Bioluminescent system for dynamic imaging of cell and animal behavior

Chikako Hara-Miyauchi, Osahiko Tsuji, Aki Hanyu, Seiji Okada, Akimasa Yasuda, Takashi Fukano, Chihiro Akazawa, Masaya Nakamura, Takeshi Imamura, Yumi Matsuzaki, Hirotaka James Okano, Takashi Fukanob, Chihiro Akazawa, Masaya Nakamura, Takeshi Imamura, Yumi Matsuzaki, Hirotaka James Okano

1. Introduction

In live imaging, fluorescent proteins such as Aequorea GFPs and GFP-like proteins are widely used [1,2]. Recently, however, chemiluminescent proteins have also shown promise as powerful tools for biological imaging [3–5]. The significant advantages of bioluminescence over fluorescence imaging include a low background signal and the resultant preservation of delicate subcellular organelles and structures during long term imaging protocols. However, because of their weak luminescence, it is difficult to use chemiluminescent proteins for optical imaging of cultured living cells or in freely moving animals. With chemical and genetic modifications of either proteins or substrates brighter and more diverse colors of bioluminescent probes continue to be developed [6–9], including the recently released NanoLuc (Promega). In addition, radiationless energy transfer can improve bioluminescent probes, i.e., if a chemiluminescent probe has a high emissive rate, its excited-state energy can be used to induce the emission of an appropriately positioned fluorescent protein. For example, coupling a green-emitting variant of Aequorea GFP to the calcium-sensitive photo-protein aequorin (derived from Aequorea victoria) improves its light emission properties. This protein, GFP-aequorin, has been used to perform in vivo bioluminescence imaging of calcium signaling in the brain of Drosophila [10]. Also, a yellow-emitting variant of Aequorea GFP (EFYP) and the Renilla luciferase have been concatenated to promote energy transfer from luciferase-bound oxyluciferin to EFYP’s chromophore [11]. However, even these new variants do not approach the temporal resolution needed for adequate live imaging.

The fusion of a fluorescent protein to a chemiluminescent protein may cause other effects, such as changes in stability or enzymatic activity that could in turn improve photon yield. In this study, we explored the effect of these modifications by concatenating firefly luciferase (Luc2, Promega) with a yellow-emitting variant of Aequorea GFP. The resulting chimERIC protein, fLuc-cp156, showed greatly improved performance in bioluminescence imaging, allowing us to observe cultured cells and transgenic mice with a high spatiotemporal resolution over extended periods of time.

Corresponding authors. Addresses: Department of Physiology, Keio University School of Medicine, Tokyo 160-8582, Japan
Laboratory for Cell Function Dynamics, Brain Science Institute, RIKEN, Saitama 351-0198, Japan
Department of Biophysics and Biochemistry, Graduate School of Health Care Sciences, Tokyo Medical and Dental University, Tokyo 113-8510, Japan
Department of Orthopedic Surgery, Keio University School of Medicine, Tokyo 160-8582, Japan
Division of Biotechnology, The Institute of Chemical Research, Kyoto University, Kyoto 611-0011, Japan
Department of Advanced Medical Initiatives, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8808, Japan
Department of Molecular Medicine for Pathogenesis, Ehime University Graduate School of Medicine, Toon, Ehime 791-0295, Japan
Core Research for Evolutional Science and Technology, The Japan Science and Technology Corporation, Tokyo 135-8550, Japan
Division of Regenerative Medicine Riken Keio University Joint Research Laboratory, Brain Science Institute, RIKEN, Saitama 351-0198, Japan
Life Function and Dynamics, ERATO, JST, 2-1 Hiroawa, Wako-city, Saitama 351-0198, Japan
RIKEN Keio University Joint Research Laboratory, Brain Science Institute, RIKEN, Saitama 351-0198, Japan

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ABSTRACT

The current utility of bioluminescence imaging is constrained by a low photon yield that limits temporal sensitivity. Here, we describe an imaging method that uses a chemiluminescent/fluorescent protein, fLuc-cp156, which consists of a yellow variant of Aequorea GFP and firefly luciferase. We report an improvement in photon yield by over three orders of magnitude over current bioluminescent systems. We imaged cellular movement at high resolution including neuronal growth cones and microglial cell protrusions. Transgenic fLuc-cp156 mice enabled video-rate bioluminescence imaging of freely moving animals, which may provide a reliable assay for drug distribution in behaving animals for pre-clinical studies.

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without any excitation light. Since the spectrum of Luc2’s emission does not overlap with the spectrum of Venus’s absorption, there was no radiationless energy transfer between them. However, the new probe created by the concatenation of Luc2 and the circularly permuted Venus appears to have enhanced stability or enzymatic activity.

2. Material and methods

2.1. Construction of ffluc

To generate the ffluc construct, we inserted a restriction site (GAATT, EcoRI) between variants of Venus and firefly luciferase (Luc2). The deletion mutant of Venus lacked 11 C-terminal flexible amino acids; the circularly permuted Venus had been interchanged and reconnected by a short spacer between the original termini [12].

2.2. Measurement of bioluminescent spectra

The emission spectrum of each ffluc construct, expressed in HEK293T cells, was measured using a spectroscopic photometer (F4500, Hitachi).

2.3. Relative intensities of the ffluc bioluminescence

Bioluminescent signals of ffluc-expressing HEK293T cells were measured by photon counting in a Kronos instrument (ATTO). The signal intensity was normalized to cell number.

2.4. Bioluminescence imaging of cultured cells expressing ffluc-cp156

Transfection was performed using Lipofectamine 2000 transfection reagent; 4 μg of plasmid DNA was used for cells in a 35 mm glass bottom dish. Transfected HeLa cells, microglial cells, or neurons were observed using an inverted microscope (Olympus IX71) with a 60× objective lens (N.A. 1.45), a 0.5× TV lens, and a back-illuminated cooled CCD camera (Cascade512B, Roper Scientific). The microglial cells and neurons were prepared from primary cultures of rat brain. Prior to imaging, luciferin solution (Promega, Beetle luciferin potassium salt) was added to the culture medium at a final concentration of 1 mM.

2.5. Animal experiments

The experimental procedures and housing conditions for animals were approved by the Institute’s Animal Experiments Committee and all the animals were cared for and treated humanely in accordance with the Institutional Guidelines for Experiments using Animals.

2.6. Bioluminescence imaging of mice using a digital camera

A luciferin solution (150 mg/kg) was administered intraperitoneally to a CAG-flLuc-cp156 transgenic mouse (5 months old). Images were taken in a dark room using a common digital camera (E-330, Olympus) with a 4 s exposure time, 3.5 F value, 1600 ISO sensitivity, and 29.0 mm focal length.

2.7. Bioluminescence imaging of anesthetized CAG-flLuc-cp156 transgenic mice

CAG-flLuc-cp156 transgenic mice were anesthetized with a mixture of isoflurane and oxygen. The mice received luciferin (150 mg/kg BW) intravenously, intraperitoneally, or orally. Immediately after luciferin administration, time-lapse or consecutive bioluminescence imaging was initiated, using the IVIS Imaging System 100 Series (Xenogen, Alameda, CA).

2.8. Bioluminescence imaging of freely moving CAG-flLuc-cp156 transgenic mice

Images of moving mice were taken using an in vivo imaging system (LumazoneFA, Roper Japan) with a Cascade 512B cooled CCD camera (Roper Scientific). Three administration methods were attempted. First, luciferin (150 mg/kg) was administered to a mouse intraperitoneally. Bioluminescent (BL) images were consecutively collected every 33 ms. Second, luciferin was orally administered voluntarily, as follows: a mouse was water-restricted for several hours, then placed in the system along with 1.5% low-melting agarose that contained luciferin (0.75 mg/mL). BL images (500 ms exposure time) and fluorescent (FL) images (100 ms exposure time) were acquired in alternation; paired BL/FL images were acquired every 5 s. Finally, luciferin was administered percutaneously: a mouse with a depilated back was placed in the system, and vaseline containing 50 mg/g of luciferin powder was applied to its back BL images (1 s exposure time) and FL images (100 ms exposure time) were acquired in alternation; paired BL/FL images were acquired every 5 s.

2.9. Visualization of CAG-flLuc-cp156 embryos in the mother mouse

A wild-type female mouse was crossed with a CAG-flLuc-cp156 transgenic male mouse (homozygous). The pregnant mouse was given an intraperitoneal injection of luciferin (150 mg/kg BW); bioluminescence and fluorescence images were captured by a cooled CCD camera (PIXIS-2048B, Roper Industries) with a lens of 50 mm focal length (EF50 mm, F1.2L USM, Canon). To take the fluorescence images, the mother mouse was illuminated with LEDs at 470 nm (Luxeon K2 LXX2-PB14-Q00, Lumileds), and the fluorescence from the sample was detected via a band-pass filter (ET535/30nm, Chroma Technology).

3. Results and discussion

3.1. Concatenation of Luc2 and Venus to yield ffluc

A modified North American firefly Photinus pyralis luciferase, codon-optimized for expression in human cells (Luc2, Promega), was fused with Venus variants containing either a deletion or a series of circular permutations [12] to create flLuc-del or flLuc-cps, respectively (Fig. 1A). After transfection into cultured cells, the bioluminescent spectra of the constructs were measured with a spectrophotometer. The new flLuc constructs showed nearly the same emission spectra, peaking at 620 nm as Luc2 (Fig. 1B). However, the bioluminescence intensities were higher in cells expressing flLuc constructs than in cells expressing Luc2. When quantitated using a photon-counting device (Kronos, ATTO), cell samples containing flLuc-cp156 were several times brighter than samples containing Luc2 (Fig. 1C). Normalization to protein expression level revealed that bioluminescent activity per molecule is similar between flLucs and Luc2 (Supplementary Fig. S1 online), but flLucs provided reliably brighter signals from both fluorescence and bioluminescence. flLuc-cp156 was used in further studies.

3.2. Visualization of bioluminescence in single cells using flLuc-cp156

flLuc-cp156 was transfected into cultured HeLa cells. After the addition of luciferin (1 mM), we observed bioluminescent signals from single cells using a standard fluorescence microscope system equipped with an oil-immersion objective lens and a
back-illuminated EM tip-equipped CCD camera. We first adjusted
the focus on cells using phase contrast (PC) images (Fig. 2A), and
then selected transfected cells using fluorescence images (Fig. 2B).
Next, a 500 ms exposure of the camera yielded a bioluminescence
image wherein individual transfected cells were sufficiently re-
solved (Fig. 2C). When the camera exposure was extended to
5 s, the image quality was greatly improved (Fig. 2D). Further-
more, use of ffLuc-cp156 enabled us to capture a series of biolu-
minescence images of actively migrating microglial cells (Fig. 2E;
Supplementary Movie 1 online) and extending growth cones of
rat hippocampal neurons (Fig. 2F; Supplementary Movie 2 online)
over several consecutive hours. Although the exposure time could
be shortened to a few seconds while preserving image quality, we
adopted a 30 s exposure as a standard, in order to limit the total
amount of data collected over long-term imaging experiments.

Bioluminescent signals from cultured cells were sustained for
>2 days after the initial addition of luciferin, demonstrating the
possibility of long-term in vivo observation of cell proliferation
and/or differentiation.

3.3. Strong bioluminescence from CAG-ffLuc-cp156 transgenic mice

To examine the utility of ffLuc-cp156 in living animals, we gen-
erated transgenic mice using a construct that consisted of a CAG
promoter-driven combination cassette bearing a neomycin-coding
sequence sandwiched by two loxP sequences and the ffLuc-cp156
gene (Fig. 3A). We cross-bred the line carrying the reporter gene
(CAG-neo<sup>inlp</sup>/loxP-ffLuc) with a line ubiquitously expressing Cre
recombinase (CAG-Cre mice) [13], to yield a mouse line ubiqui-
tously producing ffLuc-cp156. When illuminated with blue light,
the CAG-ffLuc-cp156 mice emitted yellow fluorescence. After dis-
section, we investigated the fluorescence of various organs and tis-
sues using a fluorescence stereomicroscope. Fluorescence from
Venus was observed in almost all tissues examined, with the

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**Fig. 1.** ffLuc constructs. (A) ffLuc was constructed as a fusion of a variant of Venus (a deletion mutant or a circularly permuted variant: cp144, cp156, cp172, and cp194) and humanized firefly luciferase (Luc2). GAATTC, the restriction site (EcoRI). (B) Bioluminescent spectra of transfected cells. (C) Relative intensities of bioluminescence signals from cells expressing ffLuc-cp156, ffLuc-del, and Luc2.

**Fig. 2.** Bioluminescence imaging of ffLuc-cp156 at the single-cell level. (A–D) Bioluminescence images of ffLuc-cp156 expressing HeLa cells. Images were acquired using an inverted microscope (Olympus IX71) (A, phase contrast image; B, fluorescence image; C, bioluminescence image with 500 ms camera exposure; D, bioluminescence image with 5 s camera exposure). [E and F] Bioluminescence images of a cultured microglial cell (E) and neuronal growth cones (F, red arrow). Images were acquired with 30 s camera exposure. Scale bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
exception of spleen and red blood cells (Supplementary Fig. S2 online).

We examined the bioluminescence from an anesthetized CAG-ffLuc-cp156 transgenic mouse (5 months old) in the dark box of an IVIS system (Xenogen). Within 1 s after administration of luciferin (150 mg/kg BW) through the tail vein, extremely strong bioluminescence arose throughout the body, lasting for >6 h. Even with the shortest exposure time of the system (500 ms), the bioluminescent intensity exceeded the camera's saturation level. Next, we performed intraperitoneal administration of luciferin (150 mg/kg) on another anesthetized mouse (5 months old). Although rapid and strong bioluminescence developed, we acquired a bioluminescence image within the working range of the system by placing the mouse as far as possible from the objective (Fig. 3B). Bioluminescence was visible to the dark-adapted naked eye, and the image could be acquired using an ordinary digital camera (E-330, Olympus) (Fig. 3C). Considering the imaging parameters of IVIS system described in previous reports, these observations suggested that the overall bioluminescence of the CAG-ffLuc-cp156 transgenic mouse was over three orders of magnitude brighter than that of transgenic mice that expressed firefly luciferase (ffLuc) under the control of the promoter/enhancer of the major immediate-early gene of the human cytomegalovirus (CMV-Luc) [14].

We also monitored the development of bioluminescence after oral administration of luciferin to an anesthetized mouse. As soon as a luciferin solution was injected into the mouth, strong bioluminescence was observed from the stomach (Fig. 3D, 0 min). Initially (<3 min), the stomach and intestine emitted stronger luminescence than other body parts (Fig. 3D, 2 min). Subsequently, other tissues in the trunk and limbs gradually became bioluminescent (Fig. 3D, 10 min). The spatiotemporal patterns of the spreading bioluminescence are shown in Supplementary Movie 3 online. After reaching saturation levels, these bioluminescent signals were detectable for about 6 h.

3.4. Bioluminescence imaging of freely moving CAG-ffLuc-cp156 transgenic mice

Next, we performed bioluminescence imaging experiments using awake mice. We used the in vivo imaging system (LumazineFA, Roper Japan), which allowed much shorter camera
exposures. After intraperitoneal administration of luciferin (150 mg/kg BW), we successfully visualized bioluminescence in a freely moving CAG-ffLuc-cp156 mouse at video-rate (33 ms exposure time) (Fig. 3E; Supplementary Movie 4 online). The bioluminescence from the mouse’s body was bright enough to visibly illuminate the inside of the box.

We next studied oral administration of luciferin. A thirsty mouse was placed in a dark box containing wet agarose containing luciferin. After the mouse chewed the agarose, both sides of the abdomen began glowing (Fig. 3F; Supplementary Movie 5 online). Using CMV-Luc, Hiler et al. showed that voluntary drinking of luciferin resulted in a similar spatial pattern of luminescence to that was achieved by intravenous luciferin administration [15]. In comparison to this earlier study, CAG-ffLuc-cp156 mice enabled immediate detection of drinking and analysis of the rapid kinetics of absorption which may allow its use in drug distribution studies in awake animals.

Finally, we examined whether we could visualize luciferin penetration through the skin. Immediately after attachment of a luciferin-containing Vaseline poultice to the back, the skin started to emit bioluminescent signals (Fig. 3G; Supplementary Movie 6 online). The luminescent signals appeared first as dots, and then expanded to cover the whole area of treated skin. These signals remained on the body surface during the observation time period (~1 h), despite the ubiquitous expression of ffluc-cp156, suggesting that the luciferin did not penetrate deeper into the body.

It is possible to administer luciferin in different ways to CAG-ffLuc-cp156 transgenic mice, which ubiquitously express ffluc-cp156, in order to observe spatiotemporal patterns in the development of bioluminescence, i.e., the build-up of oxyluciferin. We believe that these approaches will provide reliable readouts of absorption and infiltration of drugs in conscious animals. Important applications would include real-time analysis of uptake and bioactivatable cleavage of luciferin-transporter conjugates [16]. It should be noticed that one shot of luciferin (150 mg/kg BW) caused intense and long-lasting (>4 h) bioluminescent signals. Furthermore, the CAG-ffLuc-cp156 mice were tolerant to repeated bioluminescence imaging experiments; one mouse continued to look healthy after being subjected to three consecutive experiments over a few days. It is thus unlikely that high levels of biophotonic activity have grave, negative effects on animal physiology [17].

3.5. Labeling CAG-ffLuc-cp156 embryos in pregnant mice

The strong bioluminescence from ffluc-cp156 in transgenic mouse embryos in utero could be detected outside their mother’s body. We crossed a wild-type female mouse with a CAG-ffLuc-cp156 transgenic male mouse carrying two copies of the ffluc-cp156 gene. Luciferin was administered intraperitoneally (150 mg/kg BW) to the mother mouse at a late stage of pregnancy. The heterozygous embryos could be clearly identified as discrete objects with low background in bioluminescence images, although individual embryos were not fully resolved (Fig. 4, BL). By contrast, these embryos were not identifiable in fluorescence images (Fig. 4, FL). Thus, our bioluminescence imaging system can be used to non-invasively monitor gestation states.

3.6. Neuronal stem/precursor cells from CAG-ffLuc-cp156 transgenic mice

We investigated whether bright cell populations could be obtained from CAG-ffLuc-cp156 transgenic mice. We prepared neuronal stem/precursor cells (NS/PCs) from CAG-ffLuc-cp156 transgenic mice according to established methods [18] and counted photons per second (photon/s) emitted from cultured cells using the IVIS system. The photon/s measurements increased linearly with the number of cells (Supplementary Fig. S3A online); $1 \times 10^4$ cells yielded approximately $1 \times 10^8$ photon/s. As a comparison, we performed the same NS/PC preparation and quantification using the widely-used transgenic mouse line, CMV-Luc, in which fLuc is expressed ubiquitously [15]; in this case, $1 \times 10^6$ cells from CMV-Luc mice produced only $7 \times 10^4$ photon/s (Supplementary Fig. S3B online). Thus, NS/PCs from CAG-ffLuc-cp156 mice produce >10,000 times more photons per unit time than those from CMV-Luc mice.

NS/PCs can be maintained in vitro as cell aggregates, called neurospheres that can be used for cell transplantation experiments and therapies. To assess the utility of bioluminescence in a transplantation preparation, we prepared neurospheres from CAG-ffLuc-cp156 mice and verified the even distribution of Venus fluorescence in each sphere (Supplementary Fig. S4A online). We then transplanted the cells into the injured spinal cord of a wild-type mouse. In situ survival of the labeled cells was verified by bioluminescence imaging 1 and 2 weeks after transplantation (Supplementary Fig. S4B and C online).

3.7. Visualization of the neural crest lineage-specific expression of ffluc-cp156

Via genetic manipulation, it is possible to express ffluc-cp156 in specific cell-types or tissues. To visualize the neural crest-derived cells, the ffluc-cp156 reporter mice were crossed with transgenic mice expressing Cre recombinase under the control of either the Wnt1 promoter/enhancer [19] or the P0 promoter [20] to yield Wnt1/ffLuc-cp156 (Supplementary Fig. SSA online) and P0/ffLuc-cp156 (Supplementary Fig. SSB online), respectively. After intraperitoneal administration of luciferin (150 mg/kg BW), bioluminescent signals were observed exclusively in the neural crest-derived cells of neonatal mice, mostly in the cephalic region, reflecting the endogenous gene expression of pattern of Wnt1 and P0 genes [21]. These results highlight the effectiveness of ffluc-cp156 reporter mice for imaging specific cell populations in intact awake animals that may be bred with different Cre transgenic mouse lines to achieve cell type, tissue-specific visualization of bioluminescence and fluorescence.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.141.

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