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Imaging β -Galactosidase Activity In Vivo Using Sequential Reporter-Enzyme Luminescence

Georges von Degenfeld, Tom S. Wehrman, and Helen M. Blau

Abstract

Bioluminescence using the reporter enzyme firefly luciferase (Fluc) and the substrate luciferin enables noninvasive optical imaging of living animals with extremely high sensitivity. This type of analysis enables studies of gene expression, tumor growth, and cell migration over time in live animals that were previously not possible. However, a major limitation of this system is that Fluc activity is restricted to the intracellular environment, which precludes important applications of in vivo imaging such as antibody labeling, or serum protein monitoring. In order to expand the application of bioluminescence imaging to other enzymes, we characterized a sequential reporter-enzyme luminescence (SRL) technology for the in vivo detection of β -galactosidase (β -gal) activity. The substrate is a “caged” D-luciferin conjugate that must first be cleaved by β -gal before it can be catalyzed by Fluc in the final, light-emitting step. Hence, luminescence is dependent on and correlates with β -gal activity. A variety of experiments were performed in order to validate the system and explore potential new applications. We were able to visualize non-invasively over time constitutive β -gal activity in engineered cells, as well as inducible tissue-specific β -gal expression in transgenic mice. Since β -gal, unlike Fluc, retains full activity outside of cells, we were able to show that antibodies conjugated to the recombinant β -gal enzyme could be used to detect and localize endogenous cells and extracellular antigens in vivo. In addition, we developed a low-affinity β -gal complementation system that enables inducible, reversible protein interactions to be monitored in real time in vivo, for example, sequential responses to agonists and antagonists of G-protein-coupled receptors (GPCRs). Thus, using SRL, the exquisite luminescent properties of Fluc can be combined with the advantages of another enzyme. Other substrates have been described that extend the scope to endogenous enzymes, such as cytochromes or caspases, potentially enabling additional unprecedented applications.

Keywords

β -galactosidase; luminescent imaging; in vivo pharmacology; G-protein-coupled receptor

1. Introduction

1.1. Bioluminescence Imaging of Firefly Luciferase

Bioluminescent imaging based on firefly luciferase (Fluc) activity is now a well-established method that has proven to be a valuable tool for the investigation of biological and pharmacological questions (1–5). The characteristics, strengths, and limitations of the different luciferases and their respective luminescent substrates have been described and discussed in detail elsewhere (6). In principle, bioluminescence has the potential to provide unsurpassed sensitivity due to the absence of endogenous luciferase expression in mammalian cells and to the exceedingly low background luminescence emanating from animals.

1.2. Luminescence Imaging of Other Peptidases and Proteases by Sequential Reporter-Enzyme Luminescence

We have developed a technology that increases the versatility of luminescence imaging by making it possible for the first time to non-invasively image the activity of enzymes other than luciferases, while capitalizing on the advantages of luciferase-based bioluminescence (7). The method is based on “caged” luciferin conjugates that cannot be cleaved by Fluc due to the presence of a bulky side group. Following cleavage of the side group at a cleavage site specific to the target enzyme, free D-luciferin is generated that, subsequently, is catalyzed by constitutively expressed firefly luciferase to produce light (7). Fluc no longer acts as a *bona fide* reporter enzyme, but rather as a secondary detection system that makes it possible to visualize the activity of the enzyme of interest. Hence, enzymes for which luminescent substrates are either not known or not applicable to live cells or animals become amenable to bioluminescent imaging. Such a technique has the potential to greatly expand the scope of bioluminescent imaging applications.

The technique was first tested in a series of proof-of-principle experiments to image β -gal, a well-known reporter enzyme, using the luciferin conjugate 1-*O*-galactopyranosyl-luciferin (Lugal) as described below. This substrate was first described by Miska & Geiger, and applied to the highly sensitive detection of bacterial contamination of food stocks (8–10). We provided the first evidence that Lugal has the ability to penetrate living cells without causing overt toxicity (7). As a result, it can be used to image intra as well as extracellular β -gal in cell cultures as well as in living mice, as described below.

Importantly, a wide variety of novel luciferin conjugates have been developed, which make it possible to detect and quantify other proteases and peptidases, including endogenous enzymes. The following list shows selected examples of such substrates:

- cytochromes P450 (differentiating between specific isoforms CYP1A1, –1A2, –1B1, –2C8, –2C9, –2C19, –2D6, –2J2, –3A4, –3A7, –4A11, –4F3B, –4F12, and –19) (11) (available from Promega, Madison, WI)
- caspases 3/7, 8, and 9 (12)
- alpha-chymotrypsin (13)
- carboxylic esterase (10)
- arylsulfatase (10)
- alkaline phosphatase (10)
- kinases, e.g., carboxypeptidases A, B, and N (10,14)

Based on the principle exemplified by β -gal, it should be possible to generate substrates containing suitable peptide sequences that make it possible to detect the activity of a wide variety of peptidases or proteases via bioluminescence imaging, enabling novel applications in the fields of toxicology and pharmacology as well as to study of organ and tissue physiology and pathology. To date, however, only β -gal has been tested and shown to be amenable to bioluminescent imaging of living cells and live animals. Recently, caspase activity was imaged in living mice that, however, died later in the process of imaging, apparently due to substrate toxicity (12). Different substrate conjugates may differ from Lugal with respect to toxicity, plasma stability, ability to penetrate the membrane of intact cells, and with respect to their pharmacokinetics following intraperitoneal (or intravenous) injection. Consequently, each substrate will need to be rigorously characterized and optimized in order to assess its applicability to in vivo imaging. For β -gal, we have conducted a series of proof-of-principle

experiments that demonstrate the suitability of the Lugal substrate for in vivo imaging of intra- and extracellular β -gal activity repeatedly over time.

1.3. Characterization and Applications of β -Galactosidase Bioluminescent Imaging

β -gal is one of the most widely used reporter enzymes in life sciences. The bacterial enzyme, encoded by the LacZ gene, possesses remarkable stability, retaining high activity through tissue fixation protocols and harsh chemical treatments, making it in many ways an ideal reporter system. It can be used as a reporter in cells and in transgenic animals or as a protein that can be linked to a wide variety of chemical and biological molecules. We have performed the following experiments to establish the feasibility and validity of using β -gal for in vivo bioluminescent imaging:

Differentiation of LacZ expressing cells by luminescence *in vitro*—Lugal was applied to living cells expressing Fluc alone (Fluc cells) or LacZ and Fluc (LacZ-Fluc cells): LacZ cells incubated with Lugal were shown to produce a luminescent signal that was specific for LacZ-Fluc cells (i.e., not detectable in Fluc cells) which was linear with increasing cell number (7).

Differentiation of LacZ expressing cells by luminescence *in vivo*—LacZ-Fluc and Fluc cells were implanted into the muscle or subcutaneously in nude mice and imaged by intraperitoneal injection of Lugal and bioluminescent imaging. LacZ-Fluc cells were shown to produce robust luminescence with a high signal-to-noise ratio compared to Fluc cells (Fig. 20.1).

Imaging of inducible LacZ expression in transgenic mice *in vivo*—Myf-5-LacZ mice were crossed with mice expressing Fluc in all cells, muscle damage was induced by notexin and imaging was performed repeatedly over a period of 9 days by intraperitoneal injection of Lugal. Inducible tissue-specific, gene expression was clearly detected.

Detection of antibodies to extracellular or membrane proteins in living mice—Antibodies to a membrane protein, CD4, coupled to β -gal revealed the lymph nodes and spleen (Fig. 20.2). The activity of β -gal outside the cells cleaved the Lugal substrate releasing luciferin that entered neighboring cells, serving as a luminescent substrate for intracellular luciferase.

Detection of protein-protein interactions in live animals through imaging of β -gal complementation—Cells engineered to express a GPCR fused to a small fragment and β -arrestin2 fused to the weakly complementing β -gal fragment were injected into nude mice (15). Upon agonist binding, β -arrestin2 bound to the activated GPCR, resulting in complementation of β -gal and an increase in enzyme activity (Fig. 20.3). Intraperitoneal injection of agonist and subsequent imaging using Lugal resulted in a robust luminescence induction, showing that β -gal complementation can be imaged and used to monitor GPCR activation in live animals.

The experiments performed using Lugal have shown that β -gal can be imaged using bioluminescence. However, this is still a very recent technique, the optimization of numerous parameters is still ongoing and the ultimate value of the method as a research tool in life sciences remains to be shown. The following technical description focuses on the general aspects of in vivo methods of β -gal imaging using Lugal. If other enzymes are to be imaged, a different set of tests might be required to establish the method.

2. Materials

2.1. Mice

1. Nude mice are, in general, best suited for optical imaging experiments because fur leads to partial extinction and scattering of the emitted light, leading to loss of signal and spatial resolution. BALB/c nude mice are available from several vendors, e.g., Taconic (Germantown, NY) and Jackson (Bar Harbor, ME) and are especially well suited to this application because they also are T cell-deficient. Hence, immune response to bacterial β -gal, triggering a slight, localized accumulation of immune cells in immune-competent mice, can be avoided (unpublished observation).
2. If nude mice cannot be used, mice with white or light fur are preferred. *See* Note 1.
3. Depending on the question addressed, the source of β -gal that is to be imaged is diverse, including engineered cells, gene transfer into adult animals, transgenic mice, and cells or antibodies labeled with recombinant β -gal protein. It would clearly go beyond the scope of this chapter to describe protocols for each experiment performed to date, which might be of interest to other researchers.
4. In the case of myoblasts retrovirally transduced with the LacZ and the Fluc genes, we refer the reader to the expert protocols published (16,17).

2.2. Lugal

1. The substrate 1-*O*-galactopyranosyl-luciferin (Lugal) is commercially available from different sources. The authors have used the substrate obtained as a special order from Promega (Madison, WI), but it is also available from Marker Gene Technologies (Eugene, OR).
2. Lugal is the core ingredient of the BetaGlo[®] Kit, an assay system for β -gal quantification. Because the kit is designed to be used as a terminal measurement in cell culture, it contains detergents and other unspecified agents that lead to cell lysis. Hence, the kit cannot be directly used as sold on living cells or for injection into living animals. An alternative approach is described in the Notes section (Note 2).

2.3. Imaging Device

Systems for bioluminescent in vivo imaging that include a cabinet, a CCD camera, as well as data processing and storage software are commercially available from at least three companies. The authors have used the IVIS[®]-100 and IVIS[®]-Spectrum systems (Xeno-gen-Caliper Life Sciences, Hopkinton, MA), but a similar system (NightOWL II LB 983) is available from Berthold Technologies (Bad Wildbad, Germany).

As an example, the IVIS[®]-100 consists of the following components:

- A light-tight imaging chamber.
- A heated stage (to avoid cooling off of the anaesthetized animals). The stage is sized to hold five mice or three rats, an important prerequisite for “relatively high

¹In some instances (e.g., specific transgenic or knockout mice), black mice have to be used instead of BALB/c nude or white mice. Luminescence and spatial resolution can be improved by shaving the mice over the region of interest if needed.

²As an alternative to Lugal, the solid component (“cake”) of the BetaGlo[®] Kit can be used, although its precise composition and, in particular, the concentration of Lugal contained are not disclosed by the manufacturer. In this case, the “cake” is not dissolved in the liquid buffer provided in the kit (which contains detergents), but rather in PBS. A variety of doses were used for in vivo experiments. In theory, the use of the solid component of the BetaGlo[®] kit might provide an advantage because it also contains active firefly luciferase, ATP, and various inorganic salts, which may locally “burn off” any free D-luciferin contaminating the solution that would contribute to background luminescence. Whether this protocol reduces background, as expected, has not yet been established.

throughput” imaging. Included are gas anesthesia connections and a full gas anesthesia system (e.g., isoflurane).

- A charged-coupled-device (CCD) camera (2048×2048 pixels) cryogenically cooled to -90°C by a closed-cycle refrigeration unit to minimize electronic background and maximize sensitivity.
- The system is operated via a computer using the Living Image[®] 3.0 software. The software also serves to store the data, for image display and analysis.

3. Methods

1. Lugal can be easily dissolved in PBS, aliquoted and frozen for later use (Lugal stock).
2. Before use, an aliquot of the Lugal stock is thawed and diluted in PBS to a final volume of 100 μL per mouse.
3. The Lugal dose used in most of our experiments was between 0.1 and 0.2 mmol/kg body weight.
4. Mice are anesthetized. Injectable compounds (e.g., Ketamine/Xylazine or Avertin) can be used. The authors, however, prefer inhalation anesthesia (e.g., isoflurane), because it allows prolonged imaging sessions if necessary without the need for reinjection (which would interrupt the imaging procedure and result in altered body position).
5. Anesthesia is induced in an induction chamber with 3–4% isoflurane.
6. Mice are weighed and placed into the imaging chamber, where anesthesia is maintained through isoflurane administered through a nose cone (typically 1.75%). The position of the animal is chosen according to the localization of the organ of interest. *See Note 3.*
7. Based on body weight, the total dose of Lugal is determined and diluted in PBS to a final volume of 100 μL per mouse, and filled into a 1 mL insulin-type syringe fitted with a 27–29-gauge needle.
8. For intraperitoneal injection of Lugal, mice are briefly removed from the nose cone. It is important to inject head down into the lower left quadrant of the abdomen to minimize the risk of injury of internal organs. *See Note 4.*
9. Optimal settings of sensitivity (“binning”) and duration of exposure need to be determined for each experiment. We have used exposures of up to 180 s in experiments with low luminescence. In other instances, e.g., if β -gal was imaged in transgenic mice ubiquitously overexpressing Fluc, luminescence generally was high and exposure, hence had to be short (few seconds). Note: Binning causes reduction in spatial resolution, but this does not limit the quantitative readout of the signal.

³Anesthesia can be tricky in the setting of in vivo imaging, because the animals cannot be continuously surveyed while the images are acquired. It is recommended to regularly check on the animals every couple of minutes in between the acquisition of images.

⁴Lugal, like the standard firefly luciferase substrate D-luciferin, is readily resorbed following intraperitoneal injection. This may sound like a detail but, in practice, dramatically reduces the requirements on time and technical skills. Indeed, it is not possible for a single operator to intravenously inject five mice simultaneously, because this technique typically takes up to several minutes per mouse. In contrast, five anesthetized mice can be easily injected intraperitoneally within a few seconds (“simultaneously”), placed in the imager and imaged. Thus, the option of intraperitoneal injection of the substrate is a prerequisite for “relatively high-throughput” imaging (if two imagers are available, up to 30 mice can be imaged per hour by a single operator). Similar to Lugal, the caspase-3-substrate (Caspase-Glo, Promega, Madison, WI) can be delivered intraperitoneally (12). If other conjugates are to be used, it will be necessary to determine first whether they are absorbed following intraperitoneal administration (as is the case for D-Luciferin and Lugal) or need to be injected intravenously.

10. Collect serial images until the peak signal is achieved, typically after 20–30 min.
See Note 5.
11. Images are stored by the software (e.g., Living Image[®] 3.0) for later analysis.
Luminescence is represented by a color map over a gray-scale picture of the mouse and can be easily quantified by drawing regions of interest.

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⁵Importantly, background luminescence was seen in all experiments, most likely due to “spillover” of free D-luciferin. Indeed, in the presence of active firefly luciferase (e.g., in the transplanted cells) that acts as the “helper enzyme,” any free luciferin will produce light. Lugal appears to have limited stability in mouse in plasma and, furthermore, to be partially degraded during freeze/thaw cycles, generating free luciferin. This problem can be circumvented in part by using exclusively images acquired within the first few minutes following Lugal injection, thus enabling reliable imaging of β -gal activity. In later images background luminescence was found to increase, apparently due to the generation of free luciferin, until, eventually, β -gal activity was no longer distinguishable at all. An additional reason for unspecific luminescent signal might be the presence of endogenous, mammalian β -gal, leading to cleavage of Lugal; such signal might be reduced or prevented if alternative models such as the β -gal knockout mice were used, or by using other reporter enzymes that are not unspecifically expressed in mammals. Further studies are necessary to establish the stability of Lugal and to optimize the protocol. Chemical modification most likely could improve the stability in serum. If other substrates are to be used, this caveat needs to be cautiously monitored and characterized.

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Fig. 20.1.

Luminescent imaging of β -gal activity in living mice using Lugal. β -gal expressing cells can be imaged in a living subject using Lugal. One million cells expressing Fluc were injected into the left tibialis anterior (TA) leg muscle of a BALB/c nude mouse; the same number cells expressing Fluc and β -gal were injected into the right TA. Lugal was injected 6 h later, showing a clear luminescent signal over the right leg injected with cells expressing β -gal and FLuc, whereas the left leg implanted with cells expressing Fluc, but not β -gal, showed only minimal luminescence (*left panel*). Luciferin was injected 24 h later, showing that equivalent cell numbers expressing Fluc were present in both legs (*right panel*). The results are representative of five independent experiments. Bioluminescent images are quantified in photons/sec/cm². Reproduced with permission from Nature Methods, vol. 3, no. 4, pp. 295–301 (2006).

Fig. 20.2.

Luminescent imaging extracellularly labeled cells and of lymphocyte distribution in vivo using anti-CD4 antibodies labeled with β -gal. (A) Injected cells extracellularly labeled with the β -gal reporter enzyme can be imaged using SRL. Untransduced C2C12 myoblasts, not expressing FLuc, were labeled with biotin, followed by an avidin- β -gal conjugate, and injected into a transgenic mouse constitutively expressing FLuc. Lugal was injected 24 h later, resulting in a robust signal localized to the site of implantation. (B–E) Detection of endogenous CD4⁺ T-cells by injection of anti-CD4 antibodies conjugated to β -gal in wild-type mice transplanted with the bone marrow from transgenic mice constitutively expressing FLuc. (C) Firstly, the pattern of FLuc activity from the FLuc-bone marrow transplanted mice was determined by Luciferin injection. Luminescence intensity and distribution were similar in both mice and revealed only minimal enhancement over organs containing high densities of blood-derived cells, e.g., the liver (“blood pool”). (D) The following day the same mice were injected with an anti-CD4 antibody conjugated to β -gal or a control anti-rat antibody similarly labeled with β -gal. Four hours after antibody injection, Lugal was injected intraperitoneally, revealing markedly different antibody distributions in both mice. A clear luminescent signal emerged over the cervical lymph nodes and the spleen of the mouse injected with the CD4 antibody (arrows, right panel), whereas only weak regional luminescence was seen in the mouse having received the control antibody (left panel). Bioluminescent images are quantified in photons/sec/cm². (E) Quantification of luminescence after Lugal injection in regions of CD4⁺ T-cell enrichment. No difference was observed between the animals over the right thorax, an area containing relatively few blood cells. However, the signal over the liver was slightly enhanced in the mouse injected with the anti-CD4 antibody. A three- to fivefold higher signal was seen over the spleen and both cervical lymph nodes of the mouse injected with the anti-CD4 antibody in comparison to the control, highlighting the organs known to contain high densities of CD4 lymphocytes. Reproduced with permission from Nature Methods, vol. 3, no. 4, pp. 295–301 (2006).

Fig. 20.3.

In vivo imaging of β_2 -adrenergic receptor activation using β -gal complementation in conjunction with sequential reporter-enzyme luminescence. Cells expressing the β_2 AR construct were transduced to express Fluc and K-Ras and injected in subcutaneous location into the back of BALB/c nude mice (4×10^6 cells/injection). (**A**) Seven to fourteen days later, when cells had grown into small tumors, baseline luminescence was imaged by injection of Lugol. Isoproterenol (6 mg/kg ip) or vehicle was injected and luminescence imaged again after 1, 8, 24, and 36 h. (**B**) Robust increase in luminescence was seen 1 h after isoproterenol injection, which subsequently returned to baseline within 24 h. (**C**) Quantification shows that signal increase was approximately fourfold over baseline (*red line*: mice treated with isoproterenol; *blue line*; vehicle-treated controls) (mean $\pm$ SEM; $n = 9$ /group). Reproduced with permission from FASEB J vol. 21, no. 14, pp. 3819–3826 (2007).