Recent progress in luminescent proteins development

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Abstract

Bioimaging requires not only high sensitivity but also minimal invasiveness. Bioimaging using luminescent proteins is potentially free from problems such as photo-induced damage or an undesirable physical reaction to the sample, which are often caused by illumination with an external light required in fluorescence imaging. The recent development of several luminescent proteins and substrates have greatly improved the brightness of luminescence imaging, facilitating its application by many researchers. In this short review, we summarize recent advances in development of luminescent proteins, substrates, and indicators.

1. Introduction

Advances in fluorescent probe technologies such as fluorescent proteins have allowed visualization of functional processes inside animals and plants. In addition, advances in microscopic methods such as multi-photon microscopy and super-resolution microscopy have contributed to improvements in live imaging. However, these fluorescence-based imaging techniques can potentially cause photo-induced damage and auto-fluorescence of samples; these problems remain unsolved. Bioluminescence or luminescence imaging techniques utilize the protein luciferase, which catalyzes the oxidation of luminescent chemicals such as luciferin, to produce light. Thus, luminescence imaging does not require external light for excitation, unlike fluorescence phenomena (Fig. 1), eliminating problems such as photo-induced damage and auto-fluorescence of samples. Despite its advantages, luminescence imaging has not frequently been used for live imaging because of its dimness. However, recent advances in luminescent proteins, their substrates, and other techniques have changed this situation. In this review, we introduce recently developed luminescent proteins, luminescent substrates, and luminescence-based functional indicators.

2. Luminescent proteins

To overcome the problem of dimness in luminescent imaging, various kinds of bright luminescent proteins have been developed (Table 1). One recently developed bright luminescent protein, Nano-lantern, employs Förster resonance energy transfer (FRET) to enhance light output [1]. FRET naturally occurs inside the sea pansy, *Renilla reniformis*, from the dim luminescent protein *Renilla* luciferase (RLuc) to the bright fluorescent protein *Renilla* green fluorescent protein (*Renilla* GFP). The probability of photon emission from *Renilla* GFP is 5.7-fold as high as that from RLuc, according to the difference in their quantum yields (Φ) for light emission ($\Phi = 0.053$ and $= 0.30$, respectively). As a result of approximately 100% FRET from RLuc to *Renilla* GFP inside the sea pansy, the Φ of the RLuc-*Renilla* GFP complex increases 5.7-fold [2]. In the case of Nano-lantern, RLuc8 [3] with an S257G mutation and Venus yellow fluorescent protein [4] were utilized as the FRET donor and acceptor, respectively. Nano-lantern exhibited 5-fold greater light output than RLuc8, and 3-fold greater light output than BAF-Y [5], the brightness of which is also enhanced by FRET. The brightness of Nano-lantern allowed visualization of the fine structure of living organelles using only a short exposure

time (1–3 s). Furthermore, Nano-lantern enabled video-rate imaging of tumors in a freely moving, unshaved mouse [1]. Color variants of Nano-lantern, Cyan-Nano-lantern and Orange-Nano-lantern, were developed by using mTurquoise2 [6] and mKO2 [7] as FRET acceptors, respectively; using these proteins, inhomogeneous expression of multiple pluripotency markers in a single colony of embryonic stem cells was successfully visualized [8].

In *ffLuc2-cp156*, firefly luciferase (FLuc) was fused with a circularly permuted variant of Venus (cp156) [9], not for enhancement of light output by FRET, but rather for enhancement of protein stability and enzymatic activity. Indeed, transgenic *ffLuc2-cp156* mice exhibited light output that was more than three orders of magnitude greater than that of transgenic FLuc mice. The whole-body luminescence signal from transgenic *ffLuc2-cp156* mice could be visualized at video-rate by intraperitoneal administration of D-luciferin without anesthetization.

Artificial luciferases (ALucs) were developed by linking the key amino acids that appeared most frequently in the luciferase proteins of 13 copepods [10]. The resulting sequences differed greatly from the original 13 copepod luciferase proteins. Some of the ALucs exhibited high thermostability, and some exhibited approximately 50-fold higher light output than *Gaussia* luciferase (GLuc) [11] or RLuc8.6-535 [12]. ALucs can be utilized for various types of bioassays such as mammalian two-hybrid assays, live-cell imaging, and in applications requiring split luciferase-based probes [13].

NanoLuc (NLuc) was developed from the deep-sea shrimp *Oplophorus gracilirostris*, with 16 amino acid mutations. NLuc exhibits 150-fold brighter luminescence than RLuc or FLuc. In addition, a novel coelenterazine analogue specially customized for NLuc, furimazine, was developed. When paired with furimazine, NLuc exhibited a 30-fold brighter luminescent signal than when paired with coelenterazine. Because of its small size (19 kDa) and its brightness, NLuc has been used to report the dynamics of influenza viral infection in living mice [14].

Recently, the smallest luminescent protein, MLuc7, was cloned [15]. MLuc7 is a non-allelic isoform of *Metridia longa* luciferase. Its molecular weight is only 16.5 kDa because its sequence lacks a variable N-terminus region. Although all other copepod luciferases contain a variable N-terminus region, it is not thought to be essential for luciferase activity, as deletion of the region can increase luciferase activity [16].

3. Luminescent protein-based functional indicators

FLuc catalyzes adenylation reaction of D-luciferin, which consumes one ATP for production of one D-luciferyl adenylate that is further oxidized to yield an oxyluciferin and a photon. The emitted photon number is positively correlated with the ATP concentration. Therefore, FLuc has been used as the indicator for cellular ATP level [17]. On the other hand, RLuc doesn't require ATP to catalyze oxidation reaction of its substrate. Because of this simple catalytic reaction of RLuc, RLuc is preferable to FLuc for the application of functional sensor.

Because of the advantages of luminescence imaging compared to fluorescence imaging, several luminescent protein-based functional indicators have been developed in recent years (Table 1). BRAC is a FRET-based Ca^{2+} indicator [18] similar to the fluorescent protein-based Ca^{2+} indicator, yellowameleon [19,20]. It is composed of RLuc8 (the FRET donor), Venus (the FRET acceptor), and calmodulin linked with M13 peptide (the sensor domain for Ca^{2+}). BRAC changes its emission wavelength upon Ca^{2+} binding, and thus BRAC was able to visualize Ca^{2+} signaling in *Arabidopsis* plant leaves upon virus infection; this phenomenon cannot be visualized using fluorescence indicators, as excitation light causes physiological signaling and photo-induced damage to the plant leaves.

Functional indicators for Ca^{2+} cyclic adenosine monophosphate (cAMP) and adenosine 5'-triphosphate (ATP) were developed based on Nano-lantern [1]. Nano-lantern-based indicators employ split-luciferase technology. In the case of the Ca^{2+} indicator Nano-lantern (Ca^{2+}), a Ca^{2+} sensor domain composed of calmodulin and M13 peptide was inserted between Gly228 and Gly229 of RLuc8-S257G. Nano-lantern (Ca^{2+}) displayed a 300% signal increase upon changing the Ca^{2+} concentration from zero to saturation. Nano-lantern (Ca^{2+}) is sufficiently bright to visualize cytoplasmic Ca^{2+} signaling at the single-cell level, such as histamine-induced Ca^{2+} oscillations at video-rate in a HeLa cell and spontaneous Ca^{2+} spikes in a dissociated rat hippocampal neuron [1]. In theory, luminescent indicators can be used with optogenetic tools such as channelrhodopsin2 (ChR2) [21], which are incompatible with fluorescent indicators because the excitation light required for fluorescent indicators can produce undesired activation of optogenetic tools. This is thought to be one advantage of luminescent indicators over fluorescent indicators. Indeed, Nano-lantern (Ca^{2+}) can be used to monitor the increase in cytoplasmic Ca^{2+} upon ChR2 stimulation without any undesired effect on ChR2. Nano-lantern (Ca^{2+}) has also been used to detect very small changes in cytoplasmic Ca^{2+} in human aorta primary smooth muscle cells (HuAoSMCs) upon nicotine treatment, to detect phenotypic changes that could not be detected using a fluorescent Ca^{2+} indicator [22].

The luminescent pH indicator, photo-inactivatable luciferase (PI-Luc), indicates the cytoplasmic pH via a different mechanism than the above-mentioned luminescent indicators [23]. PI-Luc is composed of the LOV2 domain from *Avena sativa* Phototropin1, the N-terminal fragment of FLuc, and the C-terminal fragment of click beetle luciferase, McLuc1. In the dark, PI-Luc can catalyze the oxidation reaction of D-luciferin. Upon blue light irradiation, PI-Luc can be inactivated by light-dependent conformational changes of LOV2. Once the light is turned off, the conformation of LOV2 returns to the dark state, and PI-Luc recovers its enzymatic activity. The recovery time for the conformation of the LOV2 domain is known to be highly sensitive to pH. To utilize this property, PI-Luc can monitor the intracellular pH by measuring the

recovery half time of its enzymatic activity after inactivation by irradiation with blue light. PI-Luc was used to visualize the acidification of autophagosomes by fusion with lysosomes in living HEK293 cells and acidification of the footpad of living mice in low-oxygen conditions following ischemia [23].

At the end of this section, it is better to clarify a simple question “Can we make better imaging tools such as Nano-lantern and/or functional sensors by swapping dim luciferase for brighter luciferase, NanoLuc? The answer is no. The ~150-fold photon output of NanoLuc than that of RLuc might be due to improvement of not only catalytic turnover but also Φ . If the Φ for light emission in NanoLuc is high enough, it might be difficult to further enhance the brightness by efficient FRET as Nano-lantern was done. Indeed, NanoLuc-GFP fusion protein with ~100% FRET efficiency which we made showed only 1.5 times as bright as NanoLuc (unpublished data).

4. Luminescent substrates

Advances in luminescence imaging techniques have been achieved by improvement and development not only of luminescent proteins but also of luminescent substrates (Table 2). CycLucs are synthetic aminoluciferin analogues, which can generate light through oxidation by FLuc [24-26]. Surprisingly, one of these, CycLuc1, can produce the same light output as FLuc-expressing mouse xenograft tumor cells, using a 200-fold lower dose than D-luciferin, because of its high cell permeability and lower K_m . (Michaelis-Menten constant) Another aminoluciferin analogue, CycLuc10, exhibits red-shifted emission peaks at 640 nm.

Firefly luciferin analogues that emit far-red to near-infrared light have also been reported [27]. One of these analogues exhibits a luminescence wavelength peak at 706 nm when coupled with one of the FLuc variants, x5 FLuc S284T [28].

A newly synthesized coelenterazine analogue, CoelPhos, is the first cell-membrane-“impermeable” coelenterazine, which was achieved by alkylating coelenterazine with an alkyl linker containing a terminal phosphonate group [29]. CoelPhos proved to be suitable for the visualization of an outer cell membrane-bound GLuc variant (GLucM23). However, almost no signal could be observed from ER-retained GLuc with CoelPhos.

5. Luminescent substrate-based functional indicators

Another challenge for development of luminescent substrates is the generation of functional indicators. The first caged D-luciferins were developed in 1987 for the establishment of highly sensitive enzyme-based immunoassays, as alternatives to radioimmunoassays [30]. One of the well-established caged D-luciferin substrates is Lugal (1-*O*-galactopyranosyl-luciferin), which contains D-luciferin conjugated with β -galactose (β -gal) [31]. Lugal cannot be oxidized with FLuc until it has undergone a cleavage reaction with β -galactosidase. As a result of this reaction, Lugal releases D-luciferin, which can generate light by reacting with FLuc. Because luminescence signals from Lugal are dependent on β -gal activity, Lugal allowed visualization of muscle regeneration in living mice, via inducible β -gal activity under the control of the Myf5 promoter, which is known to be activated in myoblasts in repairing the muscle after injury. In recent years, coelenterazine- β -galactose conjugates, bGalCoel and bGalNoCoel, were also developed [32]. bGalNoCoel could detect β -gal activity in living cells expressing GLuc.

Split caged D-luciferin can be used to detect two different biochemical processes [33]. Peroxy-caged luciferin-2 (PCL-2) releases 6-hydroxy-2-cyanobenzothianole (HCBT) upon reacting with H_2O_2 . z-Ile-Glu-Thr-Asp-D-Cys (IETDC) releases D-cysteine upon cleavage by caspase-8. Released HCBT and D-cysteine form D-luciferin *in situ*. Because the luminescence signals are dependent on the above two biochemical processes, this system can be used for detection of both H_2O_2 and caspase-8 activities. Using this system, endogenous H_2O_2 and caspase-8 activities were detected in living mice during acute inflammation upon lipopolysaccharide stimulation [33].

6. Other techniques related to luminescence

There are other techniques related to luminescence observation. A microscopy system specialized for luminescence imaging was developed to increase the brightness of luminescence images [34]. The degree of brightness (I) of a luminescence image is proportional to $(NA/M)^2$, where NA indicates the numerical aperture of the objective lens and M indicates the magnification, which is calculated by the ratio of the focal lengths of the imaging lens (F_i) and the objective lens (F_o), $M = F_i/F_o$. Objective lenses with a higher NA usually have a shorter focal length. However, M can easily be reduced by using an imaging lens with a short focal length. Conventional microscopes with a short-focal imaging lens instead of a long-focal imaging lens have newly been customized for luminescence imaging. This imaging system enabled capture of images of organelles in living cells using NLuc, which is compatible with image capture using fluorescent proteins.

A novel hybrid strategy, bioluminescence-assisted switching and fluorescence imaging (BASFI), was developed to identify protein-protein interactions [35]. In BASFI, RLuc8 was utilized as the FRET donor, and the photochromic fluorescent protein, Dronpa, was used as the FRET acceptor. If RLuc8 is in close proximity to Dronpa, RLuc8 can alter the conformation of Dronpa from the dark state to the bright state, via FRET. As fluorescent signals from Dronpa are

dependent on the efficiency of FRET between RLuc8 and Dronpa, BASFI can be used to detect protein–protein interactions of interest. Although intermolecular FRET using fluorescent proteins has the problem of signal bleedthrough from donor to acceptor, BASFI is free of this problem. In addition, BASFI has a great advantage over luminescence-based indicators in its brightness. Using BASFI, rapamycin-induced intermolecular FKBP–FRB interactions were successfully visualized in living HEK293T cells [35].

Light-emitting channelrhodopsins were developed to control neuronal firings not only by external light but also by FRET from nearby luminescent proteins [36]. One of the luminopsins, LMO2, which is composed of GLuc fused with *Volvox* channelrhodopsin1 (VChR1), displayed a higher photo-current than GLuc or the ChR2 fusion protein (LMO1). LMO2 activation upon coelenterazine application successfully induced action potential firing in hippocampal neurons. This technology might be useful to modulate synaptic input in deep brain regions.

7. Conclusion and Perspectives

In summary, many tools have recently been developed for luminescence bioimaging. Researchers can now combine several of these techniques to design various types of experiments. The major limitation of luminescence imaging compared to fluorescence imaging is that it requires continuous supplementation with the luminescent substrate, especially for long-term monitoring. To overcome this drawback, an autonomous bioluminescence expression system has been achieved; however, it can only be used with bacterial luciferase [37]. Application of this system to other luciferases will facilitate wider use of luminescence imaging, similar to fluorescence imaging.

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9. References

1. Saito K, Chang YF, Horikawa K, Hatsugai N, Higuchi Y, Hashida M, Yoshida Y, Matsuda T, Arai Y, Nagai T: **Luminescent proteins for high-speed single-cell and whole-body imaging.** *Nature Communications* 2012, **3**:9.
2. Ward WW, Cormier MJ: **In vitro energy transfer in Renilla bioluminescence.** *J. Phys. Chem.* 1976, **80**:2289-2291.
3. Loening AM, Fenn TD, Wu AM, Gambhir SS: **Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output.** *Prot. Eng. Des. Sel.* 2006, **19**:391-400.
4. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A: **A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications.** *Nat. Biotechnol.* 2002, **20**:87-90.
5. Hoshino H, Nakajima Y, Ohmiya Y: **Luciferase-YFP fusion tag with enhanced emission for single-cell luminescence imaging.** *Nat. Methods* 2007, **4**:637-639.
6. Goedhart J, von Stetten D, Noirclerc-Savoye M, Lelimosin M, Joosen L, Hink MA, van Weeren L, Gadella TWJ, Royant A: **Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%.** *Nature Communications* 2012, **3**:9.
7. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, et al.: **Visualizing spatiotemporal dynamics of multicellular cell-cycle progression.** *Cell* 2008, **132**:487-498.
8. Takai A, Nakano M, Saito K, Haruno R, Watanabe TM, Ohyanagi T, Jin T, Okada Y, Nagai T: **Expanded palette of Nano-lanterns for real-time multicolor luminescence imaging.** *Proceedings of the National Academy of Sciences of the United States of America* 2015, **112**:4352-4356.

**The authors developed color variants of bright luminescent protein Nano-lantern by exchanging its yellow FP moiety to cyan or orange FP, and also synthesized diacetyl coelenterazine-h. With these novel tools, the authors succeeded

- p>
visualization of focal adhesion dynamics for about four hours continuously. In addition, the authors also developed color variants of
- Ca^{2+}
- indicator based on the cyan and orange version of Nano-lantern.
9. Hara-Miyauchi C, Tsuji O, Hanyu A, Okada S, Yasuda A, Fukano T, Akazawa C, Nakamura M, Imamura T, Matsuzaki Y, et al.:
- Bioluminescent system for dynamic imaging of cell and animal behavior**
- .
- Biochemical and Biophysical Research Communications*
- 2012,
- 419**
- :188-193.
10. Kim SB, Torimura M, Tao H:
- Creation of Artificial Luciferases for Bioassays**
- .
- Bioconjugate Chemistry*
- 2013,
- 24**
- :2067-2075.
11. Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO:
- Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo**
- .
- Molecular Therapy*
- 2005,
- 11**
- :435-443.
12. Loening AM, Wu AM, Gambhir SS:
- Red-shifted Renilla reniformis luciferase variants for imaging in living subjects**
- .
- Nature Methods*
- 2007,
- 4**
- :641-643.
13. Ozawa T, Kaihara A, Sato M, Tachihara K, Umezawa Y:
- Split luciferase as an optical probe for detecting protein-protein interactions in mammalian cells based on protein splicing**
- .
- Analytical Chemistry*
- 2001,
- 73**
- :2516-2521.
14. Tran V, Moser LA, Poole DS, Mehle A:
- Highly Sensitive Real-Time In Vivo Imaging of an Influenza Reporter Virus Reveals Dynamics of Replication and Spread**
- .
- Journal of Virology*
- 2013,
- 87**
- :13321-13329.
15. Markova SV, Larionova MD, Burakova LP, Vysotski ES:
- The smallest natural high-active luciferase: Cloning and characterization of novel 16.5-kDa luciferase from copepod Metridia longa**
- .
- Biochem Biophys Res Commun*
- 2015,
- 457**
- :77-82.
16. Markova SV, Burakova LP, Vysotski ES:
- High-active truncated luciferase of copepod Metridia longa**
- .
- Biochemical and Biophysical Research Communications*
- 2012,
- 417**
- :98-103.
17. Bell CJ, Manfredi G, Griffiths EJ, Rutter GA:
- Luciferase expression for ATP imaging: Application to cardiac myocytes**
- .
- Methods Cell Biol*
- 2007,
- 80**
- :341-352.
18. Saito K, Hatsugai N, Horikawa K, Kobayashi K, Matsu-ura T, Mikoshiba K, Nagai T:
- Auto-luminescent genetically-encoded ratiometric indicator for real-time Ca^{2+} imaging at the single cell level**
- .
- Plos One*
- 2010,
- 5**
- :e9935.
19. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY:
- Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin**
- .
- Nature*
- 1997,
- 388**
- :882-887.
20. Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A:
- Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins**
- .
- Proc. Natl. Acad. Sci. U.S.A.*
- 2004,
- 101**
- :10554-10559.
21. Zhang F, Wang L-P, Brauner M, Liewald JF, Kay K, Watzke N, Wood PG, Bamberg E, Nagel G, Gottschalk A, et al.:
- Multimodal fast optical interrogation of neural circuitry**
- .
- Nature*
- 2007,
- 446**
- :633-639.
22. Yoshiyama S, Chen ZY, Okagaki T, Kohama K, Nasu-Kawaharada R, Izumi T, Ohshima N, Nagai T, Nakamura A:
- Nicotine exposure alters human vascular smooth muscle cell phenotype from a contractile to a synthetic type**
- .
- Atherosclerosis*
- 2014,
- 237**
- :464-470.
p>*The authors newly developed a photo-inactivatable bioluminescent sensor, PI-Luc. As the recovery rate from dark-state to bright-state of PI-Luc is highly pH sensitive, it enabled to visualize pH changes in living cells and living mice.
23. Hattori M, Haga S, Takakura H, Ozaki M, Ozawa T:
- Sustained accurate recording of intracellular acidification in living tissues with a photo-controllable bioluminescent protein**
- .
- Proceedings of the National Academy of Sciences of the United States of America*
- 2013,
- 110**
- :9332-9337.

24. Harwood KR, Mofford DM, Reddy GR, Miller SC: **Identification of Mutant Firefly Luciferases that Efficiently Utilize Aminoluciferins.** *Chemistry & Biology* 2011, **18**:1649-1657.
- **The authors developed luciferin analogue, CycLuc1, which improved brightness of whole-body and brain imaging in mice because of its high cell permeability and affinity with luciferases compared to original luciferin.
25. Evans MS, Chaurette JP, Adams ST, Reddy GR, Paley MA, Aronin N, Prescher JA, Miller SC: **A synthetic luciferin improves bioluminescence imaging in live mice.** *Nature Methods* 2014, **11**:393-+.
26. Mofford DM, Reddy GR, Miller SC: **Aminoluciferins Extend Firefly Luciferase Bioluminescence into the Near-Infrared and Can Be Preferred Substrates over D-Luciferin.** *Journal of the American Chemical Society* 2014, **136**:13277-13282.
27. Jathoul AP, Grounds H, Anderson JC, Pule MA: **A Dual-Color Far-Red to Near-Infrared Firefly Luciferin Analogue Designed for Multiparametric Bioluminescence Imaging.** *Angewandte Chemie-International Edition* 2014, **53**:13059-13063.
28. Law GHE, Gandelman OA, Tisi LC, Lowe CR, Murray JAH: **Mutagenesis of solvent-exposed amino acids in Photinus pyralis luciferase improves thermostability and pH-tolerance.** *Biochemical Journal* 2006, **397**:305-312.
- *The authors developed coelenterazine analogue, CoelPhos, which clearly visualized extracellular localized GLuc but not intracellular localized GLuc.
29. Lindberg E, Mizukami S, Ibata K, Fukano T, Miyawaki A, Kikuchi K: **Development of cell-impermeable coelenterazine derivatives.** *Chemical Science* 2013, **4**:4395-4400.
30. Miska W, Geiger R: **SYNTHESIS AND CHARACTERIZATION OF LUCIFERIN DERIVATIVES FOR USE IN BIOLUMINESCENCE ENHANCED ENZYME IMMUNOASSAYS - NEW ULTRASENSITIVE DETECTION SYSTEMS FOR ENZYME IMMUNOASSAYS .1.** *Journal of Clinical Chemistry and Clinical Biochemistry* 1987, **25**:23-30.
31. Geiger R, Schneider E, Wallenfels K, Miska W: **A NEW ULTRASENSITIVE BIOLUMINOGENIC ENZYME SUBSTRATE FOR BETA-GALACTOSIDASE.** *Biological Chemistry Hoppe-Seyler* 1992, **373**:1187-1191.
32. Lindberg E, Mizukami S, Ibata K, Miyawaki A, Kikuchi K: **Development of Luminescent Coelenterazine Derivatives Activatable by beta-Galactosidase for Monitoring Dual Gene Expression.** *Chemistry-a European Journal* 2013, **19**:14970-14976.
- *The authors developed caged-luciferin precursors, PCL-2 and IETDC, which release luciferin precursors upon reacting with H₂O₂ and caspase-8, respectively. As luciferin precursors naturally form luciferin in cells, they successfully detected both H₂O₂ increase and caspase-8 activity in living mice.
33. Van de Bittner GC, Bertozzi CR, Chang CJ: **Strategy for Dual-Analyte Luciferin Imaging: In Vivo Bioluminescence Detection of Hydrogen Peroxide and Caspase Activity in a Murine Model of Acute Inflammation.** *Journal of the American Chemical Society* 2013, **135**:1783-1795.
- *The authors customized optical system of a microscopy, which improves brightness of bioluminescent imaging 5- to 6-fold compared to a conventional microscopy.
34. Ogoh K, Akiyoshi R, May Maw T, Sugiyama T, Dosaka S, Hatta-Ohashi Y, Suzuki H: **Bioluminescence microscopy using a short focal-length imaging lens.** *Journal of Microscopy* 2014, **253**:191-197.
35. Zhang LY, Xu F, Chen ZX, Zhu XX, Min W: **Bioluminescence Assisted Switching and Fluorescence Imaging (BASFI).** *Journal of Physical Chemistry Letters* 2013, **4**:3897-3902.

- 1 36. Berglund K, Birkner E, Augustine GJ, Hochgeschwender U: **Light-Emitting Channelrhodopsins for Combined**
2 **Optogenetic and Chemical-Genetic Control of Neurons.** *Plos One* 2013, **8**:11.
3 **The authors developed chimeric fusion proteins of GLuc and channelrhodopsins, which allowed to modulate neural
4 activity upon coelenterazine treatment.
5 37. Close DM, Patterson SS, Ripp S, Baek SJ, Sanseverino J, Sayler GS: **Autonomous Bioluminescent Expression of**
6 **the Bacterial Luciferase Gene Cassette (lux) in a Mammalian Cell Line.** *Plos One* 2010, **5**:12.

Figure 1

Schematic illustration of Jablonski diagram to explain the difference between fluorescent and chemiluminescent process.

A) A fluorescent dye is excited from ground state (S_0) to excited state (S_1) by external excitation light with proper wavelength. Emission light from S_1 is termed as fluorescence. External excitation light causes some problems such as photo-bleaching, phototoxicity and photo-induced physiological reaction to the sample, which make long-term whole-body live imaging very difficult.

B) $S_0 - S_1$ transition of chemiluminescent substrate occurs by oxidation reaction catalyzed by a luciferase. As this process doesn't need external light for the $S_0 - S_1$ transition, chemiluminescence imaging is free from above problems.

Improvements of chemiluminescent tools can be understood by the Jablonski diagram. Brightness of chemiluminescent proteins can be improved by lower K_m with chemiluminescent substrate, catalytic rate of oxidation, stability. In addition FRET to S_1 of nearby fluorescent molecule can increase quantum yield of light output, if quantum yield at S_1 of the fluorescent molecule is higher than it of chemiluminescent substrate. Properties of chemiluminescent substrate also influence the brightness in living cell such as permeability of cell membrane, stability, lower K_m with luciferase. Beside caged-substrates allow the $S_0 - S_1$ transition of chemiluminescent substrate only in limited situation, that means they can be utilized for functional indicators.

Table 1

Luminescent proteins and luminescent protein-based indicators described in the text

Chemiluminescent protein	Origin/Backbone	Peak emission wavelength, nm	Molecular weight, kDa	Substrates	Reference(s)
RLuc	Luciferase derived from sea pansy <i>Renilla reniformis</i>	480	36	Coelenterazine	-
RLuc8	RLuc with 8 mutations	480	36	"	3,12
BAF-Y	RLuc8, EYFP	530	64	"	5
Nano-lantern	RLuc8 with S257G mutation, Venus	530	62	"	1
Nano-lantern (Ca ²⁺)	Nano-lantern-based Ca ²⁺ indicator, which employs calmodulin and M13 peptide	530	82	"	1
Nano-lantern (cAMP)	Nano-lantern-based cAMP indicator, which employs cAMP binding domain	530	80	"	1
Nano-lantern (ATP1)	Nano-lantern-based ATP indicator, which employs mBSUε	530	76	"	
BRAC	Chemiluminescent Ca ²⁺ indicator utilizing FRET between RLuc8 and Venus, which employs calmodulin and M13 peptide	480 & 530	83	"	17
GLuc	Luciferase derived from copepod <i>Gaussia princeps</i>	480	20	"	11
MLuc7	Non-allelic isoform of <i>Metridia longa</i> Luciferase	490	16.5	"	15
ALuc(s)	Artificial luciferases with a linkage of the extracted key amino acids from copepods' luciferases	500-530	19	"	10
NLuc	Luciferase derived from deep sea shrimp <i>Oplophorus gracilirostris</i> with 16 mutations	460	19	Furimazine	14
FLuc	Luciferase derived from firefly <i>Photinus pyralis</i>	560 (pH >8) & 620 (pH < 5.5)	61	D-Luciferin	-
ffLuc-cp156	FLuc, cp156Venus	620	88	"	9
PI-Luc	Chemiluminescent pH indicator, which is composed of N-terminal of FLuc, LOV-Jα, and C-terminal of McLuc1	~600	-	"	22

Table 2

Luminescent substrates and luminescent substrate-based indicators described in the text

Substrate	Origin/Backbone	Luciferase	Note	Reference(s)
Lugal	D-Luciferin	FLuc	Caged D-luciferin-β-galactose conjugate which can work as D-luciferin after cleavage by β-galactosidase.	30
CycLuc(s)	"	"	CycLuc1: Over 100 times lower dose requirements compared to D-luciferin.	23-25
Far-red to near-infrared firefly luciferin analogues	"	"	CycLuc10: Red-shifted emission peak (640 nm). Up to 706 nm emission with x5 FLuc S284T.	26,27
Split caged D-luciferin	"	"	Peroxy caged luciferin-2 (PCL-2) releases 6-hydroxy-2-cyanobenzothianole (HCBT) upon reacting with H ₂ O ₂ and z-Ile-Glu-Thr-Asp-D-Cys (IETDC) releases D-cystein upon cleavage by caspase-8. Then released HCBT and D-cystein form D-luciferin <i>in situ</i> .	32
CoelPhos	Coelenterazine	GLuc	Cell-membrane impermeable coelenterazine derivative.	28
bGalCoel, bGalNoCoel	"	"	Caged coelenterazine-β-galactose conjugates which can work as coelenterazine after cleavage by β-galactosidase.	31