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Overview
In Vivo Bioluminescence Imaging

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In vivo bioluminescent imaging (BLI) is a versatile and sensitive tool that is based on detection of light emission from cells or tissues. Bioluminescence, the biochemical generation of light by a living organism, is a naturally occurring phenomenon. Luciferase enzymes, such as that from the North American firefly (*Photinus pyralis*), catalyze the oxidation of a substrate (luciferin), and photons of light are a product of the reaction. Optical imaging by bioluminescence allows a low-cost, noninvasive, and real-time analysis of disease processes at the molecular level in living organisms. Bioluminescence has been used to track tumor cells, bacterial and viral infections, gene expression, and treatment response. Bioluminescence in vivo imaging allows longitudinal monitoring of a disease course in the same animal, a desirable alternative to analyzing a number of animals at many time points during the course of the disease. We provide a brief introduction to BLI technology, specific examples of in vivo BLI studies investigating bacterial/viral pathogenesis and tumor growth in animal models, and highlight some future perspectives of BLI as a molecular imaging tool.

BLI: An Overview

Advances in molecular and cell biology techniques have led to the development of new in vivo imaging strategies. In vivo bioluminescent imaging (BLI) is a sensitive tool that is based on detection of light emission from cells or tissues. The utility of reporter gene technology makes it possible to analyze specific cellular and biological processes in a living animal through in vivo imaging methods.

Bioluminescence, the enzymatic generation of visible light by a living organism, is a naturally occurring phenomenon in many non-mammalian species (4, 19). Luciferases are enzymes that catalyze the oxidation of a substrate to release photons of light (8). Bioluminescence from the North American firefly (*Photinus pyralis*) is the most widely studied. The firefly luciferase gene (*luc*) expression produces the enzyme luciferase which converts the substrate D-luciferin to non-reactive oxyluciferin, resulting in green light emission at 562 nm. Another example of bioluminescence is from the sea pansy (*Renilla reniformis*). The *Renilla* luciferase gene (*ruc*) uses the substrate coelenterazine to produce a blue light at 482 nm. Because mammalian tissues do not naturally emit bioluminescence, in vivo BLI has considerable appeal because images can be generated with very little background signal.

BLI requires genetic engineering of cells or tissues with an expression cassette consisting of the bioluminescent reporter gene under the control of a selected gene promoter constitutively driving the light reporter (Fig. 1). When these engineered cells are injected into the mouse, their dissemination can be tracked by detecting the location and intensity of the light signal. In order to induce light production, the substrates luciferin or coelenterazine must be provided. These substrates usually are administered by intravascular or intraperitoneal injection. To date, there have been no reports of toxicity related to repeated dosing of substrates. In addition to constitutive promoters, inducible promoters can be engineered into the expression construct, making it possible to ma-

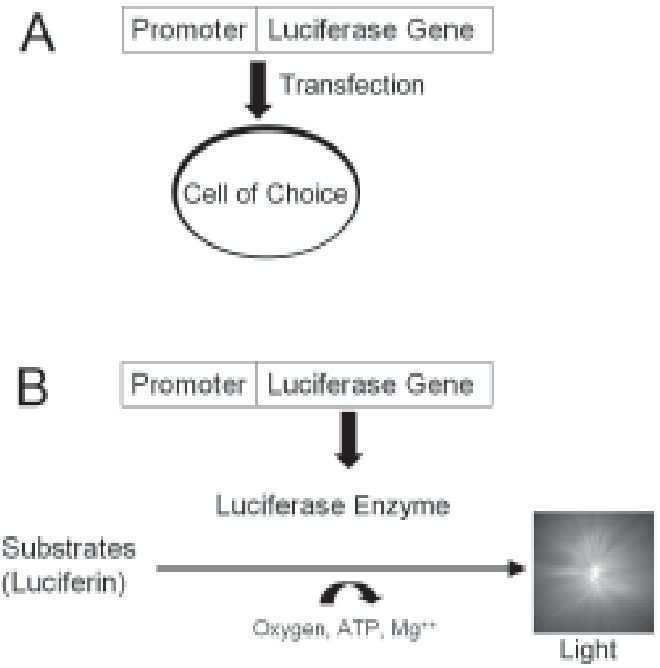

Figure 1. Bioluminescence imaging. (A) Bioluminescence expression cassette containing the luciferase gene and a promoter is transfected into the cell of choice. (B) The transfected expression cassette, when present in cells or tissues, produces luciferase enzyme inside the cell. When the luciferin substrate is added, the luciferase enzyme catalyzes luciferin substrates to emit photons of light. The emitted light then is detected by a charge-coupled device camera.

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nipulate light reporter gene expression conditionally (7).

The choice of reporter is dependent on the goals of the research, but there are some biomechanical obstacles associated with the use of *Renilla* luciferase, mostly related to the stability of the coelenterazine substrate. The firefly luciferase is a larger molecule (61 kDa), compared with *Renilla* luciferase (36 kDa) (1). Firefly luciferase requires the presence of ATP, oxygen, and magnesium, whereas the *Renilla* luciferase needs no cofactors. These two luciferases emit photons of different wavelengths: firefly luciferase emits green light at 562 nm, whereas *Renilla* luciferase emits blue light at 482 nm. Bhaumik and Gambhir examined the use of dual reporters in vivo and found that it was possible to use both in a single animal (1). They found that separate signals were distinguishable, but differences in light kinetics governed the imaging techniques. Firefly luciferase produced a stronger signal than *ruc*, and the signal persisted longer. Less coelenterazine is needed for substrate injection compared to luciferin, but coelenterazine was found to be unstable in plasma, and target site delivery was not as efficient as for luciferin. The *luc* bioassay is well defined, but potential advantages to *ruc* include its rapid light kinetics and lack of need for cofactors.

The light emitted by luciferase is able to penetrate tissue depths of several millimeters to centimeters; however, photon intensity decreases 10-fold for each centimeter of tissue depth (3). Sensitive light-detecting instruments must be used to detect bioluminescence in vivo. The detectors measure the number of photons emitted per unit area. Low levels of light at wavelengths between 400 and 1000 nm can be detected with charge-coupled device cameras that convert the light photons that strike silicon wafers into electrons (21). The software is able to convert electron signals into a two-dimensional image. The software is also able to quantify the intensity of the emitted light (number of emitted photons striking the detectors) and convert these numerical values into a pseudocolor graphic. The actual data is measured in photons, but the pseudocolor graphic enables rapid visual interpretation. Quantitative measurements within a region of interest may be necessary for more subtle differences. The use of cooled CCD cameras reduces the thermal noise, and a light-tight box allows luciferase-produced light to be optimally visualized and quantified (2) (Fig. 2).

It is useful to have the luciferase image superimposed on another type of image, such as a photograph or radiograph, for anatomical location of the emission signal. Most commercial imaging devices are equipped to generate an anatomical image, as well as the optical emission image. The software superimposes the images for visualization and interpretation. Although the detectors can measure any light source, it is necessary to have appropriate wavelength filters and a fluorescence light source if one desires the combined ability of bioluminescence and fluorescence imaging. Commercial devices are available with combined capabilities (22).

One of the challenges of in vivo small animal imaging is achieving image resolution that is fine enough to distinguish subtle details between tissues. The physical sensitivity of the optical imaging detector is one consideration, and higher sensitivity usually translates into higher equipment costs. Additional important influences on the resolution of optical imaging are the organic aspects. The sensitivity of BLI is dependent on the following organic factors: a) the number of cells expressing the reporter gene; b) the efficiency of the gene promoter; c) the availability of cofactors (luciferase only); d) the time between substrate injection and maximal signal for the assay (10); e) tissue depth; and f) potential signal impedance, such as from pigmented skin or fur. Clearly, sensitivity of BLI is difficult to define and must be established for each biological assay; however, it may be possible to image as few as several hundred to a thousand reporter cells, depending on the biological system.

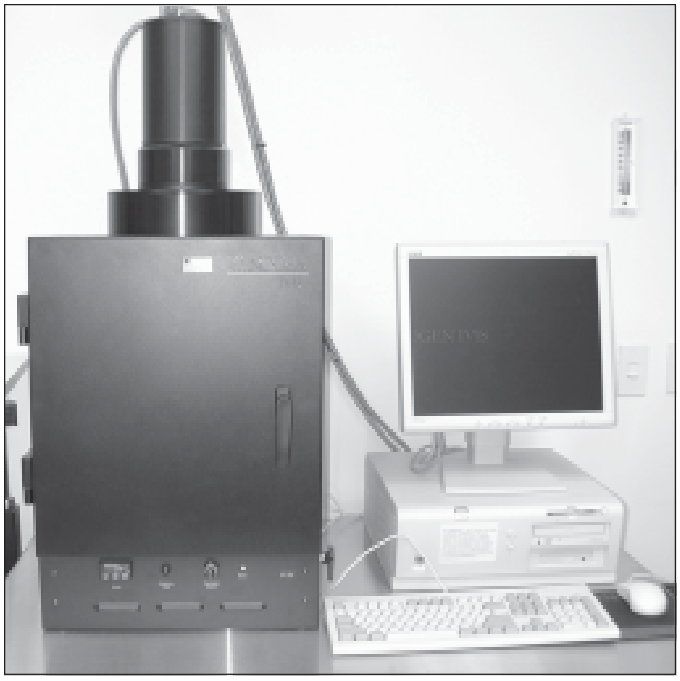

Figure 2. An imaging system consists of a light-tight box where animals are placed, the cooled charge-coupled device camera to detect light, and image processing software.

Compared with other in vivo imaging techniques, BLI is relatively inexpensive. It is possible for ambitious investigators to construct an imaging device of their own design; however, several commercial devices are available. Some of the advantages of purchasing a commercial product include the acquisition and analysis software, as well as technical support. BLI devices vary in cost and features and range in price from approximately $65,000 to $200,000. The substrates represent additional cost. The luciferin dose is 150 mg/kg, at a cost of approximately $5/mg (1). Coelenterazine is more expensive at $190/mg, with a mouse dose ranging up to 100 µg/mouse (1). With the popularity of this imaging technique increasing, the availability of these substrates is improving, and more suppliers are decreasing the costs.

The imaging times in BLI are short compared with those of other in vivo imaging techniques. Typically, a diagnostic image can be generated in a time frame of a few seconds to several minutes. In order to generate the best possible image, it is important that the subject be immobilized, and this is best accomplished with anesthesia. Animals may be anesthetized with injectable or inhalant anesthetics, depending on the set up of the imaging device.

Infection Models to Study Host–Pathogen Interactions

Luciferase imaging has been used to trace bacterial and viral infection in vivo. In vivo bioluminescence imaging was first developed using a *Salmonella typhimurium* infection model (2, 3).

Three strains of *Salmonella*, each expressing *lux* genes (bacteria-specific luciferase genes), were marked with bioluminescence through transformation with a plasmid conferring constitutive expression of bacterial luciferase (3, 6). Integration of the genes into the chromosomes of the bacteria increases the stability of light production from the labeled bacteria and represents a pronounced improvement over plasmid-based expression (2). Bacteria labeled in this manner can be detected in the tissue of mice and can reveal the location and extent of infection (2).

Labeled bacterial pathogens can be detected in the infected host in various applications. Jawhara and colleagues produced a bioluminescent *Escherichia coli* and evaluated the effects of antibiotics for the treatment of acute infections in rats (12). To identify cells that would enhance survival after stem cell transplantation, Brown and colleagues transplanted irradiated mice with hematopoietic precursor cells and then challenged them with *Pseudomonas* bacteria that constitutively expressed a bacterial luciferase (2). The bone marrow reconstitution was enhanced by myeloid progenitor pools that were capable of protection against an otherwise lethal bacterial challenge (2). BLI allowed the assessment of the location and extent of infection and thus enabled a better understanding of the host–pathogen interaction in the context of living animal models (2). Hardy and colleagues recently employed in vivo BLI to determine the location of *Listeria monocytogenes* infection in mice and reported that the organism could replicate in the murine gall bladder (9). The authors concluded that even though it is unknown whether *L. monocytogenes* replicates in the gall bladder of asymptomatic humans, the organism may be present there (9).

In vivo visualization of viral infection is also feasible using luciferase imaging. The first demonstration of a virus-mediated gene transfer to host cells using BLI was reported by Lipshutz and colleagues using an adeno-associated viral (AAV) vector in a therapy model (12, 15). In this study, the approach of detecting viral gene delivery efficiency using bioluminescence was verified as prenatal administration of luciferase expressing AAV in the mouse resulted in stable integration and bioluminescence from many tissues in these progeny mice. Luker and colleagues used BLI in living mice to monitor the herpes simplex virus type 1 (HSV-1) virus that expresses luciferase. The effects of valacyclovir treatment were monitored using BLI, and it was determined that both viral titers and imaging data showed similar dose-dependent inhibition of the virus by valacyclovir in living mice (16). Cook and Griffin used BLI to monitor *Sindbis virus* (SV) infection in a murine model of viral encephalitis (5, 6). Here, virulent and avirulent strains of SV were engineered to express firefly luciferase. Mice were infected subcutaneously in the footpad, and viral replication was detected by bioluminescence before spread to the central nervous system (5, 6).

Tumor Models to Study Growth, Metastasis, and Therapeutic Efficacy

The ability to track a small number of tumor cells expressing luciferase has allowed the study of tumor growth, metastasis, and therapeutic responses in vivo using BLI (Fig. 3). Many tumor cell lines that constitutively express luciferase have been developed. In a study characterizing the utility of bioluminescence to track tumor burden, Paroo and colleagues monitored luciferase expression in subcutaneous tumors of mice in a longitudinal study. They found that the bioluminescent tumor growth profile was similar to that observed with caliper measurements, thus further validating the utility of bioluminescence to assess tumor burden (20). BLI often has better sensitivity in detecting bone marrow micrometastasis when compared with other noninvasive imaging methods, such as radiography. In one study, Wetterwald and colleagues found that BLI was sensitive enough to detect small foci of 0.5 mm^3 of bone marrow metastasis of luciferase-expressing human mammary carcinoma cells in mice (24).

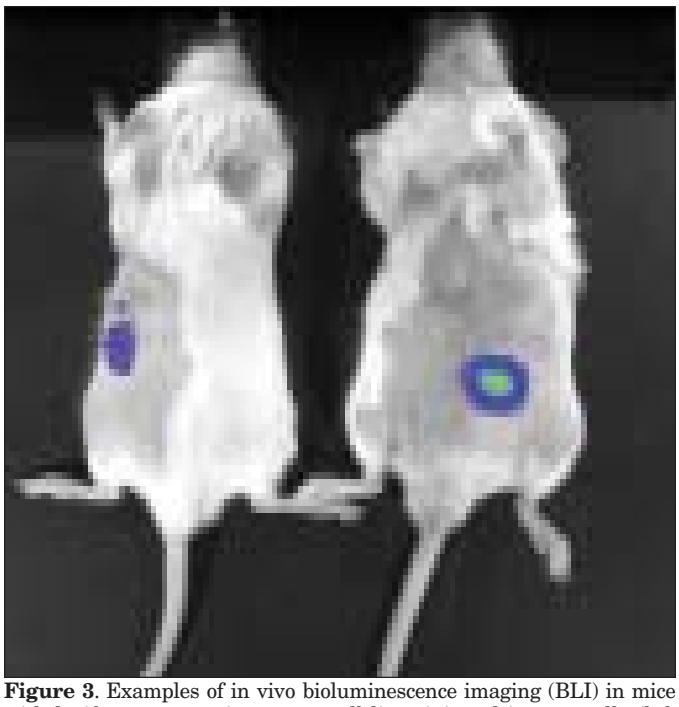

Figure 3. Examples of in vivo bioluminescence imaging (BLI) in mice with luciferase-expressing tumor cell lines injected intra-renally (left mouse) and subcutaneously (right mouse). Tumor growth can be tracked by the intensity and location of the bioluminescence. Figure courtesy of Drs. James Vasselli and W. Marston Linehan, National Cancer Institute.

The capability to longitudinally track tumor growth and metastasis is useful not only to investigate specific tumor characteristics but also can be used to better characterize genetically engineered mice with altered tumor suppressor genes. Luciferase-expressing tumor cell lines inoculated into these engineered mouse models allow the study of various aspects of the oncology disease process that are most relevant to the functions of the specific tumor genes altered and being studied in these mice (11, 17, 23). Furthermore, mice have been engineered in which the luciferase reporter is expressed on tumors spontaneously arising from engineered mice, allowing investigation of disease onset and progression (17).

BLI also has been utilized to noninvasively assess therapeutic efficacy and gene delivery. Adenovirus-induced ovarian tumor killing in mice was demonstrated effectively by using bioluminescence (13). In a study utilizing rats injected with luciferase-transfected colon carcinoma cells, the antineoplastic effects of cisplatin were effectively tracked by bioluminescence as concluded by decreased number of light emitting tumor sites and light signal intensity (25).

Assessment of Real-Time Gene Expression

BLI also has been used to study in vivo gene expression. To accomplish this, mice are engineered to express luciferase under the transcriptional control of promoters from genes of interest. Events leading to activation of the promoter, therefore, result in

the production of luciferase, which can be monitored by the level of light produced.

In one example, transgenic mice engineered with the luciferase gene under the control of the estrogen-responsive element promoter were used to detect gene activation of estrogen receptors. Here, administration of estrogen compounds led to a dose- and time-dependent activation of luciferase activity in vivo allowing the non-invasive study estrogen induction (14). In another example, mice engineered with the luciferase gene under the control of an androgen-dependent promoter were used to detect androgen-dependent gene regulation. In these mice, testosterone treatment increased luciferase expression, whereas anti-androgenic compounds decreased the bioluminescent signal (18). Bioluminescence also was used to study in vivo angiogenesis. Transgenic mice with the vascular endothelial growth factor-2 (*VEGFR2*) promoter fused to the luciferase gene were used to study *VEGFR2* gene expression, an indicator of angiogenesis, during wound healing (26).

Future Directions

BLI allows investigation of many biological and disease processes in vivo. Bioluminescence has been utilized successfully for noninvasive monitoring of bacterial or viral infection and represents an attractive approach to investigating the host–pathogen interaction in vivo. The ability to track tumor burden and metastatic disease and to assess anticancer therapy represents an important application of this technology, but new applications are rapidly evolving. For example, we are investigating neurodegeneration in the central nervous system by using BLI. The rapid diffusion of luciferin across the blood–brain barrier (4) makes possible in vivo BLI studies of neurodegeneration. Genetically engineered mice have been developed in which the luciferase gene is under the control of promoter elements of genes activated upon neuronal injury. Bioluminescence can be used to identify and track degenerating neurons in these engineered mice, whose neurons express luciferase when injured.

The capability to monitor a biological or disease process longitudinally in the same mouse is a pronounced advantage of BLI technology. Sequential chronological analysis not only reduces the number of animals needed but allows for collection of robust scientific data. In addition, the pathophysiology of disease may be better understood through the analysis of disease progression or regression in individual animals.

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