International Science Journal
(Peer-Reviewed)
ISSN: 2348 – 604X (p); 2348 – 6058 (e)

BIOLUMINESCENCE, CELL BASED ASSAY AND IMAGING: A REVIEW

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ABSTRACT
Bioluminescence is essentially the generation of electromagnetic radiation as light by the process of releasing energy from a biochemical reaction. While the generated photons can be emitted in a wide range of wavelengths from ultra violet to infrared, those that emit visible light are the most common in science. Bioluminescence and other various luminescence processes are often described as "cold light" since no external energy source is required and the chemical reaction generates photons spontaneously. There are two main types of bioluminescence assays: substrate detection and catalyst detection. Both generate little heat (loss), and have high photon generation efficiency (e.g., luciferase/ luciferin process has quantum efficiency of 0.88). The photon generation rate is a function of many variables: the molecules participating in the luminescence process, their reaction kinetics and the exact implementation of the luminescence entities in the assay. In this paper, we'll briefly explore the processes that create this *Bioluminescence*, and the function that this light performs for the creatures that produce it, as well as the applications of bioluminescence in scientific research and commerce.

KEY WORDS : Bioluminescence, luciferase, luciferin, Cell Based Assays , Imaging

INTRODUCTION

Bioluminescence is defined as the emission of light from a living organism that functions for its survival or propagation. It is a "cold" light, resulting from a specific biochemical mechanism involving chemical processes, often specific for that organism. Bioluminescent organisms occur mostly in the marine environment, and bioluminescence is one of the major communication mechanisms in the deep sea. Although less common terrestrially, observations are naturally more frequent there. Bioluminescence can be thought of as a chemiluminescence that is catalyzed by an enzyme. This light emission from an organism needs to be distinguished from other forms of luminescence, many also having biological function, fluorescence, iridescence, diffraction, etc.

Chemistry of Bioluminescence

All bioluminescence reactions involve an oxygen oxidation of an organic molecule (called the luciferin). The reaction is catalyzed by an enzyme called a luciferase and in many cases, the bioluminescence intensity is assumed to reflect the velocity of the enzyme-substrate reaction, and this intensity is used to analyze the kinetics on the Michaelis-Menten model (Figure 1-A). It was first a puzzle that the bioluminescence of aequorin and subsequently of several other like organisms, was found not to involve oxygen kinetically, and these proteins were labeled "photoproteins" (Figure 1-B). It was eventually established that the oxygen had already bound to the luciferin, and the photoprotein therefore could be more accurately thought of as a luciferase binding a stabilized reaction intermediate, a peroxy-luciferin. Many bioluminescent reactions *in vitro* require cofactors in addition to oxygen, e.g., ATP and Mg2+ for the firefly, Ca2+ for photoproteins . In the animal itself (*in vivo*), there are additional proteins involved for production and regulation, some called "accessory proteins", examples being the fatty acid reductase group of enzymes that produce the bacterial luciferin, a long-chain aldehyde, and there are luciferin-binding proteins in the dinoflagellate and Sea Pansy bioluminescence systems. Also, there are "antenna proteins" that act to modulate the color of bioluminescence, the famous Green-fluorescent protein (GFP) in the jellyfish, and the Lumazine Protein family in some types of bacteria. These are named "antenna proteins" by analogy to proteins of similar function in photosynthesis, except that they act in a reverse sense.

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$$\text{(A) luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + CO_2 + hv$$

$$\text{(B) photoprotein} + Ca^{2+} \rightarrow \text{protein-coelenteramide} + CO_2 + hv$$

Figure 1. Reaction Schemes for a luciferin/luciferase reaction**(A)**, and for a typical photoprotein reaction triggered by calcium **(B)**. The reaction product is the light (hv) emitting species, the protein-bound oxyluciferin or protein-bound coelenteramide.

Classes of Luciferins

There are five known distinct chemical classes of luciferins, namely, aldehydes, benzothiazoles, imidazolopyrazines, tetrapyrroles and flavins. An imidazolopyrazine derivative, aptly named "coelenterazine", is the luciferin found in coelenterates and many other marine bioluminescence systems.

Physics Of The Light Emission

Bioluminescence results from a chemical reaction that releases a large amount of energy which, instead of being dissipated as heat as in a normal chemical reaction, is channeled to populate the product molecule in its excited electronic state. This excited state is the same one produced in that molecule by the absorption of radiation, so that the spectral distribution of the bioluminescence is often the same as that of the product fluorescence. The color of the bioluminescence however, is sometimes "tuned" by the protein environment of the product excited state, a property evolved to suit the function of the light emission, that is for communication, defense against predation, etc.

Visible radiation corresponds to light in the wavelength range of 400-700 nm (Figure 6). Bioluminescence spectra are broad bands with widths at half-height around 60-100 nm. The bioluminescence maximum of most marine species falls within the range of 450-510 nm (7), whereas terrestrial organisms have predominantly a yellow-green bioluminescence color. In ocean water, blue to green (400-500 nm) luminescence achieves maximum transmission, whereas terrestrial species have their maximum visual sensitivity for yellow light. Visual pigments of most marine organisms are correspondingly most sensitive in the blue-green region.

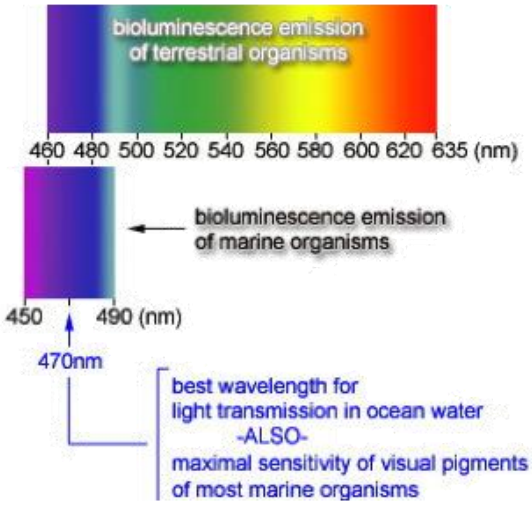

Figure 2. The end-product of bioluminescence is visible light. The visible part of the spectrum is 400-700 nm, and the emission maxima of most luminous marine organisms falls within the range of 450-490 nm.

Functions of Bioluminescence

As a result of its prevalence, bioluminescence plays an important role in the ecology of the ocean. The function of bioluminescence in the oceans is more clearly understood in the context of the essentially dark environment below about 200 m. The functions of bioluminescence are for: Defense ,Schooling of fish , Luminous lure, Feeding ,Communication (in the dark), Mating and Camouflage **Current Applications Of Bioluminescence**

Green-Fluorescent Protein

The "Green-Fluorescent Protein" or GFP, is probably the most famous protein in Biology (Nobel Prize in Chemistry, 2008). GFP was cloned in 1992 , and expressed in various organisms in 1994 . Since that time the applications of GFP have increased. In particular, GFP is now well established as an excellent gene tag or protein tag. GFP can be fused to a protein of interest, and fluorescence (and therefore the protein of interest) can be tracked within a cell to study its localization and behavior. GFP has outstanding structural stability, and with the property of being able to form the fluorescence *in situ* without the external addition of substrate, GFP becomes an excellent tool for studying cell and sub-cellular processes.

Luciferase Reporter Assay Cell based assay systems using reporter enzymes are used widely to study promoters, interactions between promoters and transcription factors, signal transduction, and other cellular activities. Cell based assays are also applied to drug screening both *in vitro* and *in vivo*. Of the reporter genes known to date, luciferases, enzymes that catalyze bioluminescence reactions, are used most frequently because their sensitivity and linear response range are superior to those of typical reporters, including β-galactosidase, chloramphenicol acetyltransferase, β-glucuronidase, and green fluorescent protein. Bioluminescence is a simple reaction that is triggered by the addition of luciferin solution and some cofactors, and the equipment for measuring light intensity is simple because it uses only a photomultiplier or a charge-coupled device (CCD) camera; thus, this assay can be applied to high-throughput screening (HTS). Luciferases are the most suitable reporter enzymes for the quantitative measurement of gene expression.

Single Luciferase Reporter Assay

All applications of bioluminescence systems are based on the principle of a chemical reaction; that is, the light intensity as the measurable product depends on the amounts of luciferase, luciferin, and cofactor(s). Using beetle bioluminescence as an example, in the presence of excess luciferin and luciferase, the bioluminescence intensity correlates with the amount of ATP, producing a beetle bioluminescence system that can measure the amount of ATP (Santos, 2003). This system can be applied to detecting bacteria in food, because bacteria contain ATP as an energy source (Venkateswaran et al., 2003). Luciferase is also used as a reporter enzyme to estimate gene expression in prokaryotic or eukaryotic cells, because the amount of luciferase correlates with light intensity in the presence of excess luciferin and ATP. Reporter enzyme assays are used widely in promoter analysis. Figure 3 demonstrates the simple luciferase reporter assay (Bronstein et al., 1994). In general, the luciferase gene containing the target promoter region of interest in the plasmid is transfected into target cells, and luciferase-expressing cells are lysed for an appropriate period, e.g., 1-2 days. The amount of expressed luciferase protein can be estimated from the light intensity *in vitro*. In the transient transfection luciferase assay, the luciferase-expressing cells are first lysed for an appropriate period. Luciferase activity in the cell extracts, measured with a luminometer equipped with a photomultiplier tube, is the most frequently used luciferase assay system because of its high sensitivity and broad linear response (up to 7-8 orders of magnitude) (Roda et al., 2009; Silverman et al., 1998).

This system is used widely for basic biological studies, including those of gene expression, post-transcriptional modification, and protein-protein interactions, and for diagnostic and drug discovery applications, because it is suited for HTS. However, we have to normalize the promoter activity using cell number or cellular internal enzyme activity, such as β-galactosidase, β-glucuronidase, chloramphenicol acetyltransferase, thus minimizing inherent experimental variability that can undermine experimental accuracy.

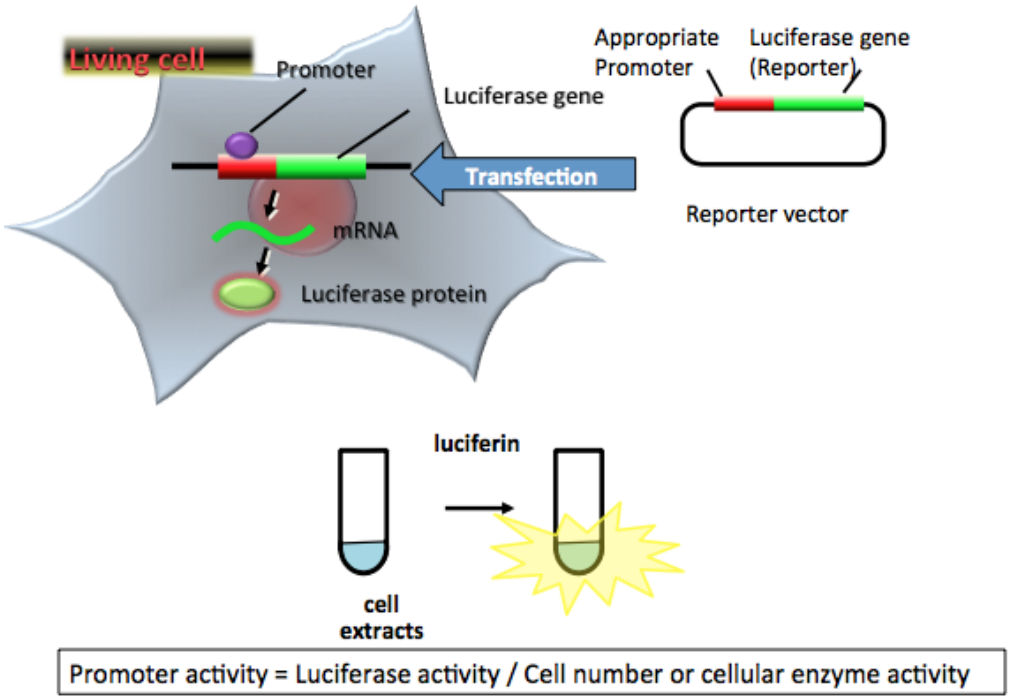

Figure 3. Principle of a simple luciferase reporter assay. The reporter plasmid vector consists of the target promoter sequence and a luciferase gene sequence. After transfection of the plasmid into target cells, the promoter region regulates the expression of luciferase gene in living cells. The expressed luciferase protein catalyzes a reaction with luciferin to produce light. In the transient transfection luciferase assay, luciferase-expressing cells are lysed for an appropriate period. The amount of expressed luciferase protein can be estimated from the light intensity, which indicates the promoter activity in living cells. In this case, the promoter activity is normalized by cell numbers or cellular enzymatic activity. (see: Bronstein et al., 1994)

Dual Non-Secreted Luciferase Reporter Assay

Advances in the transient transfection assay system have added an additional luciferase as an internal control reporter (dual-reporter assay), thereby minimizing inherent experimental variability that can undermine experimental accuracy, such as differences in the number and viability of cells used, the efficiency of cell transfection and lysis, and so on (Grentsmann et al., 1998). In Figure 2, firefly luciferase from *Photinus pyralis* (FLuc) is used as the experimental reporter, and Renilla luciferase is used as the internal control reporter, which connects to a constitutively expressed promoter, such as the herpes simplex virus thymidine kinase promoter, cytomegalovirus (CMV) immediate-early promoter, or simian virus 40 (SV40) promoter. The general assay system that uses two combinations of reporters is the Dual-Luciferase Reporter Assay System from Promega, in which humanized firefly

luciferase and Renilla luciferase are used as the experimental reporter and the internal control reporter, respectively. In this system, both luciferase activities are measured sequentially from single extracts on the basis of their bioluminescent substrate specificity. Firefly luciferase activity is measured first by adding firefly D-luciferin, and then Renilla luciferase activity is measured by adding coelenterazine (another name of Renilla luciferin) with the concomitant quenching of firefly luciferase luminescence. Finally, firefly luciferase activity is normalized by Renilla luciferase activity as a promoter activity.

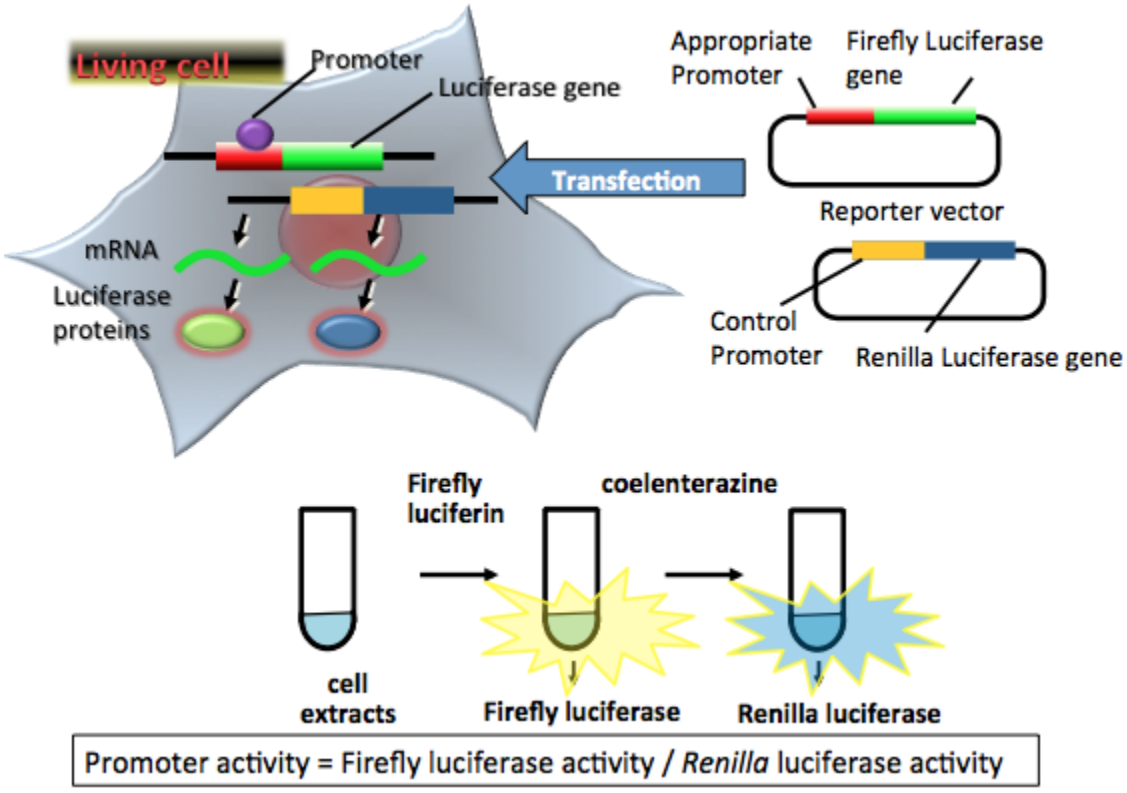

Figure 4. Principle of a dual nonsecreted luciferase reporter assay.

Two reporter plasmid vectors consist of the target promoter sequence, the firefly luciferase gene sequence, and the constitutive promoter sequence, and the Renilla luciferase gene sequence as a control. After transfection of the two plasmids into target cells, the promoter region regulates the expression of the luciferase genes in living cells. Firefly luciferase protein catalyzes a reaction with firefly luciferin to produce a yellow-green light. Renilla luciferase protein catalyzes a reaction with coelenterazine (another name of Renilla luciferin) to produce blue light. In the transient transfection dual luciferase assay, luciferase-expressing cells are lysed for an appropriate period. The amounts of expressed luciferase proteins can be estimated from the light intensity.

The light intensities indicate the target promoter and control promoter activities in living cells. In this case, the target promoter activity is normalized by the control promoter activity. (see: Grentsmann et al., 1998)

Bioluminescence

Imaging

Among known optical imaging techniques, molecular imaging using either fluorescent or bioluminescent reporters is one of the most sensitive methods, and uses the most cost-effective and simplest procedure. Fluorescence imaging using a fluorescent dye molecule or a genetically encoded fluorescent protein and its derivatives has contributed immensely to the advancement of cell biology, and provides a powerful tool to image an extensive array of samples, ranging from single molecules to whole organisms (Giepmans et al., 2006; Ghasemi et al., 2009; Day and Davidson, 2009; DiPilato and Zhang, 2010). However, because fluorescent reporters require exogenous illumination to emit light, this technique is not appropriate for long-term and quantitative imaging for the following reasons: (1) the fluorescent reporter is bleached by repetitive illumination; (2) repetitive exogenous light illumination causes phototoxic damage to cells; and (3) exogenous illumination perturbs the physiology in light-sensitive tissue such as the retina. In contrast, bioluminescence imaging using luciferase reporters does not need exogenous light illumination, and the luminescence reaction is quantitative, and has an extremely low background. This imaging method is particularly useful for longitudinal and quantitative imaging.

***In vitro* Bioluminescence Imaging**

The spatiotemporal resolution of bioluminescence imaging was improved by the development of the enhanced green-emitting beetle luciferase from *Pyrearinus termitilluminans* (ELuc) (Nakajima et al., 2010), whose light signal in mammalian cells is more than 10-fold greater than that of firefly luciferase. Although a luciferase reporter is currently used for bioluminescence imaging, including at the single-cell level, it can be difficult to perform subcellular or organelle imaging because of inadequate signal intensity in viable cells, and because a greater luminescence intensity is required for subcellular imaging with a higher-magnification lens. Figure 8 shows the subcellular localization of ELuc with high resolution in mammalian cells in cytosol, peroxisome, and nucleus (Nakajima et al., 2010).

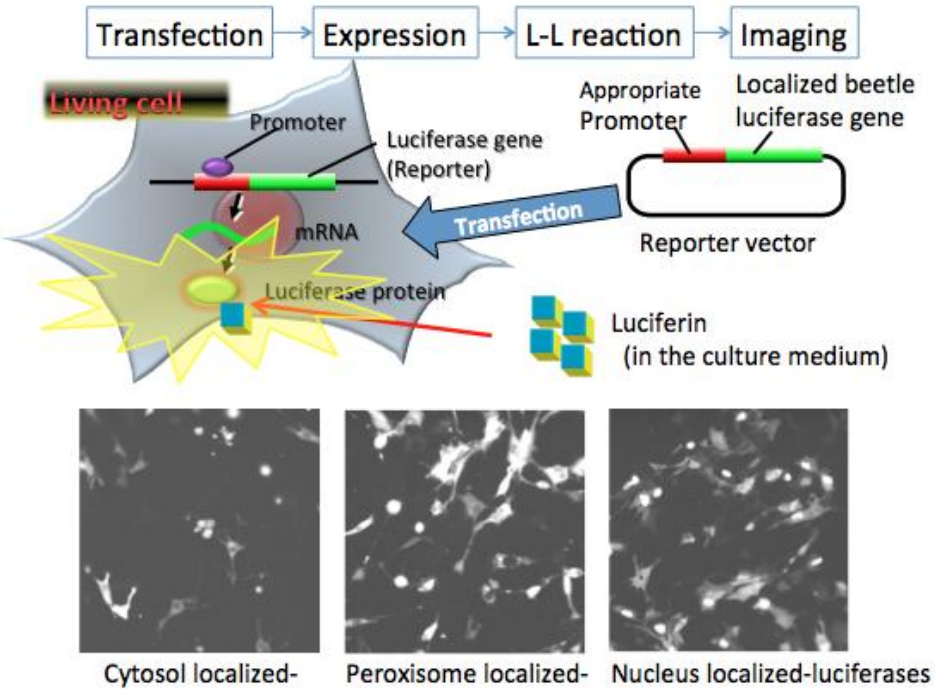

Figure 5. *In vitro* bioluminescence imaging for organelles in living cells. The reporter plasmid vectors consist of the constitutive promoter sequence and the organelle targeting luciferase gene sequence for cytosol, peroxisome and

nucleus in mammalian cells. After transfection of the plasmid into target cells, the promoter region regulates the expression of luciferase genes in living cells. For the imaging experiment, firefly luciferin added to the medium enters into the organelle, where it is catalyzed by the expressed firefly luciferase to generate light. The localization of expressed luciferase protein can be visualized by the light signal, which indicates the locality or mobility of organelles in living cells. In this case, the bioluminescence imaging is measured by special equipment using a CCD photon imaging system. (see: Nakajima et al., 2010)

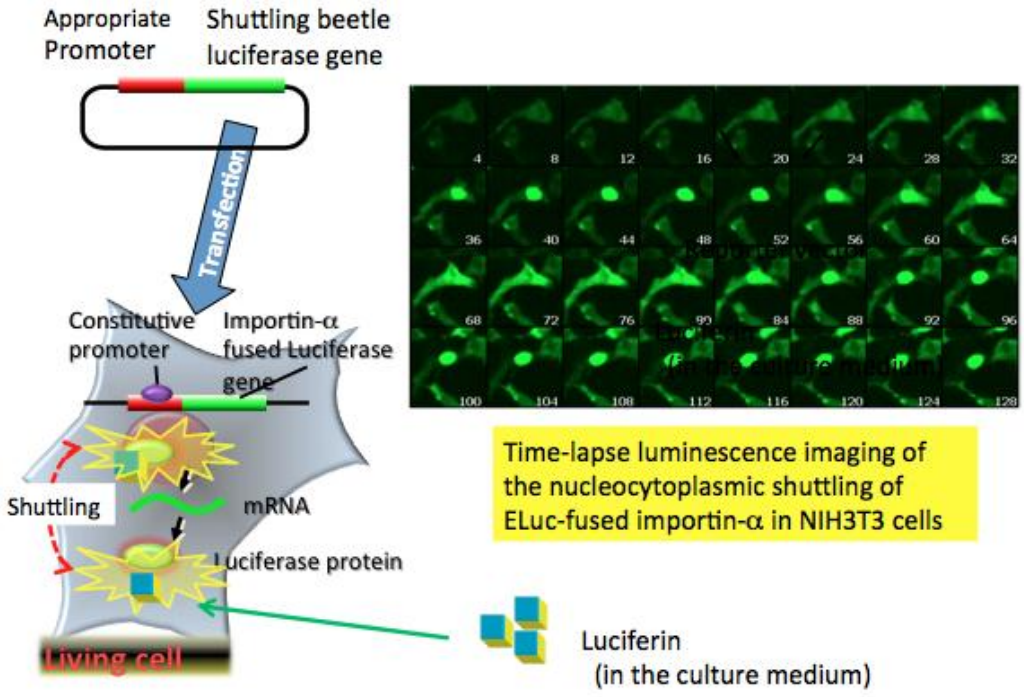

Figure 6. Time-lapse bioluminescence imaging of the nucleocytoplasmic shuttling.

The reporter plasmid vector consists of the constitutive promoter sequence and an importin-α fused luciferase gene sequence. After transfection of the plasmid into target cells, the promoter region regulates the expression of luciferase genes in living cells. For the time-lapse bioluminescence imaging experiment, firefly luciferin that is added to the medium enters the importin-α luciferase expressed cells, where it is catalyzed by the expressed firefly luciferase to generate light. The light signal indicates the nucleocytoplasmic shuttling of importin-α in living cells. In this case, the bioluminescence imaging is measured by special equipment using a CCD photon imaging system. (see: Nakajima et al., 2010)

***In vivo* Bioluminescence Imaging**

In *in vivo* bioluminescence imaging, one of the most frequently used applications is cell tracking (Rehmtulla et al., 2000). In this application, luciferase-expressing cancer cells, immune cells, stem cells, or other types of cells can be imaged repeatedly in small animals. In this method, luciferase gene transfects to a suitable cell line, and establishes the luciferase expressed stable cell line. The luciferase-expressed cells are injected into an animal and imaged at appropriate times (Figure 10). This method provides information about the number and spatial distribution of the cells

in the animal.

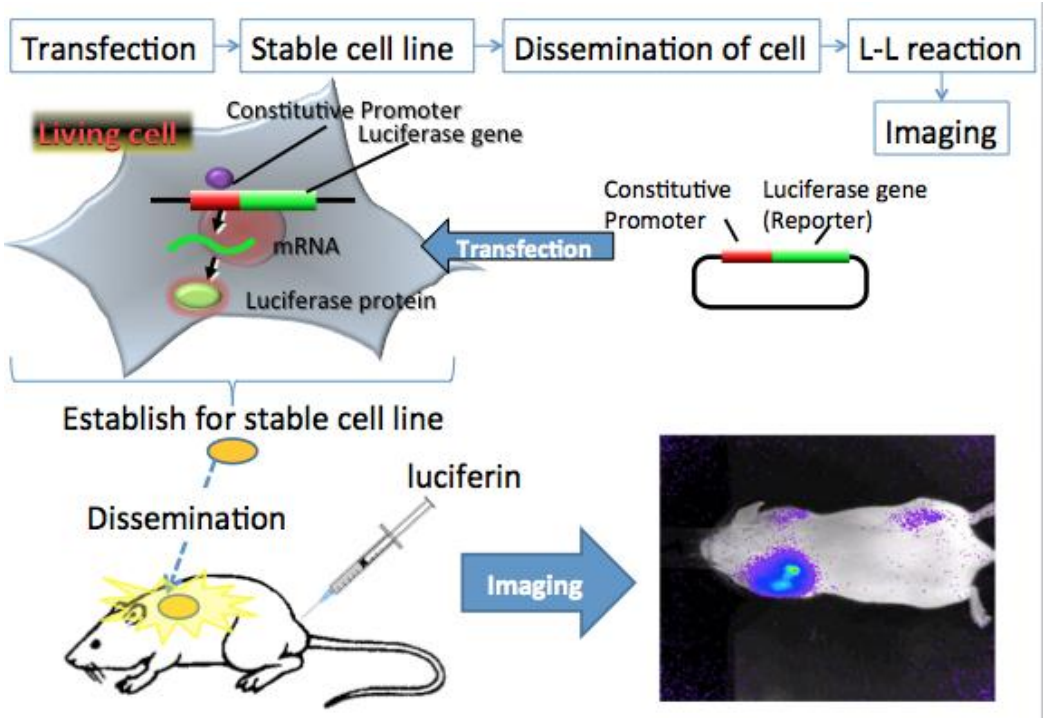

Figure 7. *In vivo* bioluminescence imaging using luminescent living cells. The reporter plasmid vector consists of the constitutive promoter sequence and a luciferase gene sequence. The reporter plasmid vector also consists of the antibiotic resistance sequence. After transfection of the plasmid into target cancer cells, the promoter region regulates the expression of the luciferase gene in living cells. The luciferase expressed stable cells are transplanted into the mouse. After an appropriate period for cancer cell growth, luciferin is injected into the body. For the *in vivo* imaging experiments, firefly luciferin enters into the cells from the blood, where it is catalyzed by the expressed firefly luciferase to generate light. The light signals indicate the location and size of cancer cells in the body. In this case, the bioluminescence imaging is measured by special equipment using a CCD photon imaging system. (see: Rehmtulla et al., 2000). Another approach for *in vivo* bioluminescence imaging is based on a luciferase protein probe. The antibody-fused luciferase visualizes disease-specific antigens on cell surfaces in a living body after the conjugation of luciferase and antibody. The purified probe is injected into mice. Twenty-four hours after the administration of the antibody fused luciferase probe, luciferin was injected, and the bioluminescence images were obtained using a CCD photon imaging system (Figure 8). For this technique, Wu et al. (2009) developed a far-red luminescence imaging technology for the visualization of disease specific antigens on cell surfaces in a living body. First, they conjugated a far-red fluorescent indocyanine derivative to biotinylated Cypridina luciferase (CLuc). This conjugate produced a bimodal spectrum that has long-wavelength bioluminescence emission in the far-red region, as a result of bioluminescence resonance energy transfer. To generate a far-red luminescent probe with targeting and imaging capabilities for tumors, they then linked this conjugate to an anti-human Dlk-1 monoclonal antibody via the biotin-avidin interaction. This far-red luminescent probe is a convenient analytical tool for the evaluation of monoclonal antibody localization in a living body.

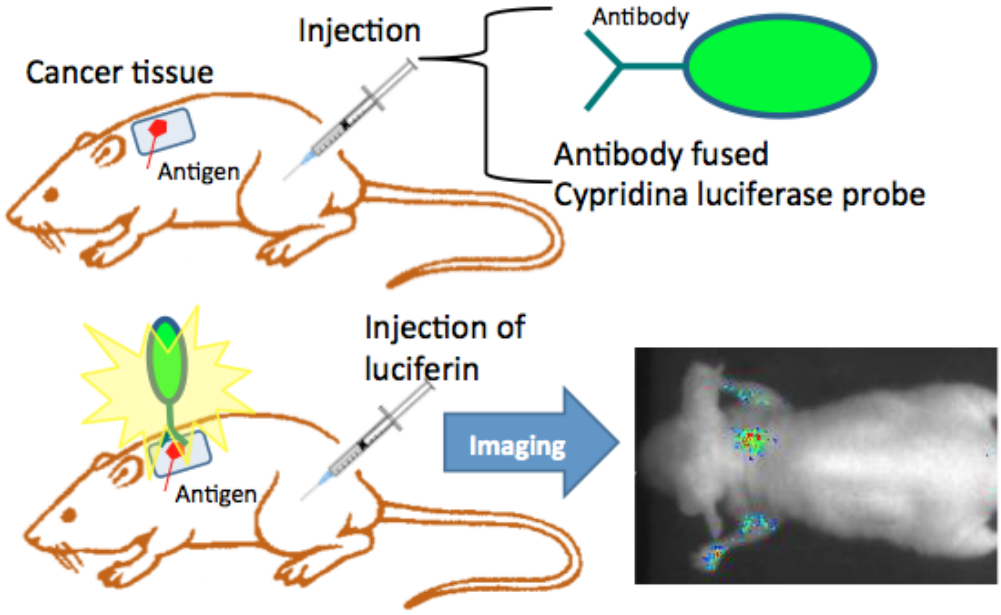

Figure 8. *In vivo* bioluminescence imaging using an antibody-fused luciferase probe.

Cypridina luciferase is expressed in a suitable expression system, and is purified by chromatography. An antibody fused Cypridina luciferase consists of Cypridina luciferase and antibody protein for an antigen on the cell membrane via, e.g., biotin-avidin conjugation. The targeted antigen expressed cell line was implanted into the back of mice. Tumor growth was monitored until it reached an acceptable size. For *in vivo* imaging, the antibody fused Cypridina luciferase was injected into mice intravenously. To obtain a bioluminescence image, the mice were given injections of Cypridina luciferin at appropriate times.

Bioluminescence imaging was performed by special equipment using an intensified CCD camera. (see: Wu et al., 2009).

CONCLUSION

In the post genome era, we must clarify quantitatively and spatially the complex phenomena of biological systems in real time. Bioluminescence based on the diversity of luminescent molecules has great potential as an assay tool, because the light is produced by enzyme reactions inherent within the physiological systems of living organisms. Such assays can be used to analyze the complex phenomena in biological systems, which can be detected as light signal outputs that correspond to specific biological events. , bioluminescence imaging is a highly sensi-tive, non-toxic analytical technique that is particularly well suited to long-term, longitudinal studies in cultured cells and living organisms. It has had a particularly strong impact in studies of tumor progression in living mice and in studies of circadian rhythmicity at the level of whole organisms, explanted tissues, and even single cells. As described in this review, bioluminescence has many applications and bioluminescence assays are expected to become essential research tools. Finally, it is essential to consider the underlying principle and the characteristics of bioluminescent reactions, and to select a suitable bioluminescence system depending on the assay purpose.

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