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Recent progress in luminescent proteins development

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

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Introduction

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

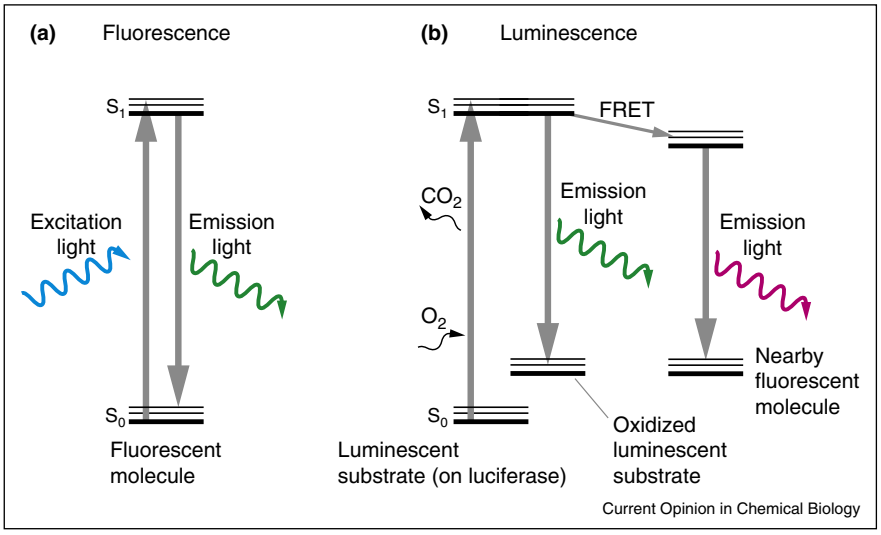
Luminescent proteins

To overcome the problem of dimness in luminescent imaging, various kinds of bright luminescent proteins have been developed (Table 1). One recently developed bright luminescent protein, Nano-lantern, employs FRET to enhance light output [1]. FRET naturally occurs inside the sea pansy, *Renilla reniformis*, from the dim luminescent protein *Renilla* luciferase (RLuc) to the bright fluorescent protein *Renilla* green fluorescent protein (*Renilla* GFP). The probability of photon emission from *Renilla* GFP is 5.7-fold as high as that from RLuc, according to the difference in their quantum yields (Φ) for light emission (Φ = 0.053 and =0.30, respectively). As a result of approximately 100% FRET from RLuc to *Renilla* GFP inside the sea pansy, the Φ of the RLuc-*Renilla* GFP complex increases 5.7-fold [2]. In the case of Nano-lantern, RLuc8 [3] with an S257G mutation and Venus yellow fluorescent protein [4] were utilized as the FRET donor and acceptor, respectively. Nano-lantern exhibited 5-fold greater light output than RLuc8, and 3-fold greater light output than BAF-Y [5], the brightness of which is also enhanced by FRET. The brightness of Nano-lantern allowed visualization of the fine structure of living organelles using only a short exposure time (1–3 s). Furthermore, Nano-lantern enabled video-rate imaging of tumors in a freely moving, unshaved mouse [1]. Color variants of Nano-lantern, Cyan-Nano-lantern and Orange-Nano-lantern, were developed by using mTurquoise2 [6] and mKO2 [7] as FRET acceptors, respectively; using these proteins, inhomogeneous expression of multiple pluripotency markers in a single colony of embryonic stem cells was successfully visualized [8••].

In *ff*Luc2-cp156, firefly luciferase (FLuc) was fused with a circularly permutated variant of Venus (cp156) [9], not for enhancement of light output by FRET, but rather for enhancement of protein stability and enzymatic activity. Indeed, transgenic *ff*Luc2-cp156 mice exhibited light output that was more than three orders of magnitude greater than that of transgenic FLuc mice. The whole-body luminescence signal from transgenic *ff*Luc2-cp156 mice could be visualized at video-rate by intraperitoneal administration of D-luciferin without anesthetization.

Artificial luciferases (ALucs) were developed by linking the key amino acids that appeared most frequently in the

Figure 1



Schematic illustration of Jablonski diagram to explain the difference between fluorescent and luminescent process. **(a)** A fluorescent dye is excited from ground state (S_0) to excited state (S_1) by external excitation light with proper wavelength. Emission light from S_1 is termed as fluorescence. External excitation light causes some problems such as photo-bleaching, phototoxicity and photo-induced physiological reaction to the sample, which make long-term whole-body live imaging very difficult. **(b)** S_0–S_1 transition of luminescent substrate occurs by oxidation reaction catalyzed by a luciferase. As this process does not need external light for the S_0–S_1 transition, luminescence imaging is free from above problems. Improvements of luminescent tools can be understood by the Jablonski diagram. Brightness of luminescent proteins can be improved by lower K_m (Michaelis-Menten constant) with luminescent substrate, catalytic rate of oxidation, stability. In addition Förster resonance energy transfer (FRET) to S_1 of nearby fluorescent molecule can increase quantum yield of light output, if quantum yield at S_1 of the fluorescent molecule is higher than it of luminescent substrate. Properties of luminescent substrate also influence the brightness in living cell such as permeability of cell membrane, stability, lower K_m with luciferase. Beside caged-substrates allow the S_0–S_1 transition of luminescent substrate only in limited situation, that means they can be utilized for functional indicators.

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

NanoLuc (NLuc) was developed from the deep-sea shrimp *Oplophorus gracilirostris*, with 16 amino acid mutations [14]. NLuc exhibits 150-fold brighter luminescence than RLuc or FLuc. In addition, a novel coelenterazine analogue specially customized for NLuc, furimazine, was developed. When paired with furimazine, NLuc exhibited a 30-fold brighter luminescent signal than when paired with coelenterazine. Because of its small size (19 kDa) and its brightness, NLuc has been used to report the dynamics of influenza viral infection in living mice [15].

Recently, the smallest luminescent protein, MLuc7, was cloned [16]. MLuc7 is a non-allelic isoform of *Metridia longa* luciferase. Its molecular weight is only 16.5 kDa because its sequence lacks a variable N-terminus region. Although all other copepod luciferases contain a variable N-terminus region, it is not thought to be essential for luciferase activity, as deletion of the region can increase luciferase activity [17].

Luminescent protein-based functional indicators

FLuc catalyzes adenylation reaction of D-luciferin, which consumes one ATP for production of one D-luciferyl adenylate that is further oxidized to yield an oxyluciferin and a photon. The emitted photon number is positively correlated with the ATP concentration. Therefore, FLuc has been used as the indicator for cellular ATP level [18]. On the other hand, RLuc does not require ATP to catalyze oxidation reaction of its substrate. Because of this simple catalytic reaction of RLuc, RLuc is preferable to FLuc for the application of functional sensor.

Because of the advantages of luminescence imaging compared to fluorescence imaging, several luminescent protein-based functional indicators have been developed in recent years (Table 1). BRAC is a FRET-based Ca^{2+} indicator [19] similar to the fluorescent protein-based Ca^{2+} indicator, yellow cameleon [20,21]. It is composed