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[Review]

Fluorescent and Luminescent Proteins Derived from Marine Organisms: Functions and Applications

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Organisms constituting a large proportion of marine ecosystems, ranging from bacteria to fish, exhibit fluorescence and bioluminescence. A variety of marine organisms utilize these biochemically generated light sources for feeding, reproduction, communication, and defense. Since the discovery of green fluorescent protein and the luciferin-luciferase system more than a century ago, numerous studies have been conducted to characterize their function and regulatory mechanism. The unique properties of fluorescent and bioluminescent proteins offer great potential for their use in a broad range of applications. This short review briefly describes the functions and characteristics of fluorescent and bioluminescent proteins, in addition to summarizing the recent status of their applications.

Keywords: Fluorescence, Luminescence, Marine, Proteins

Introduction

Light produced by living organisms is an attractive subject in multidisciplinary science. Both fluorescence and bioluminescence have been intensively investigated in bacteria, fungi, insects, and marine organisms (Lloyd, 1983; Meyer-Rochow, 2007; Haddock et al., 2010; Widder, 2010). In particular, marine organisms emitting fluorescence or bioluminescence have been intensively investigated for centuries, since the first report on the role of oxygen in bacterial bioluminescence (Boyle, 1667). The habitats of these organisms are broadly distributed on Earth, from the deep sea to the polar regions (Haddock et al., 2010). Luminescence is one of the three major light sources-sunlight, moonlight, and luminescence-for a broad spectrum of marine life, and facilitates a number of their functions, such as those of feeding, reproduction, communication, and defense (Hastings, 1983; Wood et al., 1989; Widder, 1999; Haddock et al., 2009).

Light emissions from living organisms are derived from two distinct systems: fluorescence and bioluminescence. Fluorescence is emitted by a fluorescent photoprotein, wherein a chromophore absorbs light and converts it into a longer wavelength. The green fluorescent protein (GFP) was concomitantly discovered in the jellyfish *Aequorea victoria*, when a functional study of the photoprotein aequorin was carried out (Shimomura et al., 1962; Shimomura, 1979). Later investigations identified several members of the GFP superfamily with similar protein structures, although the protein family consists of different protein classes (Yue et al., 2016). Bioluminescence systems, on the other hand, are more complicated. In general, they require a combination of luciferin and luciferase. The luciferin substrate is activated by the luciferase enzyme, which results in the excited state substrate resuming the ground state and emitting luminescence in the process. To date, only 11 systems of luciferin-luciferase pairs have been elucidated, although more than 30 bioluminescence systems have been identified

(Kaskova et al., 2016).

Early studies have revealed the biochemical mechanism of the luciferin-luciferase system and characterized the properties of GFPs (Chiesa et al., 2001; Vysotski and Lee, 2004; Shimomura, 2005). The unique properties of the light-generating systems of these proteins have gathered immense attention in a wide range

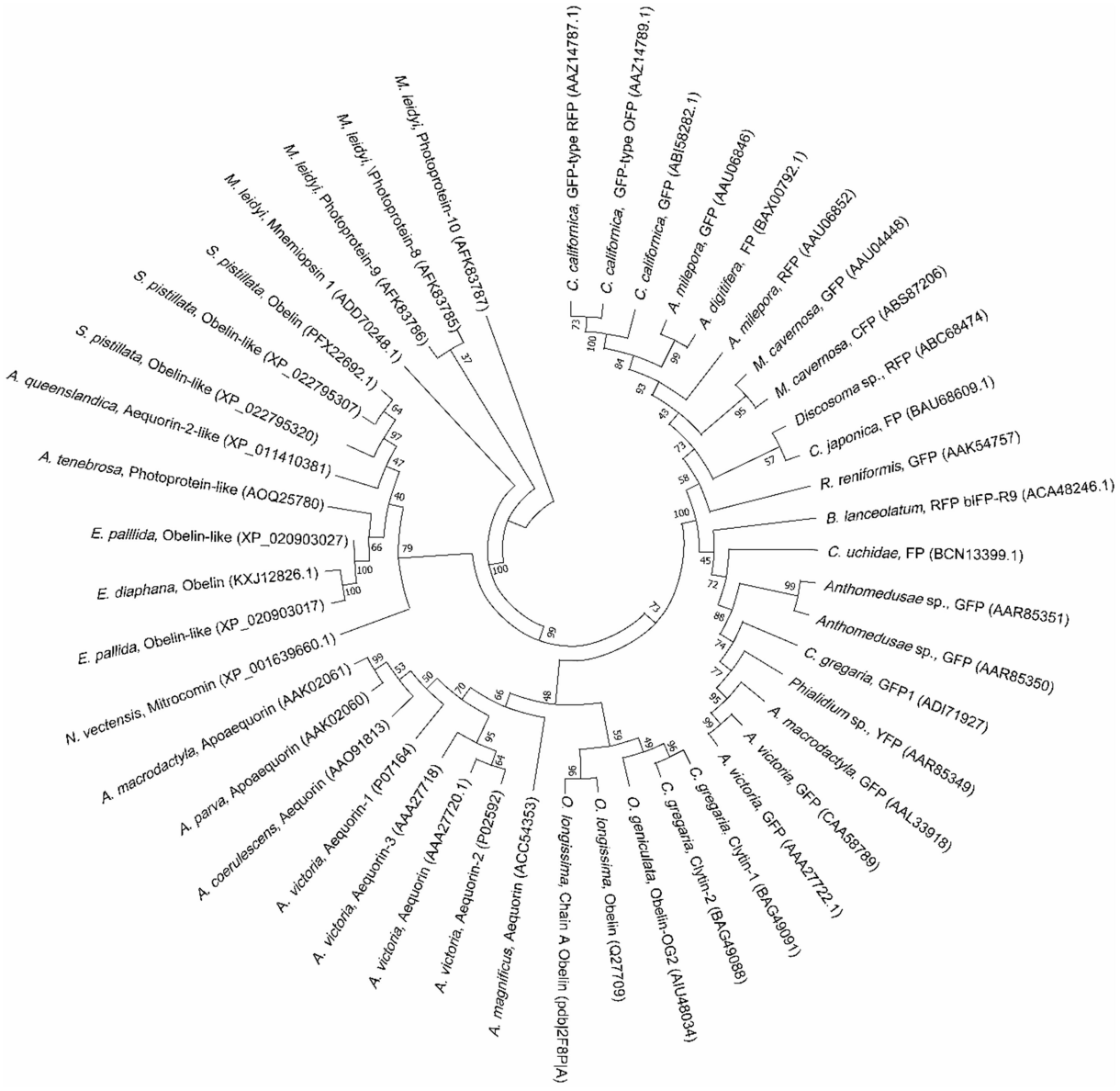

Fig. 1. Phylogenetic trees generated based on evolutionary analysis on the example protein sequences of fluorescent and luminescent proteins in marine organisms. The evolutionary history was inferred using the maximum likelihood method and Whelan & Goldman model (Whelan and Goldman, 2001). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Felsenstein, 1985). Initial trees for the heuristic search were obtained automatically, by applying neighbor joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology based on the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 5.7959)]. All positions with less than 95% site coverage were eliminated, *i.e.*, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). Evolutionary analyses were conducted using MEGA X (Kumar et al., 2018).

of multidisciplinary applications, including gene assays, macromolecule interaction assays, adenosine triphosphate (ATP) determination, biosensors, hygiene control in food industries, *in vivo* imaging in diagnosis, high-throughput screening in drug development, and clinical analysis of novel pandemic infectious diseases (Ramsaran et al., 1998; Sylvia et al., 2000; Bhaumik and Gambhir, 2002; Cook and Griffin, 2003; Kadurugamuwa et al., 2003; Cronin et al., 2008; Comps-Agrar et al., 2011; Amodio and Dino, 2014; Lundin, 2014; Karlsson et al., 2015; Lee et al., 2015; Phillips et al., 2016; Taminiau et al., 2016; Belkin et al., 2017; Morciano et al., 2017; Rincon et al., 2017; Iannotti et al., 2018; Dale et al., 2019; Hoare et al., 2019; Endo and Ozawa, 2020; Esteban Florez et al., 2020; Jonkers et al., 2020; Niu et al., 2020; Ong et al., 2020). Marine organisms are enchanting creatures that possess a wide variety of fluorescent and luminescent proteins which have tremendous value for a broad range of potential applications (Fig. 1). This review first briefly summarizes the functions of fluorescent and bioluminescent proteins found in marine organisms, then focuses on the recent status of applications of these protein systems, and finally proposes their future perspectives.

Roles of fluorescent proteins in nature

The emission of green fluorescence in the hydrozoan medusa *Aequorea victoria* depends on the chemiluminescent protein aequorin, which is composed of apoequorin and coelenterazine. Apoaequorin is a 21-kDa single polypeptide that requires Ca^{2+} and coelenterazine as prosthetic components to release blue light at the wavelength of 470 nm, which is then absorbed by GFP, resulting in the emission of a longer-wavelength green fluorescent light at the wavelength of 508 nm (Shimomura et al., 1962; Shimomura, 1979). The light conversion is driven by a chromophore derived through autocatalytic cyclization of the tripeptide 65-SYG-67 (Shimomura, 1979).

Fluorescent proteins are characterized as having an energy-dispatching function, by means of light scattering, owing to which organisms are capable of protecting themselves against photodamages, such as UVA and radiation (Salih et al., 2000). A previous study demonstrated that reactive oxygen species that occur under the condition of hyperoxia during photosynthesis in algal symbionts are quenched by GFP, thereby suggesting that it has a function of antioxidant protection in *A. victoria* (Bou-Abdallah et al., 2006). The GFP gene found in cephalochordates has also been proposed to play a role in photoprotection against oxy-radicals (Bomati et al., 2009; Yue et al., 2016). The hydrozoan jellyfish *Clytia hemisphaerica* possesses four GFPs, and its strong green fluorescence seems to protect stem cells and mitochondrial DNA against UV light (Fourrage et al., 2014).

Fluorescence is attractive to prey, and several studies have identified the function of fluorescent proteins in predation. The fluorescent tentacles of the siphonophore *Resomia ornicephala* have been shown to serve as prey attractants (Pugh and Haddock, 2010). The GFP in the tentacles of the deep sea anemone *Cribrinopsis japonica* absorbs blue light and emits green fluorescence, thereby suggesting its role in prey attraction (Tsutsui et al., 2016). Another group found that the fluorescent proteins in the tentacle tips of the hydromedusa *Olindias formosus* significantly attracted juvenile rockfish in blue light environments (Haddock and Dunn, 2015).

The intense red fluorescent body pattern observed in the diurnal fish *Cirrhilabrus solorensis* supports deep-sea vision, and the goby *Eviota atriventris* is sensitive to red fluorescence (Warrant and Locket, 2004; Michiels et al., 2008). The role of red fluorescence in vision has been discovered in over 180 species of red fluorescence-emitting marine fish till date (Gerlach et al., 2014; Sparks et al., 2014; Macel et al., 2020). In addition, rays, sharks, and reef fish can display or recognize their own fluorescence, thereby implying that they have the ability to communicate by utilizing it (Heinermann, 1984; Gruber et al., 2016). Research on several copepods and bony fish has suggested other possible roles for fluorescence in mating and camouflage (Shagin et al., 2004; Hunt et al., 2010; Gruber et al., 2016). However, the role of fluorescence in visual recognition among marine organisms remains to be elucidated through further intensive research.

Characteristics of bioluminescent proteins

More than 500 genera dwelling in the ocean, ranging from bacteria to fish, are luminous organisms (Widder, 1999; Haddock et al., 2010; Martini and Haddock, 2017; Shimomura, 2012). Diverse organisms have the ability to visually detect bioluminescence in marine environments, where sunlight or moonlight is poor or unavailable (Widder, 1999, 2002). Daylight declines by nearly 10-fold at every 5 m of depth and completely vanishes below 1,000 m (Widder, 2002). Bioluminescence is considered a major light source in areas where light is rarely available or those that are completely dark. In this context, bioluminescent light is critical for the survival of most deep-sea species, for their hunting, mating, and defense (Widder, 1999; Inouye et al., 2000; Meyer-Rochow, 2007; Stanger-Hall et al., 2007; Haddock et al., 2010).

Since Boyle reported the importance of oxygen in bacterial bioluminescence (Boyle, 1667), other researchers have also investigated the mechanisms of light production (Meyer-Rochow, 2007). In this process, Dubois and Harvey noticed the bioluminescence of the click beetle *Pyrophorus sp.* and marine bivalve mollusc *Pholas dactylus* and discovered the luciferin-luciferase system (Dubois, 1885; Harvey, 1957; Poisson, 2010; Shimomura, 2012). This finding was then extended to Shimomura's discovery of aequorin, the first bioluminescent photoprotein, in the jellyfish *A. victoria* (Shimomura et al., 1962). Functional photoproteins are composed of apoproteins, chromophoric components, and oxygen molecules (Ohmiya and Hirano, 1996). The structure of photoproteins is similar among various species that emit bioluminescence, particularly in Ca^{2+}-binding sites and spatial structures (Stepanyuk et al., 2013). Interestingly, in several dinoflagellates, the light-emitting activity of luciferase is regulated by a luciferin-binding protein, by sequestering the luciferin substrate at higher pH (Liu et al., 2004).

In a typical luciferin-luciferase system, luciferin is enzymatically oxidized and converted into an excited-state anionic species by luciferase. The excited state oxyluciferin then releases fluorescent blue light at wavelengths in the range of 454~493 nm, following which it returns to its ground state (Henry and Michelson, 1978; Shimomura, 2012). As a typical luciferin, the imidazopyrazine compound coelenterazine is oxidized to the excited state coelenteramide oxyluciferin through a dioxetanone intermediate, resulting in the release of bioluminescence (Shimomura and Johnson, 1978). Unlike insect d-luciferin, coelenterazine is not ATP-dependent for activation (Ohmiya and Hirano, 1996). It was first identified in deep-sea shrimp and copepods (Shimomura et al., 1978; Markova et al., 2019), and is utilized as a substrate for luminescence generation by approximately 15 different luciferases, including the most common luciferases, the sea pansy *Renilla reniformis* luciferase (Rluc), marine copepod *Gaussia princeps* luciferase (Gluc), and another marine copepod *Metridia longa* luciferase (Mluc) (Lorenz et al., 1991; Inouye et al., 2000; Verhaegent and Christopoulos, 2002; Markova et al., 2004; Stepanyuk et al., 2008; Takenaka et al., 2008; Titushin et al., 2008; Takenaka et al., 2012). These luciferases are smaller (~34 kDa Rluc and ~20 kDa Gluc and Mluc) than the luciferase found in terrestrial insects, Fluc (~62 kDa), which makes them appropriate for various applications (Syed and Anderson, 2021).

It is common to observe a similar protein structure for luciferases among marine species. For instance, bioluminescent marine dinoflagellates exhibit highly conserved central domains in their luciferases (Liu et al., 2004). Interestingly, the luciferin in the marine ostracod *Vargula hilgendorfii* showed a cross-reaction with luminescent fish luciferases, thereby giving rise to questions about its evolutionary origin (Thompson et al., 1989). Similar cross-reactivity has also been observed between Euphausiid krill and dinoflagellates (Nakamura et al., 1988). The discovery of more unknown luciferin-luciferase systems is an active field of research, which will broaden our knowledge of their ecological importance in marine environments and future applications.

Collaborations between fluorescent and bioluminescent proteins

Fluorescence and bioluminescence can exist simultaneously and also interact with the same organism or similar habitats. In this case, bioluminescence acts as a source of light energy for fluorescence generation. For example, when aequorin releases blue luminescent light, this energy is then absorbed by GFP, resulting in the emission of a longer-wavelength green fluorescent light (Shimomura et al., 1962; Shimomura, 1979). Aequorin, therefore, is regarded as a blue fluorescent protein. In *Renilla*, the blue light released from luciferase Rluc is transferred to the fluorophore of a nearby GFP, resulting in the emission of green fluorescent light at the wavelength of 510 nm (Wang et al., 1998). The siphonophore *Erenna sirena* converts luminescence generated from its luminescent photophore to fluorescence using tentacles and releases yellow to red light in the wavelength range of 583~680 nm (Haddock et al., 2005). Cnidarians are capable of collecting light energy by fluorescence from the blue luminescence produced in deep-sea habitats (Matz et al., 2006). Interestingly, in several dinoflagellates, luminescent organs are bifunctional. For example, luminescent photophores in the dinoflagellate alga *Gonyaulax* are considered autofluorescent organs that convert blue luminescence into green light using GFP (Johnson et al., 1985). Considering the light-limited world of the deep sea, it is not surprising to observe interactions between fluorescence and bioluminescence. Consequently, it would not be unreasonable to anticipate that there could be further undiscovered events, because the lives in the marine ecosystem are connected to each other for multiple purposes.

Applications

In basic research, fluorescent and bioluminescent proteins are utilized as reporters to trace the expression of specific genes of interest. The recombinant GFP-tagging strategy has been widely

Table 1. Examples of experimental and industrial applications of bioluminescent proteins presented on this review

Applied protein	Application	Examples of study
GFP with aequorin of coelenterazine	BRET NanoBRET	Detecting interactions between the target proteins of G protein-linked receptors and p53 involved in the oncological progress (Dudgeon et al., 2010; Comps-Agrar et al., 2011). *In vivo* protein-ligand interactions (Hoare et al., 2019).
Gluc Fluc	Split luciferase assay	Pharmaceutical validation by visualizing the interaction dynamics (Remy and Michnick, 2006). Detection of SARS-Cov-2 infection (Lan et al., 2020; Shang et al., 2020).
Fluc with D-luciferin Rluc	*In vivo* monitoring	Identifying the entry sites of encephalitis viruses into the mouse central nervous system (Phillips et al., 2016). Characterizing the HCoV-OC43 strain in the central nervous system (Niu et al., 2020).
GFP and other bioluminescent proteins	Bioreporters	Detecting various compounds including toluene, genotoxic chemicals, and heavy metals (Applegate et al., 1998; Lee et al., 2007; Charrier et al., 2011; Wang et al., 2013).
Rluc	High-throughput screening system	Quantifying viral replication of HCoV-OC43 (Shen et al., 2019).
Various proteins	Detection of ATP concentration	Monitoring hygiene control in the healthcare and food industries (Dostálek and Brányik, 2005; Amodio and Dino, 2014; Baba et al., 2018; Rodriguez and Hooper, 2019).

adopted for targeting proteins and is now a common tool in molecular biology, cell biology, and biomedicine (Shimomura et al., 1962; Mocz, 2007; Scott et al., 2011). Bioluminescence properties have been widely accepted in the field of applications (Table 1). Early studies adopted bioluminescence resonance energy transfer (BRET) assays for protein-protein interaction studies (Pfleger and Eidne, 2006; England et al., 2016; Dale et al., 2019). In this system, fluorescent and bioluminescent proteins function in a co-operative sequential reaction. An excited-state aequorin or coelenterazine serves as a donor of non-radiative energy and transfers the energy to a proximate acceptor, GFP, followed by the emission of fluorescent light. The proximal distance between the energy donor and acceptor molecules determines the fraction of the energy transfer. This system has been used to study the target proteins of G protein-linked receptors and p53 involved in the oncological progress (Dudgeon et al., 2010; Comps-Agrar et al., 2011). Moreover, the recently developed NanoBRET system has been used to identify *in vivo* protein-ligand interactions using red light-emitting acceptors (Hoare et al., 2019). The split luciferase assay is another major technology that has been used to study protein-protein interactions (Wehr and Rossner, 2016). This system is based on diverse luciferases that are divided into two functional fragments, N-terminal and C-terminal domains, each of which is designed to build a fusion protein of interest. The presence of a ligand recruits the two separate proteins into close proximity, subsequently leading to the reassembly of the two luciferase domains and restoration of the functional protein. A previous study applied Gluc to this assay system, and conducted pharmaceutical validation by visualizing the interaction dynamics (Remy and Michnick, 2006). Luciferase assays have also been used to detect various coronavirus infections in diverse cells (Zhao et al., 2013; Yang et al., 2014). In a study on SARS-Cov-2, genes of both Fluc and the viral spike glycoprotein were introduced into host cells, and the resulting recombinant viral particles containing the bioluminescent reporter Fluc were then used to identify the cellular receptor angiotensin-converting enzyme 2 (Lan et al., 2020; Shang et al., 2020). In other studies, aequorin has been used as a labeling molecule for tumor necrosis factor-alpha, Forssman antigen, cytokine, and protein A (Erikaku et al., 1991; Stults et al., 1992; Xiao et al., 1996; Zatta, 1996). It has also been shown that

a limited amount of prostate-specific antigen can be detected by means of an expression immunoassay using aequorin (White and Christopoulos, 1999; Dragulescu-Andrasi et al., 2009; Byun et al., 2019). Several other studies have reported the function of bioluminescence as sensors, for detection of acidosis, reactive oxygen species, nitric oxide, and Ca^{2+} signaling (Takakura et al., 2015; Pelentir et al., 2019; Ong et al., 2020).

Bioluminescence offers versatile applications for imaging tumor progression (Choy et al., 2003), and has been used in several cancer studies conducted on the breast, colon, and prostate using animal models (Caceres et al., 2003; Scatena et al., 2004; Zeamari et al., 2004). *In vivo* monitoring is a prominent advantage that bioluminescence offers in laboratory experiments. Light from living cells circumvents the problem of biopsy, which requires that the experimental animals be sacrificed in order to observe the effect of a certain gene expression (Syed and Anderson, 2021). For instance, Fluc and D-luciferine have been used to identify the entry sites of encephalitis viruses into the mouse central nervous system (Phillips et al., 2016). In another study, Rluc and coelenterazine were used in live mice, to characterize the HCoV-OC43 strain in the central nervous system (Niu et al., 2020). In the industrial sector, bioluminescence has been utilized in a broad spectrum of applications, with distinct advantages (Syed and Anderson, 2021). The effects of antibiotics have been evaluated over time using animal models. A study genetically engineered target bacteria with the lux operon prior to injection, thus allowing for monitoring of the effect of an antibiotic *in vivo* in the injected mice (Berger et al., 2017). This advantage can also be applied in photodynamic therapy. In a previous report, bioluminescent bacteria were loaded on the dermal abrasion site of mice treated with photosensitizers, following which the photodynamic therapy efficacy was visually evaluated under red light (Vecchio et al., 2013). There is another interesting possibility for the application of bioluminescent protein as bioreporters. Bioluminescent bioreporters have been shown to effectively detect various compounds including toluene, genotoxic chemicals, and heavy metals (Applegate et al., 1998; Lee et al., 2007; Charrier et al., 2011; Wang et al., 2013). In another study, a recombinant bacterial strain functioning as a trinitrotoluene sensor was constructed by placing a trinitrotoluene-inducible promoter in front of the GFP gene. Sensing for this system was performed using a laser-based optoelectronic system and scanner (Belkin et al., 2017). In the same context, a similar construction of a recombinant strain could be developed for the detection of terrorist compounds. Bioluminescence could be a useful tool in the pharmaceutical industry, because novel drug discovery demands high-throughput screening systems. In a recent report, Rluc was inserted into the ns2 accessory gene of the coronavirus strain HCoV-OC43, following which the recombinant strain was transfected into BHK-21 cells. The luminescent light emitted from this system upon addition of coelenterazine was then measured to quantify viral replication. Interestingly, this measurement was significantly reduced by a new antiviral drug being tested, thus demonstrating its efficacy. The group successfully tested 2,000-compound libraries using this robust screening system (Shen et al., 2019). In addition, the bioluminescence of insects is suitable for discriminating not only live and dead cells, but also healthy and diseased cells, by utilizing their sensitive detection mechanism of ATP concentration (Zhang et al., 2010; Bird et al., 2014; Lee et al., 2015; Palikaras and Tavernarakis, 2016). This unique feature of the ATP bioluminescence system has been applied to monitor hygiene control in the healthcare and food industries (Dostálek and Brányik, 2005; Amodio and Dino, 2014; Baba et al., 2018; Rodriguez and Hooper, 2019). The respiratory chain of bioluminescent bacteria is directly connected to bioluminescence output, and thus, their luminescence system is utilized as a monitoring sensor in ecotoxicology (Fukuba et al., 2011; Hassan et al., 2016; Hansen et al., 2019).

Conclusions and Future perspectives

Even though this review discusses many examples of the applications of marine fluorescence and bioluminescence, a wide spectrum of developments using these stunning lighting machineries are still possible in the future, with active basic studies on these proteins being published regularly. Fluorescent and bioluminescent proteins possess fascinating properties, which can be functionally enhanced using genetic engineering. For example, mutant luciferases of Rluc have been developed to achieve brighter and more stable enzymes, which have demonstrated enhanced BRET efficiency in *in vitro*/*in vivo* imaging and Ca^{2+} ion-detection ability (Loening et al., 2006; Takai et al., 2015; Suzuki et al., 2016). Moreover, the luciferase from the deep sea caridean shrimp *Oplophorus gracilirostris* (Oluc) has been engineered by means of mutagenesis to reveal improved light emission and thermal stability (Hall et al., 2012). With the continuous development of functionally enhanced enzymes and substrates, the application area of these proteins could be expanded. Concurrently, the discovery of new luciferin-luciferase and photoprotein systems could result in further enhanced output, by building various combinations of the substrate and enzyme, or fluorescence and luminescence. In

addition, Antarctic marine environments have diverse ecosystems, which preserve and offer tremendous potential resources for the development of future biotechnology. Consequently, it is not difficult to anticipate the discovery of novel fluorescent and bioluminescent proteins in Antarctic marine species from either shallow or deep water, which would contribute to a comprehensive understanding of the entire marine ecosystem as well as the development of beneficial applications for humans.

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Conflicts of interest

The authors declare no conflicts of interest.

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