

Coelenterazine-Dependent Luciferases

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Abstract—Bioluminescence is a widespread natural phenomenon. Luminous organisms are found among bacteria, fungi, protozoa, coelenterates, worms, molluscs, insects, and fish. Studies on bioluminescent systems of various organisms have revealed an interesting feature – the mechanisms underlying visible light emission are considerably different in representatives of different taxa despite the same final result of this biochemical process. Among the several substrates of bioluminescent reactions identified in marine luminous organisms, the most commonly used are imidazopyrazinone derivatives such as coelenterazine and *Cypridina* luciferin. Although the substrate used is the same, bioluminescent proteins that catalyze light emitting reactions in taxonomically remote luminous organisms do not show similarity either in amino acid sequences or in spatial structures. In this review, we consider luciferases of various luminous organisms that use coelenterazine or *Cypridina* luciferin as a substrate, as well as modifications of these proteins that improve their physicochemical and bioluminescent properties and therefore their applicability in bioluminescence imaging *in vivo*.

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Bioluminescence is a widespread natural phenomenon. Luminous organisms are found among bacteria, fungi, protozoa, coelenterates, worms, molluscs, insects, and fish. There are now known several thousands of bioluminescent species represented by more than 700 genera. Although luminous species can be found among terrestrial organisms (bacteria, insects, worms, and fungi), the ability for bioluminescence is still most widespread in marine dwellers (Fig. 1). At great depths, for example, where light from the surface never penetrates, more than 90% of organisms are luminous [1, 2]. It is assumed that bioluminescence is related to the visual function of organisms and is often used for interspecific and intraspecific communication, for camouflage, for scaring away predators by the use of light flashes, and for attracting prey. However, in many cases the role of bioluminescence for the functioning of certain organisms is still unclear.

Biochemical studies on bioluminescent systems of various organisms have revealed an interesting feature of bioluminescence – the mechanisms underlying visible light emission are considerably different in representatives of different taxa, despite the same final result of this biochemical process. Not only the substrates and cofactors involved in bioluminescent reactions are distinct, but

the enzymes that catalyze these reactions differ too [3]. Therefore, the terms “luciferase” and “luciferin” applied to enzymes and substrates of bioluminescent reactions are rather generalizing and functional than structural and chemical concepts.

Among the several substrates of light-emitting reactions identified in luminous marine organisms, the imidazopyrazinone-type luciferins are most frequently found (Fig. 2a). Coelenterazine was identified as a substrate of bioluminescent reactions of such taxonomically distant luminous organisms as the soft coral *Renilla* [4], the decapods *Oplophorus* [5, 6], the scyphozoan medusa *Periphylla* [7, 8], the copepods [9, 10], the ostracods *Conchoecia* [11], the cephalopods *Vampyroteuthis* [12], the fish *Benthoosema pterotum* [13], and others (Fig. 1). The hydromedusan and ctenophore Ca^{2+} -regulated photoproteins contain a 2-hydroperoxy-derivative of coelenterazine [3]. The squids *Watasenia* and *Symplectoteuthis*, as well as the mollusc *Pholas dactylus*, use coelenterazine derivatives as the substrates of bioluminescent reactions – coelenterazine disulfate in the case of *Watasenia* [14], and dehydrocoelenterazine in the cases of *Symplectoteuthis* [15] and *Pholas dactylus* [16]. *Cypridina* (*Vargula*) luciferin is a substrate of luciferases of ostracods [17], as well as of fish *Porichthys notatus*, *Apogon*, and *Parapriacanthus* [18]. Most probably, many luminous

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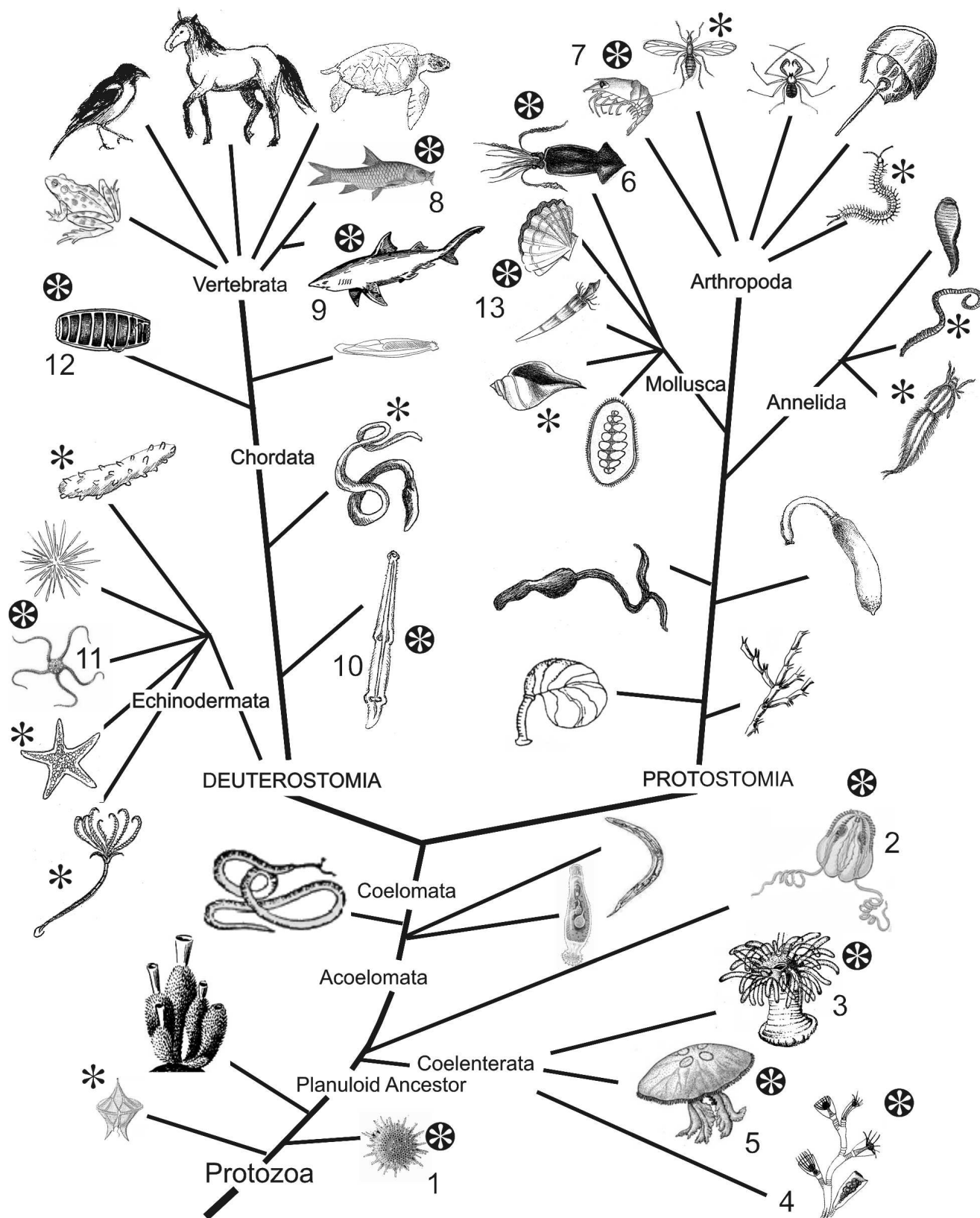


Fig. 1. Phylogenetic tree. Taxa including luminous species are marked with black asterisk. The so far identified phylogenetic groups whose representatives use imidazopyrazinone-type luciferin as a bioluminescent reaction substrate are marked with white asterisk: radiolarians (1), ctenophores (2), coelenterates (coral polyps (3), hydroids (4), scyphozoan jellyfish (5)), squids (6), crustaceans (7), some bony fishes (8) and sharks (9), chaetognaths (10), ophiurs (11), tunicates (12), molluscs (13).

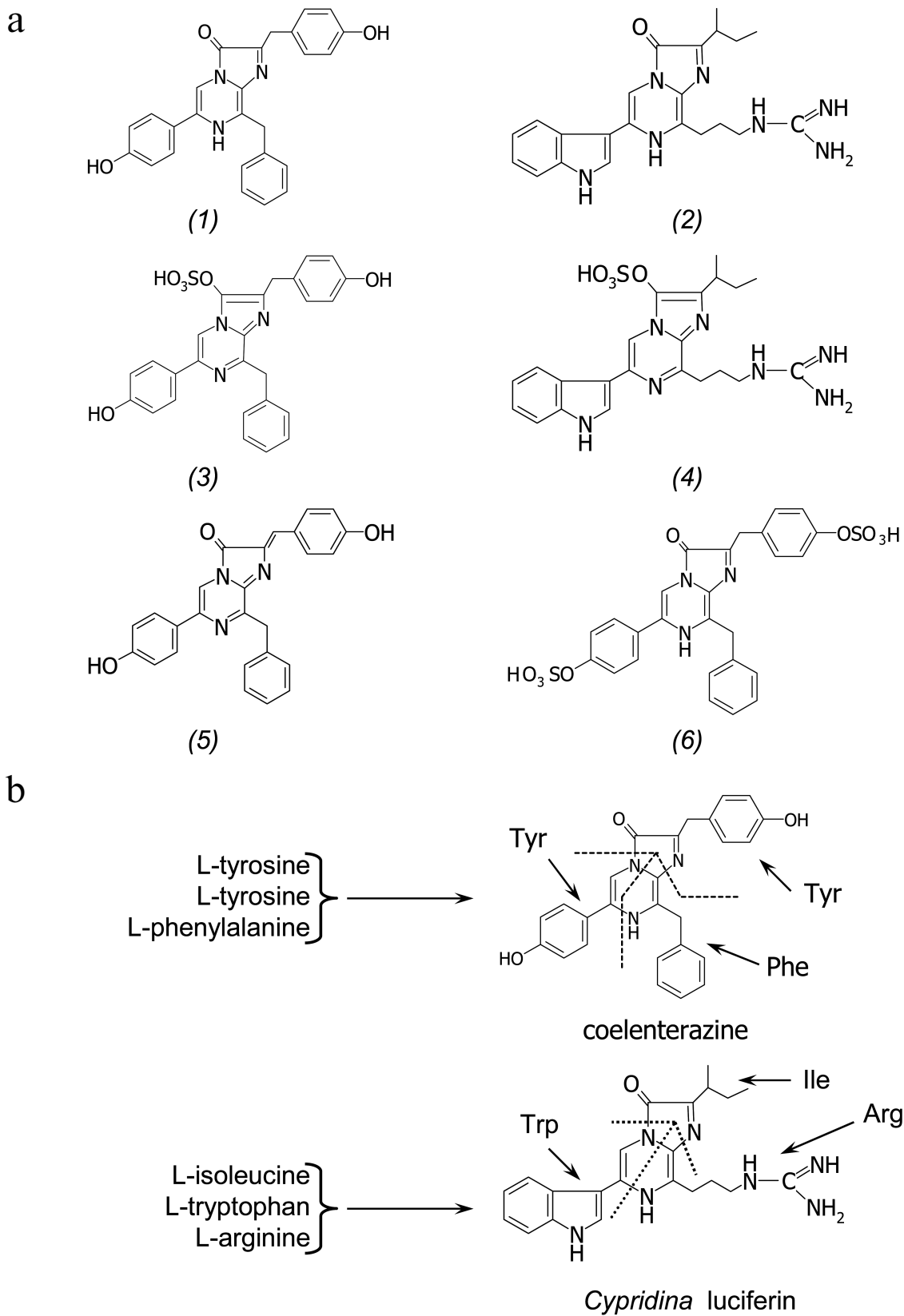


Fig. 2. a) Imidazopyrazinone-type luciferins: coelenterazine (1), *Cypridina* (*Vargula*) luciferin (2), 3-enol sulfate of coelenterazine (3), 3-enol sulfate of *Cypridina* luciferin (4), dehydrocoelenterazine (5), and coelenterazine disulfate (6). b) The hypothesized scheme of biosynthesis of coelenterazine and *Cypridina* luciferin in copepods and ostracods from L-amino acids.

organisms that use imidazopyrazinone-type luciferin for bioluminescence obtain these compounds from their diet, as do the luminous fish *Porichthys notatus* (*Cypridina* luciferin) [19, 20] and jellyfish *Aequorea* (coelenterazine) [21].

The *de novo* synthesis of coelenterazine and *Cypridina* luciferin has been shown only for luminous crustaceans – copepods and ostracods of the family Cypridinidae. With isotopically labeled L-amino acids to feed the crustaceans *Metridia pacifica* and *Cypridina* (*Vargula*) *hilgendorffii* applied, the copepods were found to synthesize coelenterazine from two tyrosines and one phenylalanine [22], while *Cypridina* luciferin in ostracods is synthesized from tryptophan, isoleucine, and arginine [23] (Fig. 2b). The imidazopyrazinone ring is apparently formed either by cyclization of a tripeptide (H_2N -Phe-Tyr-Tyr-COOH in the case of coelenterazine and H_2N -Arg-Trp-Ile-COOH in the case of *Cypridina* luciferin) that runs like the formation of the GFP chromophore [3] and includes an additional step of decarboxylation, or in a stepwise manner by nonribosomal synthesis [24], which we believe is most likely. It is noteworthy that coelenterazine was also found in rather large amounts in many non-luminous marine animals [3, 25].

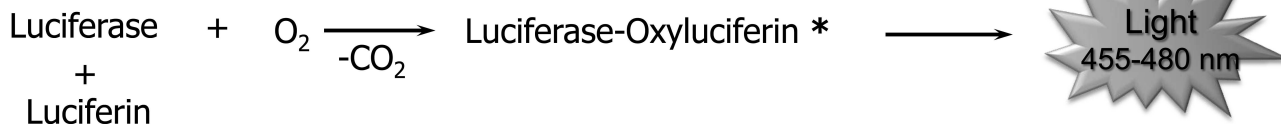
A special place among imidazopyrazinone-type luciferins belongs to 3-enol sulfate derivatives (Fig. 2a). These compounds have been found in marine luminous

coral *Renilla reniformis* [26] and ostracods *Vargula* (*Cypridina*) *hilgendorffii* [27]. The 3-enol sulfate derivatives of coelenterazine and *Cypridina* luciferin are actually pre-luciferins. These compounds are enzymatically converted into the active substrate by a sulfotransferase catalyzing sulfate group transfer from coelenterazine 3-enol sulfate (or *Cypridina* luciferin 3-enol sulfate) to an acceptor, adenosine 3',5'-diphosphate (PAP) [26, 27]. As a result, coelenterazine (or *Cypridina* luciferin) and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) are formed. This reaction is reversible, i.e. sulfotransferase can catalyze the formation of 3-enol sulfate derivatives of imidazopyrazinones as well [27]. In this case, the PAPS is the donor of a sulfate group. Since 3-enol sulfate derivatives of imidazopyrazinones are much more stable in aqueous solutions, it is assumed that these compounds provide a storage form of a substrate in marine luminous organisms that use either coelenterazine or *Cypridina* luciferin for bioluminescence.

MAIN TYPES OF COELENTERAZINE-DEPENDENT BIOLUMINESCENT SYSTEMS

Bioluminescent systems using imidazopyrazinone compounds as the reaction substrates can be divided into photoprotein and luciferase types (Fig. 3). Biolumines-

Luciferase type



Photoprotein type (Ca^{2+} -regulated photoprotein)

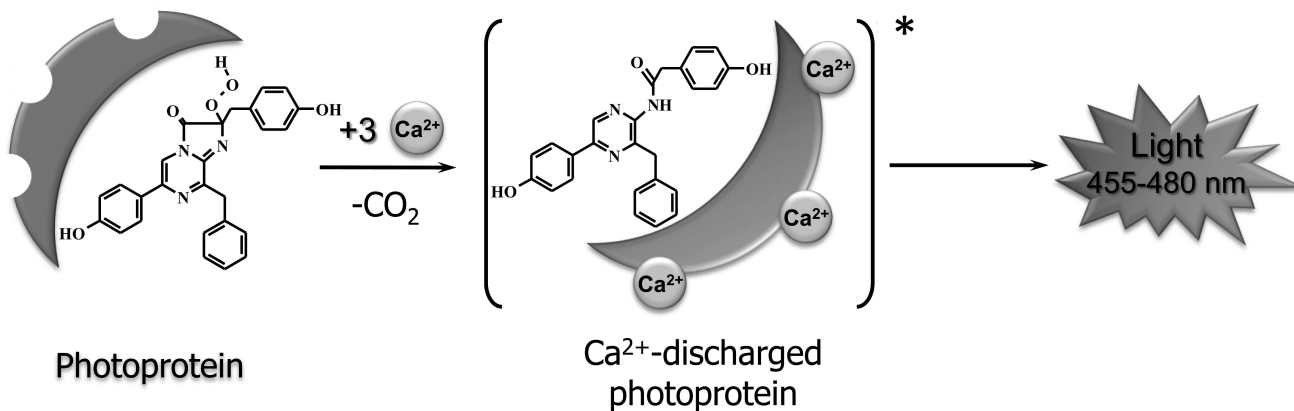


Fig. 3. General scheme of bioluminescent reactions of luciferase and photoprotein types. The reaction of Ca^{2+} -regulated photoprotein, which is a stable complex of the protein with coelenterazine activated by oxygen, 2-hydroperoxycoelenterazine, is shown as an example of photoprotein type. Bioluminescence is initiated by binding of calcium ions with protein Ca^{2+} -binding sites.

cent reactions catalyzed by luciferases are typical enzymatic reactions in which the substrate is oxidized by oxygen with generation of a product (oxyluciferin) in the excited state, whose relaxation to the ground state is accompanied by light emission. Luciferase as the usual enzyme turns over several times, completely utilizing the substrate. The bioluminescent systems of the marine copepods, shrimp *Oplophorus*, ostracods, scyphozoan medusa, and coral *Renilla* are of this type [3]. In the case of bioluminescent systems of photoprotein type, the protein forms a stable enzyme–substrate complex existing for a long time. The most studied hydromedusan and ctenophore Ca^{2+} -regulated photoproteins [3, 28–30], for example, are a complex of apoprotein and tightly but noncovalently bound 2-hydroperoxycoelenterazine, coelenterazine activated by oxygen that arises on the formation of a photoprotein from apoprotein, coelenterazine, and molecular oxygen.

A bioluminescent reaction is initiated in response to binding of calcium ions with Ca^{2+} -binding sites on the surface of the protein molecule. Binding of Ca^{2+} causes small conformational changes in a substrate-binding cavity of the protein, which disturb the hydrogen bond network that stabilizes 2-hydroperoxycoelenterazine, thereby triggering the reaction of oxidative decarboxylation leading to the formation of the product in the excited state [31]. Unlike

Ca^{2+} -regulated photoproteins, the photoproteins of squid *Symplectoteuthis oualaniensis* [15] and mollusc *Pholas dactylus* [16], simplectin and pholasin, are a complex of a protein with a covalently bound coelenterazine, which is formed upon binding of dehydrocoelenterazine with the apoprotein. The bioluminescent reaction of the photoproteins of squid and mollusc is initiated by O_2 and K^+ [15] and the reactive oxygen species [16], respectively. Since a substrate whose oxidation is necessary for light emission is already bound in photoproteins, these, unlike luciferases, can react only once, i.e. cannot turn over several times like a usual enzyme. It should be noted that since the reaction involves one molecule, the amount of the emitted light is always in proportion to the amount of the protein. This feature is very suitable for the use of photoproteins as labels in various types of bioluminescent assays [29].

DIVERSITY OF COELENTERAZINE-DEPENDENT LUCIFERASES

Despite the wide variety of luminous marine organisms that use coelenterazine as a substrate in bioluminescent reaction, genes encoding only four types of coelenterazine-dependent luciferases have been cloned thus far (Table 1). In addition, some more luciferases from differ-

Table 1. Cloned luciferases that use coelenterazine or *Cypridina* luciferin as a bioluminescent reaction substrate

Luciferase, source organism, type	Animal type (number of cloned isoforms of luciferase)	M_r , kDa	λ_{max} , nm	Reference
Substrate: coelenterazines				
<i>Renilla</i> luciferase, soft coral (intracellular)	<i>Renilla reniformis</i> (1) <i>R. muelleri</i> (2)	36	480	[35] [34, 36]
Luciferases of copepods, copepods of superfamily Augaptiloidea (secreted)	<i>Metridia longa</i> (3), <i>M. curticauda</i> (2), <i>M. asymmetrica</i> (2), <i>M. pacifica</i> (4), <i>M. okhotensis</i> (2)	18–24	485	[10, 37–39, 42, 43]
	<i>Gaussia princeps</i> (1)	20	473	[34]
	<i>Pleuromamma scutullata</i> (2), <i>P. xiphias</i> (3), <i>P. abdominalis</i> (2)	20–23	490–493	[42, 43]
	<i>Lucicutia ovaliformis</i> (3)	24	493	[42, 43]
	<i>Heterorhabdus tanneri</i> (4)	20–21	–	[42, 43]
	<i>Heterostylites major</i> (2)	23	–	[42]
Luciferase <i>Oplophorus</i> , crustacean of the family Opolophoridae (secreted)	<i>Oplophorus gracilirostris</i> (1) (decapod shrimp)	22*	454	[33]
Substrate: <i>Cypridina</i> luciferin				
<i>Cypridina</i> luciferases, ostracods of the family Cypridinidae (secreted)	<i>Vargula hilgendorffii</i> (1)	62	465	[40]
	<i>Cypridina noctiluca</i> (1)			[41]

* For the luciferase of *Oplophorus*, the characteristics of only the catalytic subunit are given.

Table 2. Partially characterized luciferases that use coelenterazine as a bioluminescent reaction substrate

Luciferase	Marine animal type	M_r , kDa	λ_{\max} , nm	Reference
Luciferase <i>Periphylla</i> (intracellular), soluble and insoluble (L)	<i>Periphylla periphylla</i> (scyphozoan medusa of the order Coronatae)	~20 ~32 (L)	465	[8]
Luciferase <i>Benthoosema</i> (intracellular)	<i>Benthoosema pterotum</i> (anchovy, fish of the family Myctophidae)	~27	475	[13]

ent taxa have been partially characterized (Table 2). All cloned coelenterazine-dependent luciferases (Table 1) belong to cofactor-independent monooxygenases as they catalyze a simple reaction (Fig. 3) involving only luciferase, substrate (coelenterazine or *Cypridina* luciferin), and molecular oxygen. Of these, only luciferase of the soft coral of the *Renilla* genus is an intracellular protein; the other luciferases cloned from phylogenetically distant groups of marine crustaceans are secreted luciferases. Luciferase and luciferin accumulated separately in a bioluminescent secretory gland of the crustacean are secreted via special pores into seawater [32] where the luciferase oxidizes substrate by the dissolved oxygen with the emission of light.

Natural luciferases of *Renilla* and ostracods of the Cypridinidae family were isolated as monomeric proteins from the corresponding luminous organisms. In contrast to these, the natural secreted luciferase of decapod shrimp *Oplophorus gracilirostris* was isolated as a tetramer consisting of two types of proteins [33], with only one revealing catalytic activity (Table 1). The natural form of secreted luciferases of marine planktonic copepods is unknown, since they have not been isolated. The first luciferases of copepods *Metridia longa* [10] and *Gaussia princeps* [34] were cloned by functional screening of cDNA gene libraries, while the cDNA genes encoding the other homologous luciferases of copepods (Table 1) were isolated using degenerate primers constructed from the known sequences [42, 43].

The sequence analysis of the cloned coelenterazine-dependent luciferases clearly shows that luciferases of all four types belonging to phylogenetically distant groups of organisms reveal no homology and are totally different proteins with different molecular masses (Table 1) and properties, although they catalyze the oxidation of the same substrate with the emission of light.

Two other coelenterazine-dependent luciferases from different taxa were isolated and partially characterized (Table 2). Cloning of the luciferase of fish *Benthoosema pterotum* should be forthcoming [13], as the pure protein that can be used to determine its sequence and consequently the following isolation of the luciferase gene has already been achieved. Taking into account the diversity of coelenterazine-dependent luciferases, it will most likely be a completely new luciferase sequence.

Luciferase of the coral *Renilla*. Luciferase of corals of genus *Renilla* (class Anthozoa) (RLuc) was one of the first cloned [35] and thus far the only cloned intracellular coelenterazine-dependent luciferase. RLuc is a single-chain protein with a molecular mass of ~36 kDa consisting of 311 amino acids. Apart from luciferase, the bioluminescence of *Renilla in vivo* involves at least two more proteins – a Ca^{2+} -regulated coelenterazine-binding protein (CBP) [44], which stabilizes the substrate, and the green fluorescent protein (GFP) [45], which is a secondary emitter (Fig. 4). The bioluminescent system of *Renilla* is well studied – all three proteins have been cloned [34-36] and characterized, and their three-dimensional structures have been determined [46-48].

Unlike Ca^{2+} -regulated photoproteins containing 2-hydroperoxycoelenterazine, the coelenterazine-binding protein contains a tightly but noncovalently bound molecule of the native coelenterazine in the inner hydrophobic cavity [47]. The binding of calcium ions by Ca^{2+} -binding sites of CBP initiates small conformational changes in the protein that are enough to make the coelenterazine molecule available for oxidation by RLuc. In the absence of GFP, the oxidation of coelenterazine is accompanied by blue light emission with a maximum at $\lambda_{\max} \sim 480$ nm (Fig. 4). In the presence of GFP, a bioluminescent reaction, both *in vivo* and *in vitro*, is accompanied by light emission with $\lambda_{\max} \sim 510$ nm, which is due to Forster resonance energy transfer (FRET) from a bioluminescent donor (luciferase) to a fluorescent acceptor (GFP) as a result of the formation of a protein–protein complex. The formation of an RLuc–GFP complex was shown in experiments *in vitro* [45]. It should be noted that GFP not only shifts the emission spectrum to longer wavelengths, but also significantly increases the quantum yield of the bioluminescent reaction. The efficiency of the luciferase reaction *in vitro* is also increased several-fold when CBP is used as a substrate instead of free coelenterazine [36], which may be due to the formation of CBP–RLuc complex during the reaction [48]. Hence, it can be assumed that the *in vivo* bioluminescent reaction of *Renilla* occurs with the formation of a triple short-lived protein–protein complex CBP–RLuc–GFP, where coelenterazine is delivered into the luciferase active site and the efficient non-radiative energy transfer from the excited product to the chromophore of GFP with following emission of green light takes place.

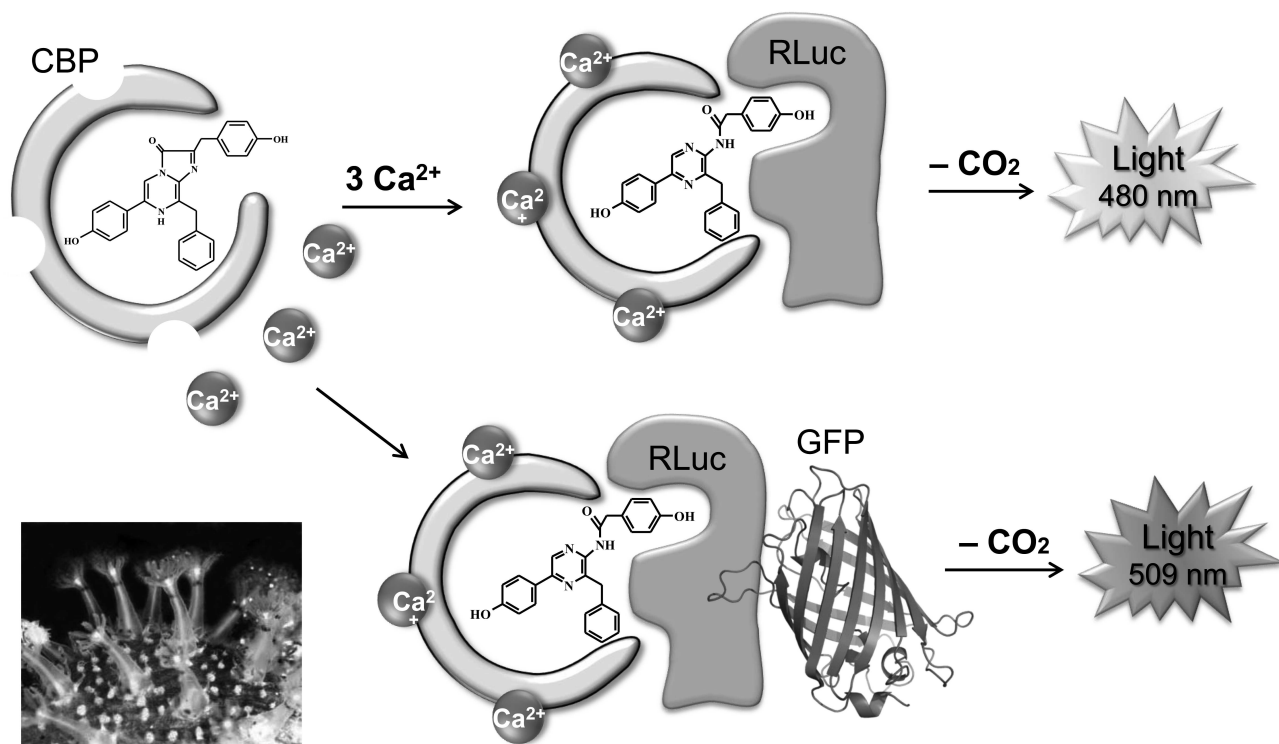


Fig. 4. Schematic representation of bioluminescent reaction of *Renilla* luciferase. After binding of three calcium ions, the CBP conformation changes so that coelenterazine becomes available for luciferase. The reaction in the presence of GFP is accompanied by emission with a maximum at ~ 509 nm; in the absence of GFP, the emission is at ~ 480 nm.

Proteins involved in the bioluminescent reaction of the coral *Renilla* are packaged in specialized cells called photocytes. Luminescence is under the control of the nervous system and appears on increase in concentration of calcium ions in photocytes in response to various stimuli [49]. Thus, CBP not only protects coelenterazine from spontaneous oxidation [50], but also performs the function of connecting the nervous and bioluminescent systems, thereby providing a quick light response of the animal to stimuli.

The amino acid sequence of RLuc has no homology with those of other coelenterazine-dependent luciferases, including photoproteins, but reveals similarities with the primary sequences of α/β -hydrolases [51]. RLuc reveals the greatest homology with the amino acid sequences of bacterial dehalogenases LinB of the α/β -hydrolases family. The degree of similarity with haloalkane dehalogenase LinB from *Sphingomonas paucimobilis*, for example, is 64% (identity – 39%) (Fig. 5a), which clearly implies evolution from a common ancestral sequence. The spatial structure of RLuc also reveals the typical α/β -hydrolase fold ($C\alpha$ RMSD ~ 1.5 Å) (Fig. 5, b and c) [46]. In this case, the difference in structures of luciferase and α/β -hydrolases is even less than that in structures of the related hydromedusan photoproteins aequorin and obelin ($C\alpha$ RMSD ~ 2.27 Å) [52] and functionally identical photoproteins of jellyfish and ctenophores ($C\alpha$ RMSD ~ 2.19 Å) [53].

RLuc is now one of the most popular bioluminescent reporters in cellular and biomedical research as it can easily be expressed in the native state in almost all cell types. The temperature optimum for the bioluminescence reaction *in vitro* catalyzed by the wild-type RLuc is 18–37°C, and the pH optimum is 6–7. However, the quantum yield of bioluminescence is quite low, i.e. ~ 0.07 at 23°C [54]. Being probably optimal for functioning together with CBP and GFP in photocytes of animals, these properties of RLuc cannot always meet the requirements for reporter proteins, e.g. for their use in mammalian cells and tissues. RLuc-based reporters with improved properties were constructed in a standard way – by random mutagenesis with the following selection of suitable variants and by site-directed mutagenesis. To select amino acids for site-directed mutagenesis, the spatial structure of RLuc was used. RLuc mutants with higher specific bioluminescence activity and increased resistance to inactivation by blood serum were obtained [51, 55]. One of the optimized variants with eight mutations (RLuc8) revealed 4-fold increase in specific activity and the 200-fold increase in resistance to inactivation by blood serum.

Animal tissues are known to significantly absorb visible light. The lowest absorption is observed in the range of 600–900 nm, the so-called “transparency window” of biological tissues [56]. Thus, luciferases as reporters emit-

ting the largest number of photons in this spectral region will provide higher sensitivity when used in intact animal tissues. The mutants RLuc-535 and RLuc-547 constructed for these purposes have the increased specific bioluminescent activity and emission spectrum shifted to longer wavelengths by up to 66 nm [57]. Another way to change the emission spectrum is the use of modified coelenterazine as a substrate. For instance, the use of coelenterazine ν shifted the bioluminescence spectrum to longer wavelengths for RLuc and its mutants by almost 40 nm

[50, 57]. The spectral maximum of light emission of the mutant RM-Y with a natural coelenterazine is at 535 nm, while that with coelenterazine ν is at 574 nm.

Luciferases of copepods. These small, secreted proteins of ~18.4-24.3 kDa, including a signal peptide for secretion, are responsible for the bioluminescence of some marine copepods. The first cloned luciferases GpLuc and MLuc of the copepods *G. princeps* and *M. longa*, respectively, were immediately and successfully applied as bioluminescent reporters *in vivo* [10, 58] and *in*

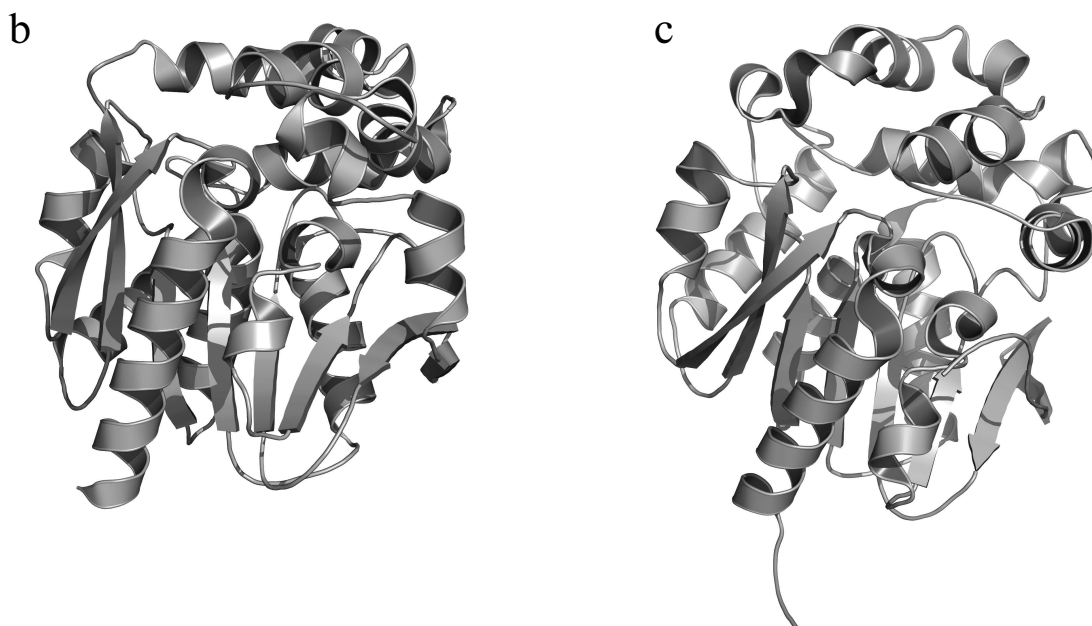
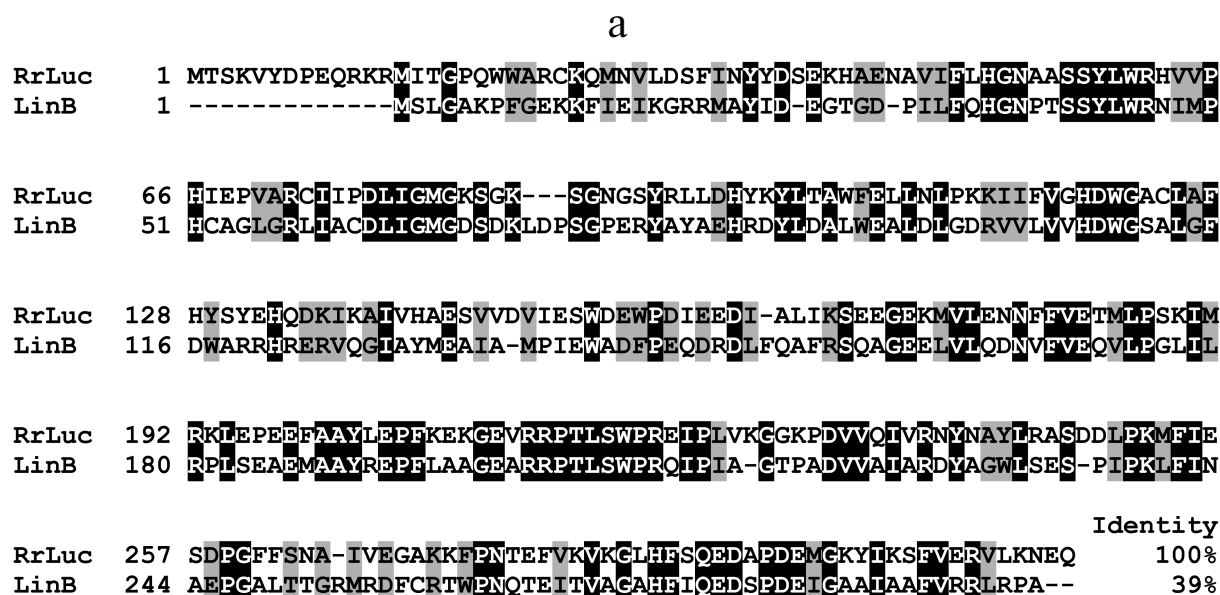


Fig. 5. a) Comparison of amino acid sequence of luciferase *R. reniformis* (RrLuc – AAA29804) with that of the homologous cellular protein haloalkane dehalogenase LinB of *S. paucimobilis* (LinB – 34810153). Black color indicates identical amino acid residues, gray – with similar properties. b, c) Structures of luciferase from *R. reniformis* (PDB file 2PSF) and haloalkane dehalogenase LinB (PDB file 1MJ5) from *S. paucimobilis*, respectively.



Alignment of repeats 1 и 2 in MLuc7: 70 aa in length, identity 26%

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25-VNRGKMPGKLPLEVLIEMEANAFKAGCTRGCLICLSKIKCTAKMKQYIPGRCHDYGGDKKTGQAGIVGA-94
96-VDIPEISGFKEMEPMEQFIAQVDLCADCTTGCLKGLANVKCSSELLKKWLPDRCAFADKIQKEAHNKG-165
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Fig. 6. Comparison of amino acid sequences of isoforms (MLuc164 – AAR17541, MLuc39 – ABW06650, and MLuc7 – AJC98141) of *M. longa* luciferase and the *G. princeps* luciferase (GpLuc – AAG54095). The identical amino acid residues are marked in black, the ones with similar properties – in gray. A signal peptide providing secretion of the luciferase and the conservative motifs within the repeats are framed; the conservative Cys residues are marked under the amino acid sequences. Deletion points of the N-terminal variable part in the truncated mutants MLuc164 [60] are labeled as M3, M4, and M5.

vitro [37, 59], and since then the scope of their application has been expanding.

Although bioluminescent species have been found among many copepods [32], so far the genes encoding luciferases have been cloned only from copepods belonging to the superfamily Augaptiloidea. The sequences of 28 full-length genes of luciferases of 12 species of luminous copepods encoding amino acid sequences with a relatively high degree of similarity have been determined (Table 1). Of the several types of luminous copepods, a few genes encoding 2-3 luciferase isoforms from one species have been isolated; for these, differences between isoforms of one species were comparable to those between the luciferases of taxonomically distant species. The sequence identity of isoforms MLuc164 and MLuc7 of *M. longa*, for example, is only 68%, which is close to the 57% sequence identity of MLuc164 and the luciferase GpLuc of *G. princeps* (Fig. 6), the copepod species from another genus of the same family Metridinidae. Such significant

differences in amino acid sequences of three isoforms of *M. longa* suggest that the luciferase of these copepods is encoded by at least three paralogous (non-allelic) genes.

Copepod luciferases can be conditionally divided into three parts: a signal peptide for secretion, a variable N-terminus, and a conservative C-terminus (Fig. 6). The variable part, sometimes constituting up to one third of the length of the amino acid sequence, does not substantially affect the luciferase bioluminescent function – mutants with “truncated” variable part (Fig. 6) reveal even higher activity than the full-length MLuc164 [60]. It should be noted, however, that the deletion of the N-terminal part has an effect on the decay kinetics of the bioluminescent signal, making it faster than that of the wild-type MLuc164. The C-terminal conservative part of the luciferase is formed by two non-identical tandem repeats of length of about 70 amino acid residues, each containing a highly conserved motif of 32 amino acids [10, 61]. Each motif contains five conservative Cys residues

(Fig. 6), suggesting the existence of up to five S–S bonds per luciferase molecule. The currently available data on the bioluminescent activity of individual tandem repeats are very contradictory. As reported in [61], each of the tandem repeats of GpLuc reveals bioluminescence activity under expression in *E. coli* cells. Hence, the authors concluded that the luciferases of copepods have two catalytic domains. Another study, however, reports on the absence of any activity of the same separately synthesized tandem repeats of GpLuc under expression in mammalian cells [62].

Despite widespread and successful application of copepod luciferases as reporters, their biochemical and bioluminescent properties are still insufficiently studied. The natural luciferase of copepods has not yet been isolated and characterized, even partially. Production of recombinant protein in native conformation using simple and inexpensive bacterial expression systems was ineffective due to the presence of numerous disulfide bonds in copepod luciferases. Thus, luciferase of *M. longa* obtained in *E. coli* cells is prone to aggregation, which leads to significant heterogeneity of the sample; the luciferase portion in a monomeric form is only a few percent of the total protein amount [37, 63]. In addition, certain properties of even monomeric highly purified recombinant luciferase from bacterial cells are very different from those of the luciferase produced in eukaryotic expression systems. For example, the specific bioluminescent activity of isoforms MLuc164 and MLuc7 obtained with a baculovirus expression system in insect cells is several times higher than the activity of these isoforms produced by bacterial expression [38, 63]. It should be noted that in addition to high bioluminescent activity, the luciferases of copepods have extremely high thermostability [39]; the isoform MLuc7 retains 50% of its activity even after boiling for 1 h [38]. The high thermostability of luciferases, similar to other secreted proteins, is apparently accounted for by the large amount of S–S bonds that stabilize their spatial structure.

The kinetics of luminescence of copepod luciferases under optimal conditions is of “flash”-type with fast decay within a few minutes [37, 58, 60]. However, some applications involving copepod luciferase as a reporter, e.g. high-throughput screening, require a bioluminescent signal that would be stable for tens of minutes. Such a signal can be obtained by changing the conditions of measurement of luciferase activity, as the kinetics are rather dependent on the buffer composition. The addition of 0.1% Triton X-100 to the buffer for measuring activity of GpLuc, for example, significantly slows the decay rate of the bioluminescent signal, but at the same time reduces the specific activity of the luciferase, thus significantly decreasing its detection limit and therefore the sensitivity of the method [64]. To overcome these shortcomings, a number of mutants of GpLuc possessing both high specific activity and slow decay of bioluminescent signal were

obtained by mutagenesis [64–66]. The decay of bioluminescence of a double mutant of GpLuc (M60L-M127L), for example, was approximately 14 times slower, and the specific activity was like that of the wild-type GpLuc [65].

Since the redox potential in the cytoplasm of all cells does not facilitate the formation of disulfide bonds, the cytoplasmic expression of copepod luciferases potentially containing up to five S–S bonds does not yield cells with high bioluminescent activity, presumably due to incorrect folding of the luciferase. However, the bioluminescent intensity of cells can be significantly increased by expression of the secreted variant of luciferase with a signal peptide at the *N*-terminus and the KDEL sequence at the *C*-terminus, which retains the protein in the endoplasmic reticulum [58]. The secretory signal peptide directs the luciferase into the endoplasmic reticulum, where the protein properly matures, but its following secretion will be stopped due to the “retention” peptide KDEL. As a result, a significant increase in the bioluminescence of cells will occur.

Luciferases of copepods are natural secreted proteins and therefore are most effective when used as secreted reporters, since this allows real-time monitoring of the intracellular events *in vivo* repeatedly without disruption of the object under study both in case of cells and in case of small laboratory animals. For GpLuc [58, 67] and MLuc [68], it was shown that the bioluminescence in cell culture medium or in the blood of the animals correlates linearly with the number of cells secreting the reporter in the range of about five orders of magnitude. This, for instance, allows the assessment of the functional state of malignant tumor, the rate of its growth and metastasis, as well as response to therapy by the level of bioluminescent activity in a small blood sample (5 μ l) regardless of the localization of the cancer cells in the body [67, 68].

The main hindrance that limits the use of copepod luciferases as reporters in imaging *in vivo* directly in tissues and intact small laboratory animals is the blue emission considerably absorbed by mammalian tissues. This attenuates the signal and consequently reduces the sensitivity. For copepod luciferases, as in the case of *Renilla* luciferase, highly active mutants with a red-shifted bioluminescence spectrum are being sought. So far, one of the best variants is the mutant GpLuc (F89W/I90L/H95E/Y97W) called “Monsta”, which is almost five times higher in activity than the wild-type GpLuc when expressed in COS-7 cells, and it has bioluminescent maximum at 503 nm [66]. Although the mutant with red-shifted emission was constructed by mutagenesis, its spectrum is still far from the “transparency window” (>600 nm) of biological tissues.

Recently, several “artificial” luciferases (ALuc) have been constructed. They demonstrate higher stability and increased bioluminescent activity, which for some of these luciferases many times exceeded that of the wild-type GpLuc, the most popular and commonly used

secreted bioluminescent reporter [69, 70]. In addition, some of the artificial luciferases revealed a significant red shift of the emission spectra (in the case of ALuc25, up to $\lambda_{\max} = 530$ nm) [69]. This property along with high bioluminescent activity makes these luciferases the most attractive reporters among the copepod luciferases for use in *in vivo* imaging in tissues and small laboratory animals. On construction of artificial luciferases, an approach was used based on the assumption that for a stable protein structure the amino acids frequently occurring in the same position are thermodynamically more favorable than the residues rarely occurring in the same position. Thus, the amino acid sequences of artificial luciferases are variants of consensus sequences derived from a comparison of the amino acid sequences of copepod luciferases [69].

Luciferase of shrimp *Oplophorus gracilirostris*.

Natural secreted luciferase of the decapod shrimp *O. gracilirostris* is a tetramer with molecular mass of ~106 kDa [71] consisting of two monomers each of molecular mass 19 kDa, and the two monomers of 35 kDa. However, it is only the protein with $M_r = 19$ kDa that reveals luciferase activity; the function of the other protein that is part of the tetramer is not yet reliably established. The quantum yield of the bioluminescent reaction catalyzed by the natural luciferase *Oplophorus* is one of the highest (0.34 at 22°C) among luciferases using luciferins of imidazopyrazinone type. The temperature optimum of bioluminescent reaction is 40°C, and the pH optimum is ~9.0. Cloning of cDNA genes encoding the luciferase subunits showed that the 19- and 35-kDa proteins are composed of 196 and 359 amino acids, respectively, including putative signal peptides for secretion [33]. The recombinant protein with $M_r = 19$ kDa produced both in mammalian and *E. coli* cells was monomeric, thermolabile, prone to aggregation, and had substantially less specific bioluminescent activity compared to the natural luciferase [72]. Based on these observations, it was suggested that the function of the protein with $M_r = 35$ kDa in the natural luciferase is stabilization of the catalytic subunit. Unlike copepod luciferases containing 10 Cys residues and presumably five S—S bonds that stabilize the structure of the molecule and are critical for activity, the catalytic subunit of luciferase *Oplophorus* of approximately the same length contains only one cysteine residue that is not essential for the activity of the luciferase [72].

As with the case of luciferases of copepods, no significant similarity is found between the amino acid sequences of the *Oplophorus* luciferase and both known and hypothetical proteins. However, when using fold-recognition programs that identify the similarity of proteins by the elements of spatial structure, a vague resemblance of the catalytic 19-kDa subunit of *Oplophorus* luciferase to the well-characterized family of intracellular lipid-binding proteins (iLBPs) was found. Introducing the N166R substitution into the 19-kDa protein, which in

accord with a spatial model must enhance the stability of the structure and increase the structural similarity to iLBP, led to increase in its bioluminescent activity ~3-fold and stability by ~50% [73]. This mutant was then used as a template for three rounds of random mutagenesis with intent to produce mutant luciferases with properties (high specific activity, slow decay of bioluminescent signal, increased solubility, and stability) optimized for applications as reporter proteins. The best mutations of each round were introduced in a template for the next round of random mutagenesis. In addition, the selected mutants were also tested with 24 coelenterazine analogs to find a substrate that would provide the highest bioluminescent activity and the lowest background signal compared to coelenterazine. As a result, a thermostable mutant named “NanoLuc” (NLuc) carrying 16 amino acid substitutions and showing several orders greater bioluminescent activity and stability than the wild-type catalytic subunit of the luciferase *Oplophorus* was produced [73]. As the best substrate for NanoLuc, the coelenterazine analog Furimazine was identified, which in contrast to coelenterazine provides a higher bioluminescent activity and stability in biological media (signal half-life exceeds 4 h) and, consequently, a lower background signal. In lysates of HEK293 cells, NanoLuc with Furimazine was reported to produce bioluminescent signals with slow decay kinetics (signal half-life > 2 h) and intensity ~2.5 million times higher than that of the 19-kDa protein with wild-type coelenterazine under similar conditions, and ~150 times higher than those of *Renilla* and firefly luciferases [73]. It should be noted, however, that the activity of NanoLuc, as shown in [74], exceeded that of 19-kDa only ~10-fold on soluble expression in *E. coli* cells, and only ~80-fold on expression in CHO-K1 cells. Also, the specific activity of NanoLuc with coelenterazine h was ~1.5 times greater than with Furimazine. A significant limitation to the use of a new artificial luciferase as a reporter for *in vivo* imaging in tissues of animals is the emission spectrum ($\lambda_{\max} = 460$ nm) corresponding to the region of strong absorption of light by mammalian tissues.

Cypridina luciferases. A natural secreted luciferase of ostracod *Cypridina (Vargula) hilgendorfi* was the first luciferase utilizing the luciferin of imidazopyrazinone type in the bioluminescent reaction that was isolated by chromatography as a highly purified protein and thoroughly characterized at the beginning of the 1960s [3]. This luciferase catalyzes the oxidative decarboxylation of *Cypridina* luciferin (Fig. 2) in a simple reaction involving only the enzyme, luciferin, and O₂. As a result of the reaction, a product in an excited state is formed, and its relaxation to the ground state is accompanied by light emission with a maximum at 448–463 nm. The light emission wavelength depends on the ionic strength of the buffer and is practically independent of pH. The temperature optimum of the bioluminescent reaction is 30°C. The

quantum yield of the bioluminescent reaction catalyzed by *Cypridina* luciferase is 0.3, one of the highest among bioluminescent reactions catalyzed by coelenterazine-dependent bioluminescent proteins [75].

Luciferase of the ostracod *Cypridina* (*Vargula*) *hilgendorffii* was one of the first luciferases using luciferin of imidazopyrazinone type for which the cDNA gene was cloned (Fig. 7) [40]. Later, the highly homologous luciferase of *Cypridina noctiluca* (identity of amino acid sequences is ~84%) was cloned [41]. Luciferase *Cypridina*

is a one-subunit protein with calculated molecular mass of 62.2 kDa composed of 552-555 amino acid residues. This luciferase comprises a signal peptide for secretion and 32 conservative Cys residues within the mature protein that form 16 S-S bonds (in the natural luciferase, free SH-groups were not found [3]), and two putative N-glycosylation sites (Fig. 7). It is the largest known coelenterazine-dependent bioluminescent protein. The bioluminescent reaction catalyzed by *Cypridina* luciferase is inhibited by EDTA and EGTA. This suggests the influ-

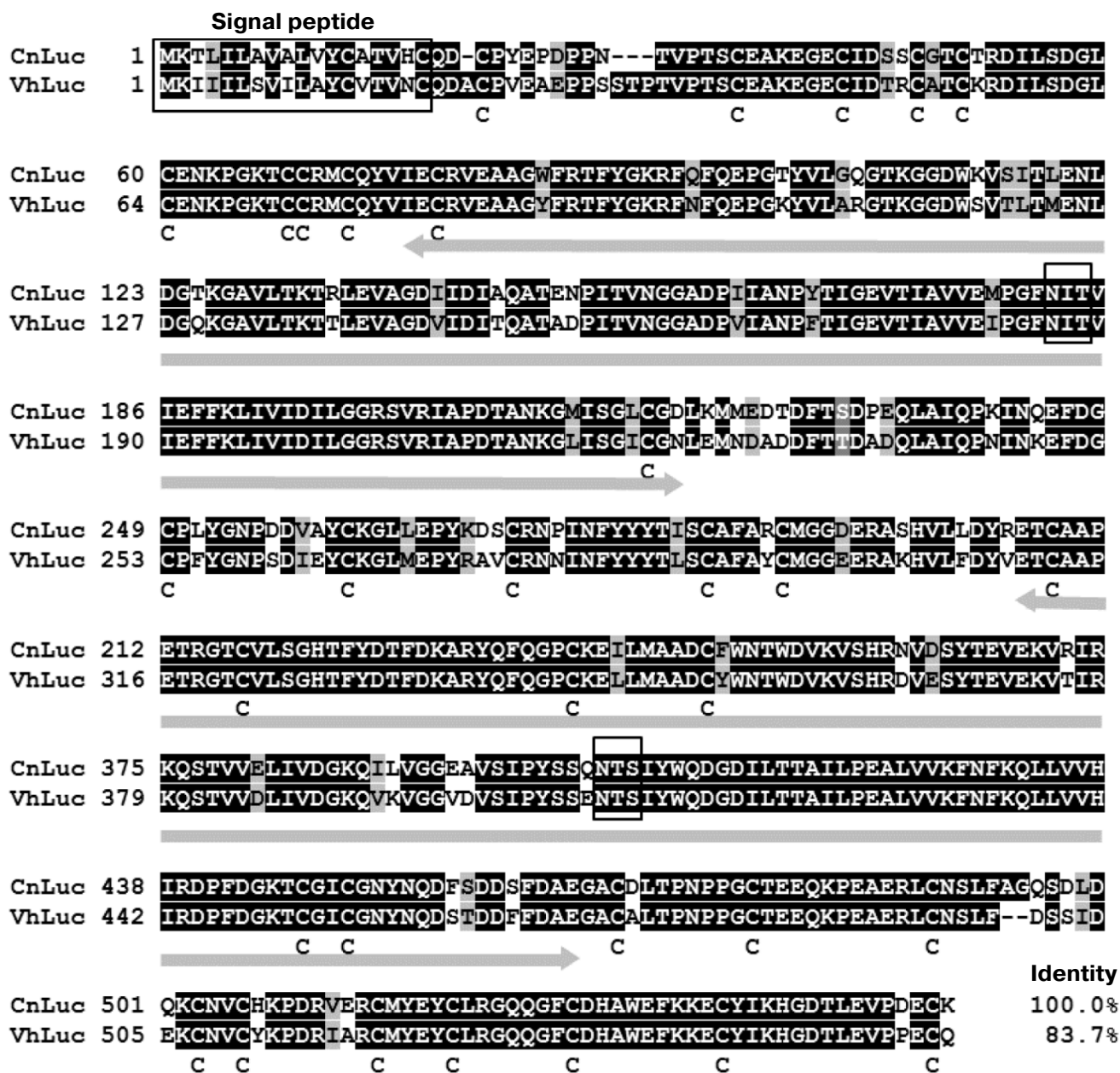


Fig. 7. Comparison of amino acid sequences of luciferases *C. noctiluca* (CnLuc – BAD08210) and *V. hilgendorffii* (VhLuc – AAB86460). Signal peptides providing secretion of the luciferases identified by the program Signal P 4.0 [76] and potential sites of N-glycosylation are framed. The conservative Cys residues are marked under the amino acid sequences. The domains of the D factor type of Willebrand (vWF-D) identified by the BLAST program are underlined with gray lines.

ence of Ca^{2+} or other divalent cations on the enzymatic activity. However, none of the amino acid sequences characteristic of Ca^{2+} -binding sites was revealed in this luciferase. Active recombinant luciferase can be obtained in secreted form only when expressed in eukaryotic cells (yeast, mammalian cells), which is apparently due to the presence of the large number of S–S bonds in the luciferase that are formed in the endoplasmic reticulum, as well as by the need for posttranslational glycosylation of the protein. It is worthy of note that despite high identity of amino acid sequences, the luciferase of *Cypridina noctiluca* reveals a much higher activity than that of *Cypridina (Vargula) hilgendorfi* under secretory expression in eukaryotic cell culture [41].

The analysis of amino acid sequences of these luciferases revealed no significant homology with any known protein, including coelenterazine-dependent proteins. However, in the sequences of the luciferases the BLAST program identifies two domains (Fig. 7) similar to domain D of the von Willebrand factor (vWF-D), the secreted multidomain glycoprotein that plays a pivotal role in blood coagulation. Domains of the vWF-D family have been found in many proteins, mostly in secreted glycoproteins such as integrins, growth factors, fibrillar collagens, proteins of the complement system, and IGF-binding proteins (sequence database on the proteins Pfam and UniProt). These domains are supposed to be responsible for multimerization of proteins and their interaction with other proteins [77]. It is not known if the vWF-D domains are relevant to the functioning of the luciferases or bioluminescent systems of ostracods.

It is interesting that luminous ostracods of the family Halocypridoidea, *Conchoecia pseudodiscophora* in particular, use coelenterazine but not *Cypridina* luciferin as a substrate for the bioluminescent reaction. It is also likely that their bioluminescence is of non-secreted type, which is accounted for by functioning of intracellular luciferase since the emission is observed strictly within the carapace (dorsal section) of the ostracods [11].

Luciferases of scyphozoan medusa *P. periphylla* and fish *B. pterotum*. The scyphozoan medusa *P. periphylla* contains soluble (L-form) and insoluble forms of luciferases, the latter being isolated as particles [7, 8]. The soluble form of luciferase with molecular mass of 32 kDa is responsible for bioluminescence and was isolated from dome mesoglea and radial lappets of the medusa. The insoluble form is accumulated as aggregates in the ovary, and its content, based on estimates of activity, exceeds 100-fold the amount of the soluble form [8]. In the presence of high concentrations of β -mercaptoethanol, the insoluble aggregates of luciferase form a mixture of active oligomers with molecular masses of 20, 40, and 80 kDa (luciferases A, B, and C, respectively). Based on electrophoresis data, it was concluded that the 40- and 80-kDa luciferases are dimer and tetramer of the luciferase A [8]. The soluble luciferase L is also a complex; the addition of

β -mercaptoethanol results in the formation of two proteins with molecular masses of 20 and \sim 12 kDa. Of these two proteins, only the one with molecular mass of 20 kDa showed luciferase activity. All the luciferases were identical in bioluminescence spectra, with λ_{max} at 465 nm, but differed in biochemical properties (temperature optimum, thermostability, and K_m). Luciferases A, B, and C obtained from insoluble particles revealed higher specific activity than the soluble luciferase L. The specific bioluminescent activity of these luciferases, according to the authors, was the highest among the luciferases using coelenterazine as a substrate. Luciferases A, B, and C are extremely resistant to denaturation; their bioluminescent activity drops only slightly at pH 1 and pH 11, and it is even enhanced in 1–2 M guanidine hydrochloride. These luciferases, however, are less stable to heating than luciferase L (32 kDa), which retains activity practically unchanged after boiling for several minutes. These luciferases differ in temperature optimum as well. While bioluminescent activity of luciferase L is maximal at 0°C and decreases almost linearly as the temperature increases, approaching zero at 60°C, luciferases A, B, and C have optimum at \sim 30°C. In addition, the value of the Michaelis constant ($K_m \sim 0.2 \mu\text{M}$) for luciferases A, B, and C is about six times lower than that for luciferase L. The reason for the differences in properties of the insoluble and soluble forms of luciferases of medusa *P. periphylla* is not clear. Despite such features as thermostability and high specific activity making this luciferase attractive for use as a reporter protein and the availability of biological material (scyphozoan medusa *P. periphylla* occurs in abundance in the fjords of Norway), the gene (or genes) encoding this luciferase has not yet been cloned.

Recently, a new coelenterazine-dependent luciferase responsible for bioluminescence of *Benthoosema pterotum* was isolated from photophores of this fish, purified by chromatography, and partially characterized [13]. The luciferase has molecular mass of \sim 27 kDa, and its Michaelis constant is 0.4 μM , which is close to values for other coelenterazine-dependent luciferases. The bioluminescence spectrum has a maximum at 475 nm, and the temperature optimum for the bioluminescent reaction is 40°C. The luciferase of *B. pterotum* is rather thermostable (retains more than 50% activity for 1 h at 65°C) and resistant to extreme pH values. An interesting feature of this luciferase is that calcium and especially magnesium ions significantly increase its activity [13]. Considering the fact it was possible to obtain enough highly purified *B. pterotum* luciferase, as well as the current state of development of protein sequencing and molecular biology, it is reasonable to expect that the gene encoding this luciferase will be cloned soon.

Thus, the known coelenterazine-dependent luciferases, despite the use of the same substrate for bioluminescence and the same chemical mechanism of its oxidative decarboxylation [3], are nonhomologous pro-

teins having no similarity with each other or other known proteins. The only exception is the luciferase *Renilla*, belonging to the group of α/β -hydrolases.

INDEPENDENT EVOLUTION OF BIOLUMINESCENT SYSTEMS IS SOURCE OF DIVERSITY OF BIOLUMINESCENT PROTEINS

Common cellular functions of all organisms living on the Earth are in the main provided by biochemical processes involving homologous proteins, which are almost the same or sometimes greatly similar, namely, up to the full match of amino acid sequences. An unusual property of bioluminescence as a function of living organisms is that the luminescence of organisms belonging to different taxa involves various proteins catalyzing different reactions. This has become especially clear after cloning of genes and determination of the primary sequences of a number of proteins, as well as elucidation of mechanisms of the bioluminescent reactions of various organisms. The presently cloned bioluminescent enzymes from taxonomically distant organisms show no similarities in their amino acid sequences even when they use the same substrate for the bioluminescent reaction. Such proteins also revealed no similarity between their spatial structures despite the fact that those are more conservative than primary sequences. For some bioluminescent proteins, it was unexpected to find close homologs among ordinary cellular enzymes that provide basic cellular functions [51, 78]. Considering these differences in bioluminescent systems and their components in various organisms, it should be assumed that the ability for bioluminescence in representatives of different taxa arose independently and repeatedly in the course of evolution, involving suitable ancestral proteins.

There are more than 40 known variants of bioluminescent systems [1] that have arisen in evolution independently and in some cases apparently very recently. The supposition on the recent origin of certain variants is supported by the "spotted" distribution of bioluminescence among taxa (Fig. 1), i.e. the presence of organisms with different biochemistry of luminescence in the same taxon, as well as the presence of luminous and non-luminous species, sometimes even closely related, in one taxon. For example, among the four species of the colonial hydroid of *Obelia* genus dwelling the White Sea, only two are luminous despite similar living conditions of all the four species. In some taxa, such as siphonophores and ctenophores, the non-luminous representatives are rare, and in some taxa, on the contrary, luminous organisms are rare, e.g. among chaetognaths (type Chaetognatha) [1].

Even close relatives sometimes differ significantly in the nature of their luminescence, like in the case of different species of squids or planktonic ostracods that use

various modifications of coelenterazine for luminescence and also different types of bioluminescence – secreted and intracellular.

Since the ability to produce light occurred repeatedly during evolution (Fig. 1), it can be reasonably assumed that first, bioluminescence is very important for organisms and second, it can appear rather easily. As noted above, luciferins of imidazopyrazinone type are found in many luminous and non-luminous marine organisms and can be transferred from one organism to another through food chains [3, 25], i.e. a bioluminescent substrate is more conservative than the other components of bioluminescent systems. Perhaps this is also true for other luciferins. Thus, having one available organic molecule capable of providing a light-emitting function, the organism only needs to "adapt" a suitable protein for bioluminescence to appear. It seems that various cellular proteins were often recruited to play the role of luciferase; mutations in these proteins led to the ability to catalyze the available bioluminescent substrate with the emission of light, thus forming all the observed diversity of bioluminescent systems.

Such a variety of organisms (Fig. 1) that use luciferins as substrates of imidazopyrazinone type in bioluminescent reactions and variety of proteins that catalyze these reactions suggest the existence of other numerous luminous organisms that have not yet been investigated and that also use these luciferins as substrates, but the function of luciferases is performed by completely different proteins. Why the imidazopyrazinone-type luciferins are the substrates of bioluminescent reactions in such a variety of luminous organisms remains a mystery.

CLONING OF NOVEL BIOLUMINESCENT PROTEINS

There are two approaches that can be used for cloning of genes encoding bioluminescent proteins from novel luminous organisms. The traditional method involves determination of components required for bioluminescence, primarily substrate and potential cofactors. If the substrate of a bioluminescent reaction is known and available, it is usually not difficult to reproduce the bioluminescent reaction *in vitro*. (As of today, these are apparently only bioluminescent proteins using luciferins of imidazopyrazinone type in bioluminescent reaction that fully meet this requirement.) In the next step, the bioluminescent protein is isolated from luminous organisms and its amount should be sufficient to determine at least a small part of the amino acid sequence. Then degenerate primers to the known protein sequence are constructed, which are then used for screening of cDNA library by hybridization or PCR to find clones with new genes. With this approach, the cDNA genes encoding several coelen-

terazine-dependent bioluminescent proteins were cloned: Ca^{2+} -regulated photoprotein aequorin from hydromedusa *Aequorea victoria* [79, 80], luciferase from ostracod *Cypridina (Vargula) hilgendorffii* [40], luciferases from soft corals *Renilla reniformis* [35] and *Renilla muelleri* [36], luciferase from decapod shrimp *Oplophorus gracilirostris* [33], and Ca^{2+} -dependent coelenterazine-binding protein from *Renilla muelleri* [36]. It is evident that this approach can be applied in the case if substantial quantities of the luminous organisms can be collected.

However, most luminous marine organisms are rare and can be caught as single specimens. Furthermore, many bioluminescent animals inhabit great depths, and therefore their gathering is rather complicated and labor consuming. These circumstances make the production of a sufficient amount of natural protein for its characterization and determination of amino acid sequence quite difficult, and therefore the traditional approach of cDNA gene cloning cannot be followed. The method of functional screening of clones of cDNA expression library helps get around this problem (Fig. 8). Modern PCR-based methods allow the construction of a cDNA gene expression library from a very small piece (only a few mg) of tissue. For functional screening, a bioluminescent reaction *in vitro* must only be reproduced and a sufficient amount of a substrate for screening must be available. High sensitivity of detection of the emitted light allows registration of positive bioluminescent signal from an individual clone among a large group of negative clones. With functional screening applied, the cDNA genes encoding Ca^{2+} -regulated photoproteins of hydroids *Obelia longissima* [81] and *Obelia geniculata* [82], jellyfish *Clytia gregaria* [83] and *Mitrocoma cellularia* [84], ctenophore *Beroe abyssicola* [30], as well as luciferases of

copepods *Metridia longa* [10, 37, 38] and *Gaussia princeps* [34], and GFP of jellyfish *C. gregaria* [83] were cloned. It should be mentioned that most of the cloned cDNA genes contain a stop codon (or even several stop codons) in the 5'-untranslated region. Nevertheless, this did not hinder isolation of individual colonies by bioluminescent activity, thereby demonstrating the high efficiency of functional screening on cloning of genes encoding bioluminescent and fluorescent proteins.

It is obvious that there are some limitations for applying functional screening. Since determination of the structure of the novel bioluminescent substrate is even more difficult than obtaining a highly purified protein for sequencing, this approach can be employed on cloning of novel proteins that use already known and available bioluminescence substrates, such as luciferins of imidazopyrazinone type. In addition, it is desirable that the bioluminescent protein be a one-subunit protein because the presence of another subunit would significantly complicate the search for the corresponding genes, and that the protein does not require posttranslational modifications necessary for its bioluminescent activity. It is likely that the latter of these limitations can be overcome using eukaryotic cells for screening of cDNA expression libraries.

Although the study of bioluminescent systems of various organisms is undoubtedly of fundamental importance, the main driving force that determines interest in the topic is still the usefulness of bioluminescence as an analytical tool. Presently, there is no field of biology, medical science, or pharmacology that does not widely apply bioluminescent methods or has not tested the ones showing suitability. The development of fundamental and applied research in these areas requires the elaboration of effective techniques for noninvasive imaging of molecular

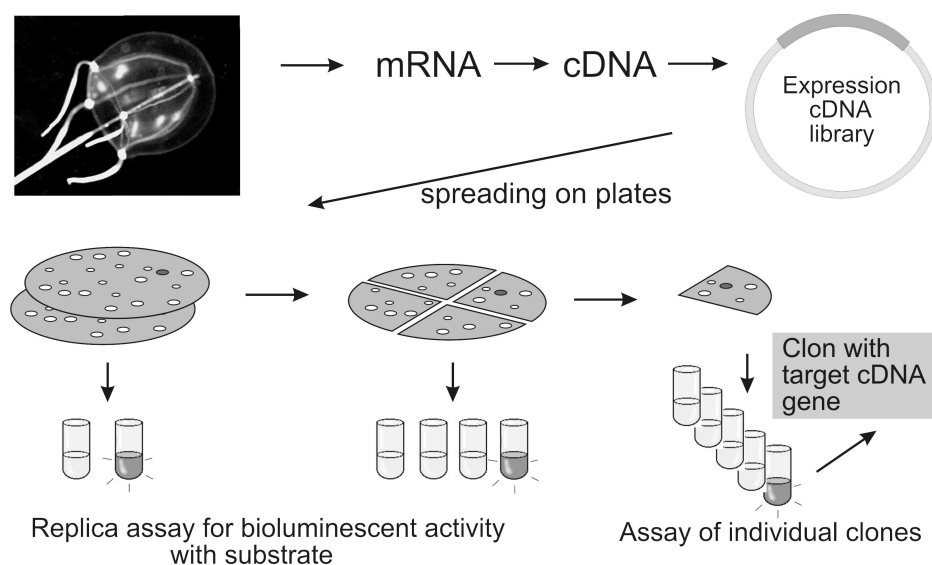


Fig. 8. Schematic representation of functional screening.

processes *in vivo* in a single living cell and in an intact living organism. Such technologies offer a good opportunity to resolve a wide range of tasks, but are primarily focused on investigation of dynamic processes *in vivo* in cells and organisms, thus giving a clearer view of the growth and metastatic activity of tumor, the response of tumor cells to therapy, migration of immunocompetent cells in the organism, and protein–protein interactions in a cell. These technologies also allow the study of metabolic activity and its regulation at the level of single cells and the intact organism, the pathways and distribution of proteins in a cell and the intact organism, regulation of gene expression, and a great deal more. In this view, reporters based on luciferases and photoproteins are very useful. Since bioluminescent reactions proceed with high quantum yield and extremely low background, technologies that use luciferases and photoproteins as reporters are capable of providing high-sensitive imaging and a wide linear dynamic range of measurements. Bioluminescent proteins and their substrates are components of bioluminescent systems of luminous organisms and are therefore nontoxic to living cells. Until recently, the use of bioluminescent imaging was limited by the imperfect methods of recording and processing of weak light signals. These problems, however, have been solved with the advent of highly sensitive cooled CCD cameras and microscopes that can provide real-time detection of changes in luminescence in a single cell.

Practically all the bioluminescent proteins available to researchers have already been tested as genetically encoded reporters for monitoring cellular processes. The most popular bioluminescent reporters today are the firefly luciferase, Ca^{2+} -regulated photoproteins, and coelenterazine-dependent luciferases of *Renilla*, copepods *Gaussia* and *Metridia*, as well as their mutant variants with improved characteristics. One of the main applications of luciferases is monitoring of gene expression and tracing of labeled cells in an organism. Luciferase activity is used to estimate cell viability, apoptosis, and various processes associated with cellular metabolism. Luciferases fused with proteins under study are used to monitor metabolism of target proteins and their interaction with other proteins. An effective way to assess protein–protein interactions *in vivo* is complementation based on the ability of luciferases split into fragments to restore their activity on complementation of these fragments fused with the interacting proteins under study. Another way of assessing protein–protein interactions *in vivo* by means of luciferase reporters are FRET technologies. This method is based on Forster resonance energy transfer from a bioluminescent donor to a fluorescent acceptor protein, which occurs only with spatial approximation of interacting target proteins fused with the reporters and is accompanied by a change in the emission spectrum.

The availability of bioluminescent proteins with different properties, including mutants with improved char-

acteristics, provides more opportunities for optimal design of experiments. Moreover, differences in substrates, kinetics of bioluminescent reactions, or bioluminescence spectra allow the use of up to three bioluminescent reporters for simultaneous registration of several different processes. Taking into account the diversity of bioluminescent proteins using luciferin of imidazopyrazinone type and continuing studies of new luminous organisms, the appearance of novel luciferases and photoproteins suited for the development of new *in vivo* imaging technologies should be expected.

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