Bioluminescent Imaging Systems for Assay Developments

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Bioluminescence (BL) is an excellent optical readout platform that has great potential to be utilized in various bioassays and molecular imaging. The advantages of BL-based bioassays include the long dynamic range, minimal background, high signal-to-noise ratios, biocompatibility for use in cell-based assays, no need of external light source for excitation, simplicity in the measurement system, and versatility in the assay design. The recent intensive research in BL has greatly diversified the available luciferase-luciferin systems in the bioassay toolbox. However, the wide variety does not promise their successful utilization in various bioassays as new tools. This is mainly due to complexity and confusion with the diversity, and the unavailability of defined standards. This review is intended to provide an overview of recent basic developments and applications in BL studies, and showcases the bioanalytical utilities. We hope that this review can be used as an instant reference on BL and provides useful guidance for readers in narrowing down their potential options in their own assay designs.

Keywords Bioluminescence (BL), luciferase, luciferin, coelenterazine, probe, imaging, BRET, protein-fragment complementation assay (PCA), assay

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1 Classification of Luminescence

Luminescence is a generic term used for the appearance of a cold body radiation of light. Luminescence comprises several sub types, which include photoluminescence (fluorescence (FL) and phosphorescence), chemiluminescence (CL), and bioluminescence (BL) (Fig. 1). The specific names were coined according to the energy sources (e.g., ultraviolet-visible rays, electrons, X-rays, or chemical energies), properties of emitted light (e.g., phosphorescence and fluorescence), and source of the luminescence (e.g., bioluminescence). These terms can be fragmented into prefix and suffix: i.e., they are prefixed by photo-, phosphor-, chemi-, or bio-, according to the emission types of light; plus, they commonly comprise a suffix “-escence,” which denotes change, action or convalescence process.  

Photoluminescence (especially, fluorescence) and bioluminescence have been broadly utilized in bioassays and molecular imaging. They are mutually complementary and advantageous for visualizing intracellular molecular events in living subjects. The corresponding technologies have rapidly advanced in the past two decades.

A unique feature of FL imaging in comparison to BL is its higher inherent brightness, which makes it possible to visualize an intriguing molecular event with optical resolution of millisecond time scale. It does not require any external cofactors, which is also advantageous over bioluminescence imaging (BLI). Therefore, FL imaging is often used for analysis of molecules and events occurring in a subcellular level, and relatively at fast dynamics in living cells. On the other hand, FL imaging has not been extensively utilized in clinical practice. The main reason is the need for concurrent excitation light in the imaging process, which produces severe light-tissue interactions (i.e., light scattering, tissue absorption, and autofluorescence), consequently resulting in poor signal-to-noise (S/N) ratio and low penetration depth.

2 Characterization of Bioluminescence

BL is a light-emitting phenomenon resulting from oxidation of a specific substrate, luciferin, catalyzed by the specific enzyme, luciferase. One of the best-known examples of BL is the light exhibited by fireflies, a class of beetles that use D-luciferin as their substrate along with the enzyme luciferase. Some researchers suggest that the evolution of luciferase in beetles is thought to have emerged from the ancestral fatty acyl-CoA synthetase (ACS), an enzyme, which is present in all insects.

As a variety of species are bioluminescent in nature, artificial integration of natural bioluminescent reactions into living systems has also become a multiplex optical readout that is widely used in molecular and cell biology.

The distinctive merits of BLI and analysis include the low background, no external light source, high S/N ratio, wider dynamic range of signals, versatility in the molecular design, and suitability of imaging in small animal models. A direct methodology to utilize luciferases in assays as an optical readout is to simply fuse a luciferase to a protein of interest, such as antibodies.

BL can occur in the presence of a minimal amount of luciferase and its specific substrate luciferin. Luciferases are nearly ideal reporters for bioanalysis for the following reasons: (i) the light generation occurs through a potentially very simple and time-efficient manner, (ii) the corresponding assays are generally very sensitive, (iii) the assays are broadly applicable to various organisms, from bacteria to large animals, (iv) all the reagents are biocompatible and are non-radioactive, and (v) some marine luciferases contain a secretion signal, enabling us to easily measure gene expression in the culture medium or blood samples of animals without destroying the cells or tissues. As such, there is no doubt that luciferases are excellent reporter proteins having great potential to be utilized in various assay systems.

Recent research on BLI has focused on three important areas: (i) Development of new luciferases and synthesis of novel luciferins; (ii) protein engineering to create genetically-encoded probes while improving the luciferases; and (iii) practical application of imaging with the probes such as investigation of cellular function and disease progression. These three technologies have deeply correlated with each other in technical progression. Luciferases with excellent optical properties enable us to design new optical probes with novel strategies. The new bioluminescent probes should facilitate better optical imaging of molecular phenomenon of interest in living subjects.

As BL is generated through oxidation of the substrate by luciferase-mediated catalyzation, most basic studies on BL have concerned the synthesis of new luciferins and mutagenesis of luciferases. cDNAs encoding luciferases can be genetically engineered to create bioluminescent probes with improved properties. The cDNA construct can also be easily expressed in living cells or animal models by a conventional gene transfer method.

In nature, there are numerous light-emitting organisms. It is known that more than 2000 species of fireflies are found on
land and the number is updated every year. Such light-emitting organisms are rather rare on land but extremely common in the oceans. Bioluminescent organisms are found in oceans at all depths with the greatest numbers found in the upper 1000 m of the vast open ocean.

Because of this surprising diversity, it is difficult to classify all the luciferases present in different organisms, since many of them have not been identified yet. However, it is relatively simple to categorize them according to the luciferin, considering many luciferases commonly share a limited number of specific luciferins. Thus, BL systems can be surprisingly simplified into a few groups with respect to their substrate luciferins. The most common luciferin for beetle luciferases is β-luciferin, whereas the common luciferin for many marine luciferases is coelenterazine (CTZ). These two luciferins claim the majority of the BL systems. The next major luciferins are bacterial and fungal ones. The remaining minor luciferins are Cypridina and Tetrapyrrole luciferins.

3 Beetle and Marine Luciferases

The luciferin-luciferase systems are broadly utilized in various bioassays as an optical readout platform. Thus, improved optical properties of luciferins and luciferases directly contribute to the sensorial performance of the bioassays. As there are optical properties of luciferins and luciferases directly contribute bioassays as an optical readout platform. Thus, improved luciferase reaction does not require accessory molecules such as Photinus pyralis substrates.

BL systems that are using D-luciferin and CTZ as their substrates. In Table 1, we focus on the recent, representative advances in the luminometers equipped with injectors to measure the transient quickly decaying bloluminescence, the measurement requires stability. As copepod luciferases reportedly emit bursting but peak luminescence. Furthermore, the CTZ substrate is prone to exert chemical instability and high autoluminescence, properties that make sample handling difficult and increase error bars in bioassays.

GLuc and MLuc were first cloned from copepods Gaussia princeps and Metridia longa respectively and successfully applied them as secreted reporters in mammalian cells. A few years later, Takenaka et al. newly established 25 kinds of new copepod luciferases, including Metridia pacifica luciferases (MLuc1, and 2). The copepod luciferase database reveals that the luciferases are highly conserved and possess two repeated catalytic domains.

A luciferase from Oplophorus gracilorostris (deep sea shrimp) is a typical marine luciferase emitting blue BL (462 nm) through the oxidation of CTZ in the presence of O2. The Oplophorus luciferase (OLuc) consists of two 35 kDa subunits and two 19 kDa subunits with the enzymatic activity in the smaller subunit. OLuc is naturally secreted and highly thermostable.

4 Modification of Luciferases

Upon emission of BL, luciferases oxidize substrate luciferins and make them into the excited state. This excited oxyluciferin emits photons when it relaxes to the ground state. This mechanism inspired researchers to modify the luciferase and/or luciferin scaffolds to improve the optical properties of BL.

To date, thousands of luminescent species have been identified from nature, but only a few of them have been characterized in detail. The native luciferases have been conventionally manipulated through a random and site-directed mutagenesis for improving the BL intensity, stability, and change in spectral properties.

4-1 Variants of beetle luciferases

The oldest and most famous luciferase is from North American firefly, which emits predominantly yellow-green light. Many efforts have been dedicated to modifying luciferases. The initial studies were directed towards the mutagenesis of natural luciferases to improve the optical properties. The crystal structures of North American FLuc and Japanese Genji-botaru (Luci crocuta) were determined. The active site of FLuc was intensively site-specifically mutated for improving the optical properties, including spectral shift towards red-shifted BL. The mutation sites in FLuc share consensus sites with the other beetle luciferases, such as click beetle (CBLuc) and railroad worm luciferases (RWLuc) (Fig. 2).

A major drawback of luciferases is the limited range of the available color palette, especially the deficiency in red-shifted colors above 600 nm. As the shorter wavelengths suffer from severe light attenuation in the tissue of living subjects, it is of importance to extend the color palette to the red and infrared side.

To address this issue, a great deal of effort has been directed towards developing red-shifted BL ranging from visible into the near infrared (NIR) region (650 – 950 nm) in order to achieve higher imaging depth with the decreased light scattering and tissue absorption of NIR photons through living tissues relative to that of visible light. The red-shifted BL has been achieved through mutagenesis of luciferases and synthesis of the specific substrates with different lumiphore structures.

One of the most successful examples is the red-shifted BL with the pair of AkaLuc (luciferase) and Akalumine (luciferin). IWano et al. established a bright mutant named AkaLuc through random mutagenesis of FLuc, which has 28 amino acid

4-2 Marine luciferases

The marine luciferases are further categorized into non-secretory and secretory luciferases. RLuc and its variants are the representative non-secreted luciferases, whereas most of the other marine luciferases like copepod luciferases and OLuc are secretory in nature.

Copepod luciferases are members of a family of secretory proteins derived from marine copepods, and exert flashin BL with CTZ as a substrate. Copepod luciferases are attractive reporters for their small size, bursting BL, and high thermostability. As copepod luciferases reportedly emit bursting but quickly decaying bloluminescence, the measurement requires luminometers equipped with injectors to measure the transient
### Beetle Luciferases
- **Firefly luciferase** (FLuc, Luc2, (61 kD))
  - Origin: Photinus pyralis
  - Substrate: d-Luciferin, Akalumine
  - Features: pH-sensitive, thermo-labile
- **Click Beetle luciferase Red2**
  - Origin: Photinus pyralis
  - Substrate: d-Luciferin, Akalumine
  - Features: Red-shifted, higher increased pH and thermostability
- **Emerald Luciferase** (Pty, ELuc)
  - Origin: Photinus pyralis
  - Substrate: d-Luciferin, Akalumine
  - Features: Red-shifted, brighter and increased affinity for ATP; high pH and thermostability
- **AkaLuc**
  - Origin: Photinus pyralis
  - Substrate: d-Luciferin, Akalumine
  - Features: Red-shifted, highly bright and stable
- **Railroad worm luciferase** (RWLuc, SLR)
  - Origin: Photinus pyralis
  - Substrate: d-Luciferin
  - Features: Green or red

### Marine luciferases
- **Renilla reniformis** (Sea Pansy)
  - Origin: Renilla reniformis
  - Substrate: CTZ
  - Features: Thermo-stable
- **Cypridina luciferase** (CLuc)
  - Origin: Cypridina luciferin
  - Substrate: CTZ
  - Features: Secreted, thermo-stable

### Artificial Luciferases
- **Firefly luciferase (FLuc, Luc2, (61 kD))**
- **Click Beetle luciferase Red2**
- **Emerald Luciferase** (Pty, ELuc)
- **AkaLuc**
- **Railroad worm luciferase** (RWLuc, SLR)

### Bacterial Luciferase
- **Bacterial Luciferase**
  - Origin: Alivibrio fischeri, Vibrio harveyi, and Photrobudah luminescens
  - Substrate: FMNH₂ and long-chain aliphatic aldehyde
  - Features: Bacterial expression

### Other Luciferases
- **Table 1**

| Luciferase (size) | Origin (λ_max, nm²) | Substrate | Features | Ref. |
|------------------|---------------------|-----------|----------|------|
| Firefly luciferase (FLuc, Luc2, Ppy, (61 kD)) | Photinus pyralis (560 nm with d-Luciferin, 677 nm with Akalumine) | d-Luciferin, Akalumine | pH-sensitive, thermo-labile | 30, 119 |
| Ppy GR-TS (Mut. of FLuc) (61 kD) | Photinus pyralis (547 nm) | d-Luciferin, Akalumine | Temperature and pH-stable variant | 120 |
| Ppy RE-TS (Mut. of FLuc) (61 kD) | Photinus pyralis (612 nm) | d-Luciferin, Akalumine | Red-shifted, increased pH and thermostability | 120 |
| Ppy RE9 (Mut. of FLuc) (61 kD) | Photinus pyralis (620 nm) | d-Luciferin, Akalumine | Red-shifted, highly increased pH and thermostability | 121 |
| PLR3 (Mut. of FLuc) (61 kD) | Photinus pyralis (614 nm) | d-Luciferin, Akalumine | Red-shifted, brighter and increased affinity for ATP; high pH and thermostability | 122 |
| AkaLuc (Mut. of FLuc) (61 kD) | Photinus pyralis (650 nm) | d-Luciferin | Red-shifted, highly bright and stable | 29, 123 |
| Click Beetle Green (CB green) (65 kD) (Mut. of CBLuc) | Pyporphorus plagiophthalamus (540 nm, Green) | d-Luciferin | Red-shifted | 124 |
| Click Beetle Red (CB red) (65 kD) (Mut. of CBLuc) | Pyporphorus plagiophthalamus (615 nm, Red) | d-Luciferin | 9.8-fold increased activity; pH insensitive, increased thermostability | 125 |
| PhRED-TS (65 kD) (Mut. of CBLuc) | Pyrophorus plagiophthalamus (623 nm) | d-Luciferin | High thermostability, pH-insensitive, codon-optimized version termed ELuc | 33 |
| Emerald Luciferase (Ppy, ELuc) (65 kD) (Mut. of CBLuc) | Pyreryminis termitilluminans (538 nm for Green) | d-Luciferin | Mutated Ppy | 126, 127, 128 |
| Click Beetle luciferase Red2 (CBR2, CBR2opt) (65 kD) (Mut. of CBLuc) | Pyreryminis termitilluminans (628 nm for Red) | d-Luciferin | Green or red | 126, 127, 128 |
| Thermotable Railroad worm luciferase (RWLuc red) (60 kD) for Red | Photiosrix vivani and Photiosrix hirtus (546 nm for Green or 630 nm for Red) | d-Luciferin | Thermo-stable | 27 |
| Renilla luciferase (RLuc) (36 kD) | Renilla reniformis (Sea Pansy) (480 nm) | CTZ | Thermo-stable | 13 |
| Renilla luciferase 8 (RLuc8) (36 kD) (Mut. of RLuc) | Renilla reniformis (Sea Pansy) (487 nm) | CTZ | Long lifetime in cells, enhanced serum stability and thermostability, improved quantum yield | 35, 6 |
| Renilla luciferase 8.6-535 (RLuc8.6-535) (36 kD) (Mut. of RLuc) | Renilla reniformis (Sea Pansy) (535 nm) | CTZ | Bright and red-shifted | 35 |
| Renilla luciferase 7-571 (RLuc7-521) (36 kD) (Mut. of RLuc) | Renilla reniformis (Sea Pansy) (521 nm) | CTZ | Bright and red-shifted | 36 |
| Gaussia luciferase (GLuc) (20 kD) | Gaussia princeps (470 nm) | CTZ | Secreted | 14 |
| Monsta (20 kD) (Mut. of GLuc) | Gaussia princeps (513 nm) | CTZ | Secreted, variable λ_max values according to buffer conditions | 8 |
| GLuc (20 kD) (Mut. of GLuc) | Gaussia princeps (495 nm) | CTZ | Secreted | 129 |
| Oplphorus luciferase (OLuc) (106 kD) | Oplphorus gracilicristos (Deep sea shrimp) (454 nm) | CTZ | A complex of large and small subunits | 18 |
| NanoLuc (NLuc) (19 kD) (Mut. of a small subunit of OLuc) | Oplphorus gracilicristos (Deep sea shrimp) (456 nm) | Furimazine | High pH and thermostability, very bright, stable and prolonged light emission | 41 |
| teLuc (19 kD) (Mut. of NanoLuc) | Oplphorus gracilicristos (Deep sea shrimp) (502 nm) | Diphenylterazine | Enhanced activity with diphenylterazine | 45 |
| yeLuc (19 kD) (Mut. of NanoLuc) | Oplphorus gracilicristos (Deep sea shrimp) (527 nm) | Selenoterazine | Enhanced activity with selenoterazine | 45 |
| Cypridina luciferase (CLuc) (61 kD) | Cypridina noctiduca (460 nm) | Cypridina luciferin | Secreted, thermo-stable | 16 |
| Metridia longa luciferase (MLuc, MLuc7) (24 kD) | Metridia longa (480 nm) | CTZ | Secreted, small molecular weight | 15 |
| Benthosema pterotum luciferase (BLuc) (27 kD) | Benthosema pterotum (475 nm) (Lanternfish) | CTZ | Thermo-stable | 130 |
| Artificial Luciferase 16 (ALuc16) (23 kD) | Copepoda luciferase database (496 nm) | CTZ | Secreted, bright, λ_max is variable according to buffer conditions | 38, 105 |
| Artificial Luciferase 23 (ALuc23) (23 kD) | Copepoda luciferase database (503 nm) | CTZ | Secreted, bright, strain-sensitive | 38, 105 |
| Artificial Luciferase 47 (ALuc47) (21 kD) | Copepoda luciferase database (487 nm) | CTZ | Secreted, highly specific to CTZ | 39 |
| Artificial Luciferase 49 (ALuc49) (21 kD) | Copepoda luciferase database (490 nm) | CTZ | Secreted, specific to CTZ | 39 |

**Table 1** Optical properties of representative luciferases and their variants

**a. Maximum wavelength of bioluminescence spectrum. Mut. denotes mutant.**
substitutions across the sequence. The AkaLuc shows improved thermostability and robust BL with the emission maxima at 650 nm with the substrate Akalumine. In the process of mutagenesis, the researchers also made other variants, FLuc-8, and FLuc-10, which showed an emission maxima of 677 nm, and FLuc-24 and FLuc-27, which showed the emission maxima of 656 and 650 nm, respectively.

Inspired by the success of Akalumine, Ikeda et al. synthesized a new series of NIR-emitting luciferin substrates for beetle luciferases, NIRLucs. This strategy resulted in pH-independent luciferins emitting NIR lights with beetle luciferases. When this system was applied in mammalian cells, the luciferins were bright even at very low concentrations used for the reactions.

Another significant red-shifted BL system was accomplished with click beetle luciferase (CBLuc) variants named CBR2 and CBR2opt. This system uses two naphthyl-based luciferin analogs as substrates. The most successful peaks in the spectra achieved by this system were found at 730 and 743 nm.

4-2 Variants of marine luciferases

Random mutagenesis is a slow, tedious, and labor intensive process, which consumes a significant amount of time to produce results. Especially, in the absence of the crystallographic data, researchers have no alternative measures for engineering luciferases.

While random mutagenetic approaches are somewhat useful for establishing variants of luciferases with improved optical properties, some researchers have tried to find consensus amino acids and mutagenesis sites through aligning the sequences of the same lineage of luciferases, called “consensus sequence-driven mutagenesis strategy (CSMS)”.

Partly aided by the CSMS strategy, Loening et al. established various red-shifted variants of RLuc and applied them to animal models. The mutants were named RLuc8, RLuc8.6, RLuc8.6-535, RLuc8.6-535SG, etc., according to the number of mutations used for generating the variants with the maximal wavelengths shifted. Separately, another research group also attempted mutagenesis to fabricate bright and red-shift RLuc variants, named Super RLuc and RLuc+ (Fig. 2).

Kim et al., expended the basic concept of the CSMS to artificially construct whole amino acid sequences (named Artificial luciferases (ALuc)), whose identities are distinctive from any existing luciferases. The ALucs were made by extracting and linking frequently occurring amino acids from an alignment of many existing copepod luciferases in public databases. Copepod luciferases were selected because they are the smallest ones among luciferases with two-repeated catalytic
domains like a mirror image, besides the variable N-terminal domain including the secretion signal.\textsuperscript{40} The three domains of the extracted sequences can be further aligned in overlapping fashion for finding consensus amino acids, the method of which was named as Single-Sequence Alignment (SSA). This SSA reveals that successful mutation sites described in the research literature interestingly can be explained by their contribution to the homology. For example, the known successful mutation sites M60L (\textsuperscript{M43L} without SP), M86L (\textsuperscript{M96L} without SP), F89W, and I90L\textsuperscript{4} in the first catalytic domain of the aligned GLuc sequence are exactly superimposed on the following sites, I131, L157, W160, L161 in the second catalytic domain, respectively. Likewise, this feature is generally applicable to the other copepod luciferases such as Mpluc1 and MLuc.\textsuperscript{8}

Another marine luciferase, the OLuc, was engineered to develop the brightest NanoLuc. OLuc consists of two 35 kDa subunits and two 19 kDa subunits.\textsuperscript{25} Nanoluc (or NLuc) is established through random and direct mutagenesis of the smaller luciferase unit of OLuc (19 kDa). Although it utilizes CTZ as the substrate, a CTZ analogue named Furimazine is known to enhance the BL intensity by 25-fold.\textsuperscript{41}

To date, many luciferase variants have been established through site-directed mutagenesis as shown in Fig. 2(A). In this site-directed mutagenesis, a rational search for an effective candidate amino acid is important to reduce labor. Kim previously suggested to look for suggestions from the chemical structural similarity between the fluorophore (\textsuperscript{65SYG67}) of GFP previously suggested to look for suggestions from the chemical structure of the common substrate of marine luciferases.\textsuperscript{42} This view suggests that the success story in GFP mutation is potentially successful even for the mutagenesis of marine luciferases. For example, native GFP was successfully modified by mutating S202F, N167Y, T203I, Y66W and Y66H,\textsuperscript{43} and further modified in the sites F46L, V68L, Q99K, S72A, V163A, and T203Y.\textsuperscript{44} This view demonstrates that alteration of the fluorophore-neighboring amino acids to F, V, W, H, Y and L may be effective for improving the optical properties of marine luciferases. Kim \textit{et al.} successfully developed bright and stable variants of copepod luciferases (GLuc, Mpluc1, and MLuc) by introducing F, L, H, W amino acids in the sequence.\textsuperscript{8} This approach has the potential to save a great deal of labor in future site-directed mutagenesis.

The red-shifted BL of NanoLuc was further achieved through two pathways: one is by mutagenesis of NanoLuc and the other by organic synthesis of the specific substrates. Yeh \textit{et al.} synthesized a series of furimazine analogues exerting red-shifted BL with NanoLuc.\textsuperscript{45} They further established bright and red-shifted variants of NanoLuc named teLuc and yeLuc. The maximal intensities of teLuc and yeLuc were found to be 502 and 527 nm, respectively.\textsuperscript{45}

### 5 Synthesis of New Substrates for Luciferases

Substrates are key components for generating BL with luciferases. The role of substrates was previously explained as “luminophore.” It is because the substrates act as “luminophore” when being oxidized by luciferases to generate BL. One may say that luciferases recruit the “luminophore” substrate from the aqua phase, whereas fluorescent proteins embed the “fluorophore” inside the molecular scaffold. It is interesting to note that the chemical structure of the common substrate of marine luciferases, CTZ, is similar to the fluorophore of fluorescent proteins.\textsuperscript{42} This view suggests that the successful mutagenesis cases in fluorescent proteins may be applicable to those in luciferases.

A recent trend in luciferin studies is to append functionalities of the natural luciferin backbone using organic synthesis. The authors classify and explain the natural luciferins in the section on “passive substrates,” while the functional luciferins are summarized below in the section on “activatable substrates.”

#### 5-1 Passive substrates

Luciferin is defined as the general term of an organic compound that is biosynthesized in a luminous organism to provide energy for light emission by being oxidized by catalysis of a luciferase in the presence of O\textsubscript{2}.\textsuperscript{46} As luciferin is an absolute requirement as the source of light energy, thousands of luminescent species biosynthesize luciferins endogenously, although most of the mechanisms are unknown. In contrast to a great number of reported luminescent species to date, the luciferin is highly conserved and mostly categorized into six groups according to the chemical structures: (i) beetle luciferin (\textdelta-luciferin), (ii) tetrapyrrole luciferin, (iii) fungal luciferin, (iv) bacterial luciferin, (v) \textit{Cypridina} luciferin, and (vi) coelenterazine (CTZ).\textsuperscript{47} Considering the variety of the light-emitting species in nature, the limited luciferin repertoires are astonishing. As illustrated in Fig. 3, all the natural luciferins have no additional functions like ligand recognition and thus can be categorized into the “passive” ones.

The most widely used is \textdelta-luciferin (Group 1) that is commonly observed in luminescent organisms, including fireflies, click beetles, and railroad worms. \textdelta-luciferin was first extracted by McElroy \textit{et al.} and crystallized.\textsuperscript{48} \textdelta-Luciferin consists of a benzothiazole moiety attached to a thiazole carboxylic acid moiety.\textsuperscript{49} These two moieties have been modified for enhancing the optical intensities and red-shifted BL.

The research proves that beetle luciferases can tolerate modification to \textdelta-luciferin. One important modification of \textdelta-luciferin was achieved through alkylation and cyclic alkylation modification of the hydroxy group (\textendash OH).\textsuperscript{50} The alkyl and aminoluciferins are further modified by bulky fluorescent dyes for red-shifted emission of BL.\textsuperscript{51} Caged luciferin is another important category of \textdelta-luciferin analogues. Caged luciferin cannot luminesce until an enzymatic cleavage event occurs and thus can minimize the autoluminescence. \textbeta-Galactoside-linked \textdelta-luciferin was previously introduced, which is only active in case its galactoside moiety is removed by the co-expressed \textbeta-galactosidase.\textsuperscript{52}

Iwano \textit{et al.} previously put forward a design strategy to generate NIR BL by extending the \pi-conjugation length in the \textdelta-luciferin backbone between the benzothiazole and thiazole moieties and established the red-shifted BLI system with the pair of AkaLuc (luciferase) and Akalumine (luciferin). They accomplished a diverse hue of visible and NIR BL, whose longest emission maximum was observed at 675 nm.\textsuperscript{53} The water solubility was significantly improved with the acidic salt form of Akalumine, named TokeOni.\textsuperscript{54} Tetrpyrrole luciferins (Group 2) are utilized by Dinoflagellates and Euphausiids to generate BL. The BL occurs as flashes of light triggered by electrical or mechanical stimulation in the living organisms.

Fungal luciferin (Group 3) is an \textalpha-pyrene 3-hydroxyhispidin\textsuperscript{5} that is biosynthesized in fungi for BL.\textsuperscript{5} The fungal luciferin is oxidized by the specific luciferase from \textit{Neonothopanus nambii} (nnLuc). The reaction requires only O\textsubscript{2}, and generates green BL.
As the fungal BL system is constructed by the expression of only three genes, one can develop a luciferin-free BL system that is growing in eukaryotes.47 Bacterial luciferin (Group 4) is known as myristic aldehyde, which is oxidized in the reaction. Bacterial BL requires flavin mononucleotide (FMN), O₂, and nicotinamide adenine dinucleotide (NADH), besides the myristic aldehyde. Natural bacterial BL is blue (ca. 490 nm), but red-shifted BL is also reported with fluorescent protein-fused ones.54 The brighter version was reported by Gregor et al. and named iLux.55 These bacterial luciferin-luciferase systems are applicable to various bioanalytical studies such as cyanide measurement.56 Cypridina luciferin (Group 5) generates another blue light BL. The luciferin is a tripeptide and synthesized from tryptophan, isoleucine, and arginine.57 CTZ (Group 6) is the luciferin of a wide variety of marine luciferases including RLuc and GLuc, and is another modified tripeptide that is biosynthesized from one phenylalanine and two tyrosine residues.46 This CTZ-based imaging system naturally emits BL in blue in the presence of O₂.

It is known that marine luciferases can tolerate modified CTZ analogs. Modification of the C2 and C6 positions of CTZ has been actively studied by several research groups. Through the modification, we can expect (i) luciferase specificity, (ii) red-shifted BL, and (iii) enhanced BL. Such a high selectivity to RLuc is accomplished by introducing a double bond at the C6 position, besides the elimination of the OH group at the C2.58 It is also known that an ethynylation at the C6 position enhances the overall optical intensities of the analogs with ALucs.58 Red-shifted BL is obtained through conjugating fluorescent dyes to the C2 or C6 position of the backbone of CTZ.59 Even blue-shifted CTZ analogs can be utilized for NIR BLI in animal models through a large stock-shift of NIR fluorescent proteins (iRFPs).19 It was accomplished through combining the blue-shifted CTZ analog with RLuc8.6SG-linked NIR fluorescent proteins (iRFPs).

5-2 Activatable substrates
“Activatable substrates” are intriguing to discuss in the field of molecular imaging. Activatable substrates may be defined as substrates that can respond to a specific stimulator or environment, and emit optical signal(s) to reflect the condition in addition to the original role as the energy source for light emission. This concept has become possible as researchers synthesized new substrate analogues that carry additional functions like sensing ligands.

Fig. 3 The chemical structures of active and passive luciferins. The chemical structures are divided into six major groups according to the chemical structures. The specific substrate names are referred from the following references: CycLuc1 and 2 (bright),3 NH₂-NpLH2 (red-shifted BL),33 OH-NpLH2 (red-shifted BL),33 ILH2,4 BP-PS (caged luciferin),64 AkaLumine (red-shifted BL),30 AkaLumine-HCl (TokeOni),30 Furimazine (strong and stable BL),41 6-pi-OH-CTZ,46 6piOH-2H-CTZ (RLuc-specific),46 6et-OH-CTZ (ALuc-specific),46 6-N₃-CTZ,46 6-FITC-CTZ,46 BBBlue2.3 (CTL analog),47 and CCTZD-1, -2, -3 (caged CTZ analog).60
A prototypical one is “photoactivatable” luciferins, where the substrates are protected by a cage. These caged luciferins commonly carry a bulky group that blocks the interaction of the luciferin backbone with the enzyme. The cages in the luciferins are removed by specific wavelengths of light stimulation that exert the luciferin free through a bond cleavage for its interaction with luciferase for imaging. Some research groups have developed caged photoactivatable luciferins and succeeded in imaging by UV irradiation in animal models.60 This strategy was also applied for other stimulators (analyte), including oxygen species,61 copper ions,62 and labile iron levels.63 The balance of sulfur redox plays an important role in maintaining homeostasis of the living subjects. The d-luciferin backbone was modified to visualize endogenous hydrogen polysulfides (H₂S₅) and applied to animal models.64 Likewise, some active substrates that are sensitive to reactive nitrogen species (RNS) have also been synthesized.65

One of the most desirable functionalities of luciferin may be red-shifted BL, which can minimize the light attenuation in physiological samples or in the tissues of living subjects. Abe et al. recently synthesized a CTZ analog that emits NIR BL.66 The CTZ analogue named Cy5-CTZ was prepared by conjugating Cyanine-5 (Cy5) dye to CTZ through an acetylene linker. The acetylene linker enables through-bond energy transfer (TBET) between the energy donor CTZ and the energy acceptor Cy5. This novel derivative is intrinsically fluorescent and emits NIR-shifted luminescence in the Cy5 channel upon reacting with an appropriate luciferase, RLuc. The authors demonstrated that Cy5-CTZ is optically stable in physiological samples, rapidly permeabilizes through the plasma membrane, and luminesces NIR BL.

6 Bioluminescence Imaging (BLI) Systems

Many natural luciferin-luciferase systems have been reported from bioluminescent organisms. Although natural BL has potential as optical readouts, their direct utilization in BLI systems is limited due to their poor color palette and optical properties. To date, considerable effort has been directed towards improving the systems, optical properties, which include optical intensity, thermostability, turnover rate, and variation in colors.

Reumann et al. previously categorized the BLI probes into “static” and “activatable” ones.67 Kim et al. also categorized the BLI systems into two major families: (i) a family of genetic and transcriptional probes (GTPs), and (ii) a family of non-transcriptional and activatable probes (NAPs) (Fig. 2).68 A typical feature of GTP is the transcription of the luciferase in the imaging process. This family of probes generally requires a long ligand-stimulation time until sufficient accumulation of the reporter protein is reached. This is mainly controlled at the promoter level. A reporter-gene assay and a two-hybrid assay are grouped in this family. On the other hand, NAPs are expressed beforehand and pre-localized in adequate intracellular compartments of interest. The probe is ready to develop BL upon stimulation of a signal. Therefore, the NAPs intrinsically respond quickly to signals and generally are expected to have higher S/N ratios than GTPs. Protein-complementation assay (PCA), protein-fragment splicing assay (PSA), and single-chain probes are a few which are categorized under NAPs.

The NAPs can be further categorized into split-reporter imaging systems and split-free imaging systems. The details are explained in the following sections.

6-1 Split-reporter imaging systems

Protein-fragment complementation assays (PCAs) provide a unique experimental strategy for quantitatively imaging the dynamics of protein-protein interactions (PPIs) in animal cells. A luciferase as reporter is initially dissected into N- and C-terminal fragments and thus it temporarily loses its enzymatic activity. The fragments are then genetically fused to proteins of interest. If the two proteins interact, the reporter fragments are approximated together, are folded into the native structure of the luciferase, and its activity is reconstituted (Fig. 4(A)).69,70 Michnick et al. greatly contributed to the development of PCAs by conducting a whole cell-wide mapping of PPIs in yeast using luciferase fragments in 200871 and 2010.72 In 2010, they also presented detailed protocols for large-scale analysis of PPIs with the survival selection of dihydrofolate reductase (DHFR) reporter PCA.73 The known examples of PCAs are summarized in Table 2.

As a similar strategy to PCA, protein-fragment splicing assays (PSAs) have been introduced. PSAs make use of protein ligase intein-extein fragments, that enable us to provoke spontaneous protein splicing between two fusion proteins without energy. In the probe design, split-fragments of a luciferase are linked to a protein pair of interest via dissected intein fragments, where the split-luciferases act as the extein. The split-luciferase temporarily loses its enzymatic activity. Upon approximation of the split-fragments, the inteins trigger protein splicing, which is a self-catalyzed excision of the intein and ligation (reconstitution) of the flanking split-luciferase.74 The known examples of PSAs are summarized in Table 2.

PCAs with BL readouts are best for studies of the spatial and temporal dynamics of protein pairs. And most of the interaction cases are reversible and thus allow for the detection of kinetic and equilibrium aspects of protein complex in living cells. On the other hand, PSAs are considered irreversible spontaneous reactions, and thus the background can be drifted by time. The distinct benefits of these assays are that the probes are primed beforehand and ready to respond to ligand rapidly, and are reversible in the association. It is known that the luciferase-fusion protein is completed within a few minutes after ligand addition in cell-free conditions.75–77 This near-real-time feature is advantageous for repeatedly imaging the dynamics of the molecular events in cells. This has great significance in terms of timeframes, considering that reporter-gene assays require overnight incubation until the reporter is accumulated enough to be determined by luminometers.

A frontier study on the conditional association of protein fragments was demonstrated in 1994 with split-ubiquitin.78 The complementation of split-fluorescent protein was first examined in Escherichia coli by Ghosh et al.79 Calmodulin (CaM)-M13 binding was examined in mammalian cells with fragmented yellow fluorescent protein (YFP) in 2001.80 Intein-mediated protein splicing assay (PSA) was first introduced by Ozawa et al. using both a split-GFP in 200981 and split-firefly luciferase (FLuc) in 2001.82 BLI of PPIs in living mice was first reported by Paulmurugan et al., where both strategies of protein complementation and intein-mediated protein splicing were comparatively examined.83

In contrast to the above-mentioned PCAs and PSAs that are based on two independent fusion proteins, a studies on series of single-chain probes with split-luciferases were also reported by Paulmurugan et al.84 and Kim et al.,85 in 2006 and 2007, respectively. These single chain probes are characteristic in point that all the components for ligand sensing and light emission are integrated in a single molecular backbone.

While PCAs and PSAs provide distinctive merits as bioassays,
it is difficult to determine the optimal dissection sites of each luciferase. In practice, it is tedious and time-consuming to find optimal dissection sites of luciferases of interest. Conventionally, researchers selected the potential dissection sites among the flexible amino acids such as glycine in the luciferase sequence. To address this issue, Kim et al. previously suggested a hydrophilicity search, e.g., the scale of Kyte and Doolittle (Swiss Institute of Bioinformatics, SIB). It is because this scale generally reveals a remarkably hydrophilic region. Kim et al. suggested that this search empirically narrows down the potential dissection site region. The approach is empirically confirmed with the proven, successful dissection sites from other researchers. Many other optical dissection sites that were independently found by other researchers are also considered to be within the distinctive hydrophilic regions of the luciferases.

6.2 Full-length (split-free) reporter gene imaging systems

Luciferase has been genetically engineered to construct various optical probes. Many previous probes carry dissected luciferase fragments for a temporal loss and conditional recovery of luciferase activities. However, this methodology inevitably hampers the intrinsic enzymatic property of the luciferase and...
recovers merely 0.5 – 5% of the original luciferase activity.\textsuperscript{89,91} In addition, the luciferase dissection strategy requires a sophisticated probe design and a tedious optimization step for deciding a suitable splitting site in the luciferase.

To address the limitation, researchers have fabricated a new BLI system using full-length luciferases. The imaging probes conceptionally embed a full-length luciferase, termed “split-free reporter imaging system.” The working mechanisms of such probes are explained in the following sections.

### 6·2·1 Reporter-gene assays and two-hybrid assay systems

The most broadly utilized and conventional systems for studying various biological functions of cells are “reporter-gene assay system” and “two-hybrid assay system.” These systems commonly make use of an expression vector encoding a specific promoter sequence linked to the coding region that regulates the transcription of the reporter luciferase. A ligand-activated transfection factor binds to the promoter sequence and triggers expression of the reporter luciferase accumulation in cells, which can be measured at various time points.

#### Optimal dissection sites of luciferases for construction of bioluminescent probes and molecular imaging

| Luciferases | Optimal dissection sites (AAa) | Guest protein pairs | Working mechanism | Ref. |
|-------------|-------------------------------|---------------------|-------------------|------|
| Firefly luciferase (FLuc) | 1-415/416-550 | AR LBD and FQNLF motif | Intramolecular complementation | 75 |
| | 1-437/438-454 | IRS-1 and SH2 domain of PI3-kinase | Intein-mediated protein splicing | 82 |
| | 2-416/398-550 | FRB and FKBP12 | Intermolecular complementation | 86 |
| | 1-437/438-550 | FRB and FKBP12 | Intermolecular complementation | 89 |
| | 1-445/446-550 | FRB and FKBP12 | Intermolecular complementation | 89 |
| | 1-475/245-550 | FRB and FKBP12 | Intermolecular complementation | 89 |
| | 1-475/254-550 | FRB and FKBP12 | Intermolecular complementation | 89 |
| | 1-475/265-550 | FRB and FKBP12 | Intermolecular complementation | 89 |
| | 1-475/300-550 | FRB and FKBP12 | Intermolecular complementation | 89 |
| | 1-464/417-550 | AR and Src | Intermolecular complementation | 133 |
| | 1-464/395-550 | IBC domain of IP3-R2 | Intermolecular complementation | 134 |
| | 1-437/438-544 | IRS-1 and SH2 domain of PI3-kinase | Protein splicing using DnaE (Ssp. PCC6803) | 82 |
| | 1-437/438-544 | MyoD and Id | Protein splicing using DnaE (Ssp. PCC6803) | 135 |
| | 1-490 (H489K)/438-544 | FRB and FKBP12 | Protein splicing using Saccharomyces cerevisiae | 136 |
| | 1-439/440-542 | AR LBD and LXXLL motif | Intramolecular complementation | 91 |
| Click beetle luciferase (CBLuc) | 1-439/443-542 | AR LBD and LXXLL motif | Intramolecular complementation | 91 |
| | 1-439/437-542 | AR LBD and LXXLL motif | Intramolecular complementation | 91 |
| | 2-413/395-542 | CXC4 and β-arrestin | Intermolecular complementation | 137 |
| Emerald luciferase (ELuc) | 1-415/394-550 | GPCR and β-arrestin | Intermolecular complementation | 138 |
| | 1-411/390-550 | GPCR and β-arrestin | Intermolecular complementation | 138 |
| Rellina luciferase (RLuc) | 1-229/230-311 | Full length AR | Intein-mediated protein splicing | 139 |
| | 1-93/92-311 | Dimerization between ERK2 and ERK2 | Intramolecular complementation | 140 |
| Gausia luciferase (GLuc) | 1-229/230-311 | MyoD and Id | Intermolecular complementation | 141 |
| | 18-109/110-185 | FRB and FKBP12 | Intermolecular complementation | 142 |
| | 18-105/106-185 | CaM and M13; AR LBD and LXXLL motif; ER LBD and Src SH2 domain | Intramolecular complementation | 88 |
| ALuc16 | 1-93/94-169\textsuperscript{a} | CXCL12 and CXC4 | Intramolecular complementation | 137 |
| | 19-125/126-212 | GR LBD and LXXLL motif | Intramolecular complementation | 38, 143 |
| | 19-129/130-212 | GR LBD and LXXLL motif | Intramolecular complementation | 38 |
| | 19-141/142-212 | GR LBD and LXXLL motif | Intramolecular complementation | 38 |
| | 19-146/147-212 | GR LBD and LXXLL motif | Intramolecular complementation | 38 |
| NanoLuc and its mutant (NanoBiT) | 1-156/157-171 | FRB/FKBP; BRAF/CRAF; PKA/β-arrestin-2 | Intermolecular complementation | 144 |
| | 1-156/157-171 | Exocytosis of Gult4 | Intramolecular complementation | 145 |

The slash means the dissected position. EGF, Epidermal growth factor; IRS-1, phosphorylated insulin receptor substrate 1; FKBP12, mammalian target of rapamycin and FK506-binding protein 12; FRB, rapamycin-binding domain; AR LBD, ligand binding domain of androgen receptor; CaM, calmodulin; M13, a CaM-binding peptide; IBC, IP3-binding core domain (residues 224 – 604 AA); MyoD, a myogenic regulatory protein; Id, a negative regulator of myogenic differentiation. a. AA means Amino Acids. b. The secretion signal at the N-terminal end (1 – 18 AA) seems to be uncounted in the literature.

In addition, the luciferase dissection strategy requires a sophisticated probe design and a tedious optimization step for deciding a suitable splitting site in the luciferase.

To address the limitation, researchers have fabricated a new BLI system using full-length luciferases. The imaging probes conceptionally embed a full-length luciferase, termed “split-free reporter imaging system.” The working mechanisms of such probes are explained in the following sections.
the reporter accumulation to obtain sufficient S/N ratios. In addition, it requires nuclear trafficking of the proteins of interest, because their proximity to the transcriptional machinery is needed for expression of the reporter proteins.

### 6.2.2 Multiplex luciferase systems

Multiplex luciferase systems have been utilized in various molecular imaging aspects as an expanded tool box of bioluminescent imaging. They may be conceptionally summarized into three categories:

(i) **Orthogonal multiplex luciferase-luciferin systems**, where multiple luciferases coexist in the same context. They oxidize completely distinctive luciferins without cross-talks and thus one can easily distinguish each BL signal. Likewise, Kim et al. also developed a multiplex reporter system by combining two luciferases with specific luciferins, which was newly synthesized. Similar orthogonal luciferase-luciferin systems have been developed by Jones et al., who synthesized many luciferins to find positive binding pairs with each luciferase. These systems allow researchers to distinguish the signal from each other.

(ii) **Ratiometric luciferase-fluorescent protein systems**. One of the most direct and intuitive methods is to consecutively link a couple of fluorescent and bioluminescent reporters. A pH-sensitive BL reporter system named “pHLuc” is an excellent example of such a reporter system. pHLuc enables us to ratiometrically determine pH levels in living cells and animal models through a linkage of pH-sensitive GFP with pH-stable full-length luciferases, Antares and NanoLuc. pHLuc successfully visualized variance in the level of acidosis across the tumor.

(iii) **Multiple enzyme-based systems**. This unique luciferase-luciferin system consists of a luciferase-luciferin system combined with other reporter enzymes like β-galactosidase and β-galactosidase activities are determined with the same aliquot of cell lysates. Therefore, one can reduce manual labor and increase experimental accuracy.

### 6.2.3 BRET-based bioassays

Bioluminescence resonance energy transfer (BRET) is a technique used for determining PPIs on the basis of the nonradiative energy transfer from a bioluminescent donor (e.g., RLuc) and a fluorescent protein acceptor. The donor and acceptor are tandemly fused to proteins of interest, respectively. In case that the proteins are approximated at a distance of less than 100 Å, the BRET signal is enhanced.

BRET allows sensitive analysis of small spatial changes of proteins. It is used to interrogate PPIs and conformation changes within the protein. Different from FRET, BRET employs an endogenous light source and thus shows very low background BL. BRET also does not suffer from phototoxicity, photobleaching, and autofluorescence. BRET is useful to examine PPIs, protein folding, and protease activities because the BRET-permissive distance of less than 10 nm is very similar to the dimensions of biological macromolecular protein complexes. The first generation BRET assay, called “BRET1”, uses RLuc and an enhanced yellow fluorescent protein (EYFP) as donor acceptor combination, which yielded a spectral separation of nearly 50 nm. Figure 4 summarizes the overall molecular mechanisms of BRET probes.

To date, many versions of BRET systems have been reported, varying in designation from BRET1 to BRET8, and in which various energy donor and acceptor pairs were examined in practice. RLuc and its derivatives have been widely used in most of the BRET systems as the energy donor. *Cypridina* luciferase (GLuc), NanoLuc, and *Cypridina* luciferase (CLuc) have also been reported as energy donors in BRET assays.

The conventional BRET systems have solely depended on an overlap of the energy donor and acceptor spectra. However, recently, Kim et al. engineered a conceptually unique ligand-activatable BRET system (termed BRET9), where a full-length Artificial Luciferase variant 23 (ALuc23) as the energy donor is sandwiched between a protein pair of interest, FRB and FKBP. This fusion protein was further linked to a fluorescent protein as the energy acceptor. A specific ligand, rapamycin, activates the interaction of FRB and FKBP, which simultaneously enhance both the overall BL spectrum and the specific BRET peak. They conducted a new ligand-activatable BRET platform that efficiently reports BL signals in cells and living animal models.

As NanoLuc is a small, bright, and stable luciferase, NanoLuc-based BRET probes have been developed and named “NanoBRET” and “eNanoBRET.” NanoBRET is less likely to cause steric hindrance and allows for BRET assays to have better assay sensitivity. Although the spectrum of NanoLuc is blue-shifted, the intense BL allows energy transfer to occur with a broader range of colored fluorophores. As NanoLuc emits only blue BL, additional efforts have been made to diversify the colors and thus to fill voids in the color palette. Nagai et al. previously developed a NanoLuc-based BRET system named “Nano-lantern.” Recently, an enhanced set of colors has been achieved with the NanoLuc-based BRET system. In the BRET system, NanoLuc is designed to conjugated various fluorescent molecules via SNAPtag and HaloTag. This conjugation created diverse hues of BL.

Red-shifted BRET systems were also achieved by Cha et al. by sandwiching NanoLuc between two copies of an orange fluorescent protein (CYOFP1), and named “Antares.” The next generation NanoLuc named “teLuc” was further developed through mutagenesis and luminesced with a new furimazine analog, named DTZ, as substrate. This system with teLuc and DTZ emits robust and enhanced red-shifted BL for imaging in deep tissues.

Very recently, Nishihara et al. developed a novel BLI platform on the basis of BRET for achieving a ~300 nm blue-to-near infrared shift of the emission (NIR-BRET). The NIR-BRET system consists of a synthetic CTZ analogue, named “Bottle Blue 2.3 (BBBlue2.3)” and a unique irFP-linked RLuc8.6-535SG fusion protein as a BRET probe. The blue-shifted RLuc substrate BBBlue2.3 is of value for *in vivo* imaging because it overlaps well with the Soret band of the irFP that was chosen as a BRET acceptor. This NIR-BRET system shows very low autoluminescence in *vivo*, due to its blue emission and the large blue-to-near infrared shifts (400 ~ 717 nm) of the resonance energy.

A large shift in the resonance energy is also accomplished by employing BRET, and two-step FRET specially designed with cyanine dye FD-1029. The emission reaches to the second near infrared (NIR-II) region at 1029 nm. This imaging system is widely useful for high contrast imaging of biological events within complex tissues, such as vessels and lymphatics in mice. The basic concept of BRET was even applied to image the organelle-organelle interactions in mammalian cells. Hertlein et al. developed a new BRET-based biosensor for quantifying the physical associations between the ER and mitochondria, named “MERLIN.” This concept opens the opportunity to many other applications including various membrane contacts occurring in the intracellular contexts.

### 6.2.4 Molecular strain probes

Kim et al. previously demonstrated a unique non-transcriptional assay system based on molecular tension of a
luciferase artificially appended by PPL. They found that an artificially appended molecular tension to a full-length luciferase diversifies the enzymatic activity. For the basic probe design, a full-length luciferase was sandwiched between two component proteins of interest with the minimal length of flexible linkers between all the components. To date, they examined various luciferases, and found that RLuc8 and ALuc23 are suitable for developing the tension probes. The full-length RLuc8 is sandwiched between the ligand-binding domain of estrogen receptor α (ER LBD) and the SH2 domain of Src; this fusion protein enhances the optical intensities ligand-dependently. Likewise, full-length ALuc23 was sandwiched between FRB and FKBP, and showed excellent sensorial performance as a bioluminescent probe. The imaging system works even in a cell-free condition and in the application to a BRET system.

7 Instrumentation

The objective of the emerging body of the studies on bioluminescent assays is to develop versatile, low-cost, and portable devices for point-of-care and field monitoring of physiological and environmental samples.

Recent technological advances have allowed researchers to develop portable, field-deployable luminometers based on silicon photomultipliers (SiPM). Kim et al. also developed a unique eight-channel light sensing system for simultaneously determining a maximum of eight different light samples. This system is compact, portable, and energy-efficient, and the power is supplied through a USB cable connected to a personal computer.

Recently, another trend in BL measurements is to use smartphone cameras. Since smartphones are widespread and cost-effective platforms, developing bioassays based on smartphones are very useful. This modern device has been utilized in the determination of various optical readouts, including colors, FL, and BL.

Maddalena et al. developed a smartphone-based assay platform that determines hygiene levels of analytes on nitrocellulose papers immobilizing luciferases. They exploited smartphone photocameras as the reliable light detector for on-site bioassays. They also developed a smartphone-based bioluminescent 3D cell biosensor platform for determining the signal transduction pathways of Nuclear Factor-kappa B (NF-κB).

8 Summary

Taken together, BL has unique advantages as an optical readout for analytical scientists. For example, it provides long dynamic range, low background intensity, high signal-to-noise ratios, biocompatibility, simplicity in the measurement system, low cost, and versatility in the assay design.

Current BL studies can be summarized into three major categories. First are the studies using synthetic substrates, second are those for designing novel bioluminescent probes and bioassay systems, and third are the studies for application of bioluminescence for in vivo imaging and developing new instrumentation. This review provided an overview of the importance of all three categories along with how we can move forward on this topic. As the performance of bioluminescent probes and bioassay systems are rooted on the advanced properties of luciferins and luciferases, it is expected that future efforts will continue to be devoted to basic science studies, in addition to the parallel application in tracking biological events of cells in vitro and in vivo. Similarly, it is also expected that many BL studies will diversify to develop a wide variety of colors palettes and to develop red-shifted BL systems. The direct merits of these developments could be that one can construct multiplex bioassay systems using multiple optical readouts while the use of red and NIR BL can enhance imaging applications in physiological samples and in living subjects.

The other important research tend in BL studies is convergence and heterogenicity. BRET is a good example of such a convergence between BL and fluorescence. Likewise, BL can be adapted to various heterogenous materials like nanoparticles and fluorescent dyes. This convergence should greatly expend the optical toolbox with truly quantitative, highly sensitive, and versatile optical readouts. Such great advances in BL can be achieved from ideas inspired by nature and imagination.

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