BIOLUMINESCENT IMAGING: NEW OPPORTUNITIES

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Bioluminescent imaging methods are the most sensitive, however, their use for multicolor labeling is complicated due to the insufficient number of available ulciferin-luciferase pairs. Having a number of advantages compared to previously studied bioluminescent systems, the new bioluminescence systems of higher fungi and marine polychaete Odontosyllis could become a useful expansion of the biolumaging toolbox.

Keywords: bioluminescence, bioimaging, luciferin, luciferase

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BIOLЮМИНЕНЦЕНТNYЙ ИМИДЖИнG: НОВЫЕ ВОЗМОЖНОСТИ

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Современные биомедицинские исследования активно используют методы биолюмининга клеток, тканей и целых организмов. Многоцветный биолюмининг имеет свои преимущества в случае необходимости одновременного наблюдения разных событий на молекулярном и клеточном уровнях. Наиболее чувствительными являются методы биолюминесцентного имиджинга, однако их использование для многоцветного мечения сдерживается недостаточным количеством доступных пар люцифераза-люциферин. Удачным расширением палитры инструментов молекулярного имиджинга могут стать новые биолюминесцентные системы высших грибов и морской полихеты Odontosyllis, обладающими рядом преимуществ по сравнению с ранее изученными системами.

Ключевые слова: биолюминесценция, биоимиджинг, люциферин, люцифераза

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Bioimaging of tissues and whole organisms is an integral part of the current research routines aimed at studying molecular events in the context of the disease development investigations [1]. Fluorescent and bioluminescent proteins (luciferases) are a very popular vehicle in bioimaging. For the purposes of fluorescent bioimaging, there was developed a set of fluorescent proteins with various spectral properties, ranging from violet to far-red, photoactivated and photoconvertible, as well as derivative sensors [2]. Luciferases that can be used for bioimaging are significantly more scarce than fluorescent proteins, but the methods making use of bioluminescence deliver some of the most accurate results when applied to examine deep tissues.

Luciferase catalyzes luminescence through oxidation of luciferin, molecular substrate, which allows receiving the analytical signal without using an external source of radiation, an integral part of the routine with fluorescent proteins. Thus, bioluminescent imaging in vivo is virtually free from background noise and offers unequaled sensitivity [3]. However, luciferases as reporter proteins are not flawless. The activity of some of them is strongly dependent on the number of cofactors and can be inhibited by intracellular components or medicines [4]. Still, despite bioluminescent systems being less practical because of the need for at least two components to trigger light emission, the relevant optimization methods continue to evolve and gain popularity rapidly.

Tools for bioluminescent imaging

Multicolor bioimaging allows simultaneous observation of different events at the molecular level (e.g., gene expression or protein-protein interactions), which translates into a smaller number of animals used in the context of an experiment. There is a number of strategies to follow; some of them are: joint
use of luciferases of different organisms, mutant luciferases of one organism, use of ‘luciferase-fluorescent protein’ hybrid constructs, etc. [5].

Some of the most popular luciferases used for bioimaging are those of Photinus pyralis (62 kDa) Fluc, a North American firefly, Pyrophorus plagiophthalmus (62 kDa) (λ_max = 540–615 nm), a click beetle, and Renilla reniformis Fluc (36 kDa) (λ_max = 480 nm), both of which are marine creatures [6]. Luciferases of terricole organisms catalyze the D-luciferin-oxygen reaction in the presence of ATP and Mg^{2+} ions; luciferases of marine organisms utilize coelenterazine as a sole substrate. NanoLuc (8 kDa) (λ_max = 460 nm), an engineered luciferase derived from the small domain of Ophalophus graciilis luciferase, has been very popular since 2012, when it was developed [7]. NanoLuc makes use of a different substrate, furimazine, a synthetic analogue of coelenterazine.

Apart from the purely historical reasons, luciferases catalyzing D-luciferin are popular because of their applicability to imaging the processes involving ATP molecule [8]. Also, there is a wide range of structural analogues of D-luciferin available [3], including those emitting in the more red portion of the spectrum, like the recently developed AkaLumine-HCl (λ_max = 677 nm) [9]. It is necessary to clarify here that shifting bioluminescence to the near IR range is important because the spectral window for in vivo examination of processes in deep tissues belongs there. In addition to the improved spectral characteristics, AkaLumine-HCl distributes through the cells better than D-luciferin and saturates firefly luciferase (Fluc) more effectively. Directed mutagenesis of Fluc produced Akaluc, an artificial luciferase, the synthetic substrate of which is also the case for other marine luminescent systems. As soon as the structure of Oplophorus undecimonta luciferase, which may somewhat complicate its practical use.

Technologically, a light filter and different bioluminescent systems used together allow analysis of signals from different processes; to this effect, the standard Promega DLR assay contains both P. pyralis and R. reniformis luciferase. Another option is to introduce several mutant proteins to a single bioluminescent system. DART, a recently developed examination method, is an example of the latter. The system makes use of green (PLG) and red (PLR1) firefly luciferase mutants together with D-luciferin and its benzothiophene analogue, respectively [11]. However, the techniques described above are not flawless: the former carries a risk of light signal inhibition by luciferases, the latter can produce cross-reactions of the related luciferases with similar substrates.

Development of the fully artificial luciferases that work with standard substrates (based on the genetic sequences of known proteins) is an interesting approach to remedying the flaws, one similar to the approach that produced the aforementioned AlaBLI system. An example of the product thereof is Aluc, a recent development that interacts with coelenterazine and analogues (λ_max = 487–500 nm) [12]. Chemical modification of coelenterazine by conjugation with fluorescent dyes allowed shifting the Aluc reaction’s emission maximum to an even longer wavelength region [13]. Preliminary calculations in the context of an effort guided by a similar approach revealed several orthogonal pairs ‘D-luciferin analogue–mutant Fluc luciferase’; their activity was further confirmed in vivo [14].

Absence of cofactors in the luminescence reaction simplifies analysis and makes marine luciferases usable in extracellular imaging, which makes them a convenient vehicle. NanoLuc, an engineered luciferase, is especially popular. Its miniature size simplifies development of the new ‘luciferase–fluorescent protein’ fusion proteins used to expand the imaging palette with the help of the FRET technique. FRET is based on the Förster Resonant Energy Transfer (FRET) from luciferase to a fluorescent protein, which results in a shift of the emission maximum. NanoLuc luciferase and applicable proteins produced a whole range of chimeric proteins that have the emission maximum shifted to up to 680 nm with the help of various fluorophores [15]. The recently developed Fluc8-iRFPs chimeric proteins that also work in the long-wavelength portion of the spectrum are a similar example [16].

**Expansion of the palette: new luminescent system**

Studying new, previously unresearched luminescent systems is another approach to expanding the bioluminescent palette that shows promise. Some of the recent discoveries here include luciferin from Fridericia heliota (λ_max = 480 nm), an earthworm, and that from fungi (λ_max = 530 nm) [3]. Researchers have already obtained a recombinant luciferase for the latter [17]. The cost price of fungal luciferin is several orders of magnitude lower than that of D-luciferin while its stability is much higher. The new fungal bioluminescent system allows for a simple modification of luciferin structure, thus enabling production of the functional analogues emitting light in the longer wavelength range [18]; this property makes it a quite promising tool for bioimaging, even considering the membrane localization of the fungal luciferase, which may somewhat complicate its practical use.

In 2018, researchers isolated luciferase of Odontosyllis undecimonta, a marine polychaete [19]. This protein does not luminesce with the known luciferins of marine organisms (coelenterazine, Cypridina luciferin), which makes it the first marine luciferase belonging to a fundamentally new type of bioluminescent systems (i.e. orthogonal to all previously studied) described in a long time. In vivo, the bioluminescence of Odontosyllis peaks at around 510 nm. The reaction between Odontosyllis luciferin and luciferase requires no cofactors, which is also the case for other marine luminescent systems. As soon as the structure of Odontosyllis luciferin is deciphered and its synthesis method developed, this bioluminescent system will be actively used in bioimaging.

**CONCLUSIONS**

A huge variety of multicolor bioimaging techniques available allows choosing the one optimal for the particular task in the context of a specific medical research. The most sensitive are the bioluminescent imaging techniques, but in terms of multicolor labelling, their application is limited by the insufficient number of available luciferase–luciferin pairs. The recently discovered bioluminescent systems of higher fungi and Odontosyllis, a marine polychaete, which have several advantages over those that are currently popular, can extend the palette.
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