemoglobin, is reduced at wavelengths longer than 600 nm (12, 13).

Application as Fusion Tags for Subcellular Structures. Next, we expressed CNL and ONL as fusion proteins to examine whether they work as the luminescent fusion tags for subcellular structures (Fig 2 and Fig. S5). Fusions with histone H2B, inositol-trisphosphate 3-kinase A (ITPKA; F-tractin) (14), β-tubulin, peroxisome targeting signal 2, zyxin, fibrillarin (Fig. 2), and all tested other various proteins or peptides (Fig. S5) showed correct localization by both fluorescence and luminescence imaging, indicating that both CNL and ONL (as well as YNL) can be used as fusion tags. Furthermore, because the emission spectrum of each color variant of Nano-lantern has a distinct peak that is distant enough from the others (Fig. 1B), the luminescence signals from these Nano-lantern color variants were easily separated by optical filtering (Fig. 2B). It should be noted that the samples were repeatedly illuminated with blue to green excitation light for CFP, YFP, or GFP excitation for fluorescence imaging. The images shown in Fig. 2 and Fig. S5 were first taken with excitation illumination (fluorescence) and then, without illumination (luminescence). Thus, our Nano-lanterns would be fully compatible with optogenetic tools.

To show live-cell imaging of subcellular structures, we examined three subcellular structures stained with ONL: lysosomes, peroxisomes, and focal adhesions (Movies S1–S3). The rapid dynamics of peroxisomes and lysosomes were examined, and their trajectories were tracked for several minutes (Fig. S6 and Movies S1 and S2), showing that the rapid dynamics of submicrometer structures can be investigated by luminescence imaging with ONL.

It should be noted that luminescence imaging is free from photobleaching and photodamage. Therefore, long-term luminescence imaging is only limited by the depletion of the substrate. Adding too much substrate coelenterazine-h, however, can increase the background luminescence because of autooxidation (15). We, therefore, synthesized diacetyl coelenterazine-h, where autooxidation is inhibited by protective acetyl groups. This inhibition of autooxidation allows diacetyl coelenterazine-h to be added at higher concentrations without increasing the background autoluminescence. Diacetyl coelenterazine-h is reverted to coelenterazine-h by esterase, which serves as a constant supply of the substrate. Thus, we can observe the luminescence signal without replenishing the substrate for several hours. With this condition, the slow dynamics of focal adhesion were observed for about 4 h continuously with 1-s exposure imaging, which is difficult with fluorescence imaging because of photobleaching (Movie S3).

Application as Gene Expression Reporters. LPs, such as firefly luciferase (FLuc), have been widely used as a reporter for gene

![Fig. 1. Development of multicolor Nano-lanterns and their characterization. (A) Schematics of multicolor Nano-lanterns. Numbers represent the relative amino acid position in the original protein. (B) Emission spectra of LPs were measured in triplicate, and data are presented as means. (Inset) Luminescence of recombinant Nano-lantern proteins. (C) Luminescence intensities of equimolar amounts of LPs. Intensities were measured in triplicate, and data are presented as means ± SDs. One-way ANOVA followed by posthoc Tukey’s honestly significant difference test compared with RLuc. ****p < 0.0001.](https://example.com/figure1.png)
expression with cell lysate-based assays (16, 17). However, the luminescence signal from FLuc is much weaker than that from FPs. Extremely long exposure time (1–10 min) and low spatial resolution (4 × 4 to 8 × 8 binning, resulting 2.5–5 μm/pixel) were required to collect enough photons. Thus, monitoring the dynamics of gene expression at single-cell level using FLuc has been difficult.

We, therefore, examined whether Nano-lanterns could be applied as gene expression reporters (Fig. 3 and Fig. S7). Reporter plasmids were constructed by introducing cDNA of Nano-lanterns with a nuclear localization signal (NLS) and destabilization tag (hCL1-PEST) (18) downstream of Wnt-responsive promoter 7xTcf (19). The cell line harboring this plasmid responded to Wnt-3a.

Fig. 2. Luminescence imaging of intracellular microstructures. (A) Luminescence and fluorescence images of subcellular structures: nuclei in interphase (CNL-H2B and ONL-H2B), F-actin [inositol trisphosphate 3-kinase A (ITPKA) -ONL], microtubules (β-tubulin-ONL), peroxisomes [peroxisome targeting signal 2 (PTS2) -CNL], and focal adhesions (zyxin-ONL). (B) Subnuclear structures visualized by dual-color luminescence imaging: CNL-fibrillarin (nucleoli) and ONL-H2B. Each luminescence signal was separated by linear unmixing. (Scale bars: 10 μm.)

Fig. 3. Live single-cell luciferase assay for the expression of Wnt target gene. (A) Schematic of Nano-lantern–based Wnt signal reporter. (B) Luminescence of NLS-tagged Nano-lantern expressed in HEK293A cells harboring Wnt reporter construct. Images were acquired with a 495- to 540-nm band-pass emission filter at 16 h after the addition of 200 ng/mL Wnt-3a protein or 10 mM LiCl. Luminescence signals were overlaid with the bright-field image. (Scale bars: 10 μm.) (C) Percentages of the positive cells plotted as a bar graph. Cont, control.
as well as LiCl (Wnt signaling activator through inhibition of GSK3β). Response was sensitively detected at the single-cell level as the luminescence signal in the nucleus, even with 0.5 s of exposure time.

We next tried multiple gene expression monitoring. A stable line of murine ES cells was established, which have three gene expression reporters: Oct4 by CNL, Nanog by YNL, and Sox2 by ONL. Multi-channel luminescence images of differentiating murine ES cells showed the expression level of these three genes in each cell and also, heterogeneity of the differentiation state, even within a single colony (Fig. 4). Here, imaging took only 10 s for each channel without binning (0.5 μm/pixel). This spatial resolution is high enough to distinguish single cells in a dense colony, and the temporal resolution is fast enough to monitor the dynamics of gene expression.

Application as Ca2+ Indicators. As another application of multicolor luminescence imaging, we constructed Ca2+ indicators by applying the complementation of split luciferase technology as reported previously for YNL (3). CNL- and ONL-based Ca2+ indicators were expressed as fusion proteins with a mitochondria localization tag and histone H2B, respectively. After stimulation with 20 μM histamine, acute Ca2+ spikes followed by Ca2+ oscillations with smaller amplitudes were observed in both mitochondria and the nucleus (Fig. 5 and Movie S4), thereby showing the same result as previously for YNL (3). CNL- and ONL-based Ca2+ indicators were also used for functional imaging, especially combined with optogenetic tools, such as channel rhodopsin or photoactivatable Ca2+ releaser (21), both of which are activated by blue light.

Discussion

Through the optimization of the BRET pairs and the linker in between them, we have succeeded in making bright cyan and orange LPs, CNL and ONL, in addition to the original YNL. The chemical energy liberated by the oxidation of the substrate coelenterazine is transferred to the FP, which emits photons with high QY. This mechanism does not only enhance the QY of luciferase but also, changes the color of the emitted light. Thus, the three color variants of Nano-lantern are about 20 times brighter than the wild-type RLuc, and their emission spectra are essentially same as those of the acceptor FPs. The enhanced brightness has enabled luminescence imaging of subcellular organelles with submicrometer and subsecond resolution. For the gene expression reporters, our proof-of-principle experiments show that Nano-lanterns can monitor the expression of three different genes at single-cell resolution with an image acquisition time shorter than 1 min in total. Furthermore, Nano-lanterns can be applied as indicators for functional imaging, such as Ca2+ imaging.

Regardless of these successes, luminescence imaging with Nano-lanterns still has some drawbacks compared with fluorescence imaging with FPs. First, the signal intensity of Nano-lanterns is still more than 100 times weaker than that of FPs. A single FP molecule emits more than 1,000 photons per 1 s under conditions for single FP imaging in living cells, whereas a single Nano-lantern emits less than 10 photons per 1 s. Therefore, the application as the fusion tag for low-abundance proteins or the reporter for rapid dynamics (video rate or faster) is a remaining challenge to be overcome by additional improvements in brightness.

Second, luminescence imaging lacks optical sectioning capabilities. FPs emit signals only when illuminated with the excitation light. Thus, the signals are stronger near the focal plane with fluorescence imaging. This optical sectioning effect can be further enhanced by confocal optics, multiphoton excitation, or light-sheet illumination. Contrastingly, all of the luciferases in the sample emit signal. The signals of the thick samples are blurred by the haze from the out-of-focus luciferases.

Third, different colors of FPs can easily be separated by the combination of excitation and the emission filters. For example, a pair of FPs with emission spectrum overlap can often be separated by filtering the excitation wavelength. With luminescence imaging, the signals from different colors of Nano-lanterns are separated only by the emission spectrum, and therefore, we have used a linear unmixing algorithm to achieve better separation (Fig. S8). Nonetheless, the unmixing algorithm can be much simpler than with fluorescence imaging, because autofluorescence signals are negligible.

Fourth, luminescence imaging requires substrate instead of excitation light. The supply and the consumption of the substrate limit the imaging. Unlike FLuc, our Nano-lanterns do not require ATP for its reaction, and therefore, the consumption of intracellular ATP does not compromise its application. However, a constant supply of the substrate coelenterazine is still necessary...
for stable long-term imaging. We have, therefore, synthesized diacetyl coelenterazine in this study; however, long-term imaging, especially in vivo, is still compromised by its supply. Re-constitution of synthesis or recycling of substrate would help to overcome this difficulty.

Thus, for many applications, fluorescence imaging still has many advantages over luminescence imaging. However, our three color variants of Nano-lantern are the first, to our knowledge, to enable multicolor luminescence imaging with significantly higher spatial and temporal resolution compared with conventional luciferases. Although the temporal resolution of luminescence imaging (0.1–10 s in this paper) is currently one to three orders of magnitude lower than that of fluorescence imaging (typically, 10–100 ms), the spatial resolution achieved here is comparable with cultured cells (Fig. 2 and Fig. S5). Our proof-of-principle experiments reported in this paper clearly show that luminescence imaging has become a practical alternative when the side effects by the excitation light are not negligible, such as when the samples are very sensitive to photodamage. The combination with optogenetic tools would be the most effective future application of luminescence imaging, because the external light illumination can be reserved for optical stimulation.

Materials and Methods

General. DNA oligonucleotides used for gene cloning and construction were purchased from Invitrogen or Greiner. KOD-plus-Neo (TOYOBO) was used for PCR. Products of PCR and restriction enzyme digestion were purified by agarose gel electrophoresis followed by the FastGene Gel/PCR Extraction Kit (Nippon Genetics). Restriction enzymes were purchased from New England Biolabs, and the ligase was purchased from TOYOBO (ligation high version 2) and used as recommended by the manufacturer. Plasmids were prepared from the bacterial liquid culture by using either the Fast-n-Easy Plasmid Mini-Prep Kit (Jena Bioscience) or NucleoBond Xtra Midi Plus (Machery-Nagel). The DNA sequences were read by dye terminator cycle sequencing using the Prep Kit (Jena Bioscience) or NucleoBond Xtra Midi Plus (Machery-Nagel). The DNA oligonucleotides used for gene cloning and construction were PCR-amplified, and cDNA of Venus in pcDNA3-NYL was replaced by the PCR products. Actual amino acid sequences used in this study were mTurquoise2 (1–222 aa), mKusabiraOrange2 (1–218 aa), LSS-mOrange (1–226 aa for FL and 1–226 aa for LcM10), mEmiRed (1–218 aa), tdTomato (1–221 aa), mCherry (1–226 aa). TurboFP635 (1–222 aa), mKatu2 (1–222 aa), mOrange2 (1–226 aa), TagRFP (1–225 aa), mRuby2 (1–225 aa), and TurboFP650 (1–224 aa). The cDNA of TurboFP650 was generated by PCR-based mutagenesis using cDNA of TurboFP635 as a PCR template. For pRLuc8.6- and pRLuc8.6-based BRET constructs, cDNA of pRLuc8.6 and pRLuc8.6-S542N3, respectively, was generated by PCR-based mutagenesis using cDNA of RLuc8 as a PCR template, and cDNA of RLuc8-257GaN3 in pCDNA3-YNL was replaced by these PCR products.

Construction of Gene Expression Reporter Genes and Ca²⁺ Indicators. We used a bright red-excitable fluorescent protein, mCherry (to A.T.), 24659092 (to Y.O.), 25113723 (to Y.O.), and 25293046 (to Y.O.); part of this work was supported by KAKENHI Grant-in-Aid for Scientific Research (Young Scientists) 25871128 (to A.T.), 24659092 (to Y.O.), 25113723 (to Y.O.), and 25293046 (to Y.O.); Riken Incentive Research Grant (to A.T.); Ministry of Education, Science, Sports and Culture Grants-in-Aid for Scientific Research on Innovative Areas “Spinning minority in biological phenomena (no. 3309)” 21517721 (to Y.O.) and 23115003 (to T.N.); and the Strategic Programs for R&D (President’s Discretionary Fund) of RIKEN (Y.O.). Precursory Research for Embryonic Science and Technology (T.N.), and Development of Systems and Technology for Advanced Measurement and Analysis (T.N.) from Japan Science and Technology Agency.