In vivo bioluminescence for tracking cell fate and function

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In vivo bioluminescence for tracking cell fate and function. Am J Physiol Heart Circ Physiol 301: H663–H671, 2011. First published June 10, 2011; doi:10.1152/ajpheart.00337.2011.—Tracking the fate and function of cells in vivo is paramount for the development of rational therapies for cardiac injury. Bioluminescence imaging (BLI) provides a means for monitoring physiological processes in real time, ranging from cell survival to gene expression to complex molecular processes. In mice and rats, BLI provides unmatched sensitivity because of the absence of endogenous luciferase expression in mammalian cells and the low background luminescence emanating from animals. In the field of stem cell therapy, BLI provides an unprecedented means to monitor the biology of these cells in vivo, giving researchers a greater understanding of their survival, migration, immunogenicity, and potential tumorigenicity in a living animal. In addition to longitudinal monitoring of cell survival, BLI is a useful tool for semiquantitative measurements of gene expression in vivo, allowing a better optimization of drug and gene therapies. Overall, this technology not only enables rapid, reproducible, and quantitative monitoring of physiological processes in vivo but also can measure the influences of therapeutic interventions on the outcome of cardiac injuries.

bioluminescence imaging; heart; gene therapy; stem cell therapy; in vivo cell tracking

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Cell-based therapies have rapidly emerged as a potential therapeutic approach for heart disease. After the initial work to characterize putative endothelial progenitor cells (1) and their potential to promote cardiac neovascularization and to attenuate ischemic injury, a decade of intense research has examined several novel approaches to promote cardiac repair in adult life. A variety of adult stem and progenitor cells from different sources have been examined for their potential to promote cardiac repair and regeneration: bone marrow-derived cells (55, 72), circulating and mobilized CD133+ and CD34+ stem and progenitor cells (36), mesenchymal stem cells (56, 65, 83), cardiac resident stem cells (43, 62), and skeletal myoblasts (54, 97). However, many questions such as the optimal type and number of progenitor cells to be administered, the route of administration, and the best time to administer cells after injury remain unresolved. This is particularly true in vivo where it is difficult to assess cellular activity in the heart in real time. Therefore, it would be helpful to have practical tools to accurately track cell location and their functional status over time in vivo.

Histopathological examination has been the main approach for ex vivo evaluation of the distribution, the engraftment, and the differentiation of injected cells. However, histopathology precludes the evaluation and monitoring of these parameters in real time and in vivo. Molecular and cellular imaging has provided various techniques for identifying and tracking transplanted cells in cardiovascular research (15). Magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and single photon emission computed tomography (SPECT) offer deep tissue penetration and high spatial resolution (64, 70, 94a). However, in small animal studies, these techniques are more costly and time consuming to implement compared with optical imaging. Among the optical imaging tools, bioluminescence imaging (BLI) is a promising technique that is especially useful in small animal models and does not require the use of radionuclides with their associated hazards. BLI is a high throughput technique that can provide unmatched sensitivity because of the absence of endogenous luciferase expression in mammalian cells and the low background luminescence emanating from animals. BLI can be very useful in evaluating the delivery efficiency of therapeutic genes and their expression levels in vivo. By the use of different cellular promoters to drive the expression of a luminescent reporter gene, BLI allows monitoring of the transplanted cells’ differentiation status as well as their location and functional characteristics in vivo (39, 51, 72, 78, 94).

Overall, BLI of reporters cloned into promoter/enhancer sequences or engineered into fusion proteins has demonstrated the modality’s ability to monitor fundamental processes such as transcriptional regulation, signal transduction cascades, pro-

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tein-protein interactions, protein degradation, oncogenic transformation, cell trafficking, and targeted drug action under in vivo spatial registration. In this review, we will discuss recent advances and applications of BLI, specifically as they apply to cardiovascular research.

Principles of Bioluminescence

In nature, numerous luminous species exist in more than 700 genera, of which 80% are marine species (93). Luciferase enzymes have been cloned from both marine (e.g., *Renilla* luciferase) and terrestrial (e.g., firefly and click beetle luciferase) eukaryotic organisms and are commonly used as reporters for in vitro and in vivo studies. They emit long wavelengths of bioluminescence (>600 nm) in the red and near-infrared regions of the spectrum and are efficiently transmitted through mammalian tissues (20, 86). These wavelengths can avoid absorbing and scattering environment of mammalian tissues (69), thus can be efficiently detected outside a small animal’s body using BLI.

BLI is based on the detection of light emitted by cells that express light-generating enzymes such as luciferase. In bioluminescent reactions, luciferase generates visible light through the oxidation of enzyme-specific substrates such as D-luciferin for terrestrial organisms (29, 30) and coelenterazine for marine organisms (31, 53). Luciferases from different organisms can be distinguished by their abbreviations: *lux* (bacterial), *luc* (firefly), and *lcf* (dinoflagellate). To track cells in vivo by BLI, the cells of interest need to be genetically modified to express luciferase. The animal recipient of the cells receives the luciferase substrate either intraperitoneally or intravenously and is placed in a light-tight dark box where luminescence is detected (Fig. 1). In a bioluminescent reaction, the generation of light depends on several factors. Firefly luciferase requires ATP, Mg$^{2+}$, and oxygen to catalyze the oxidation of its substrate D-luciferin and generates CO$_2$, AMP, inorganic pyrophosphate, oxyluciferin, and a yellow-green light at a wavelength that peaks at 562 nm (Fig. 2). By comparison, *Renilla* luciferase catalyzes the oxidative decarboxylation of coelen-
terazine in the presence of dissolved oxygen to yield oxyluciferin, CO₂, and blue light that peaks at 480 nm (49). Bioluminescence generated by the luciferase reporter reaction is captured by a cooled charge-coupled device camera that can detect very low levels of visible light emitted from internal organs (19). Charge-coupled device camera-imaged bioluminescence can then be superimposed on photographic images of the mouse to detect quantitatively and repetitively the bioluminescent signal from a given location (95). Imaging can be conducted to 10 min after intraperitoneal injection of α-luciferin (reporter probe), with relatively stable light emission levels for 30 to 60 min, depending on the experimental conditions. The sensitivity of detection depends on the wavelength of light emission, expression levels of the enzyme in the target cells, the location of the source of bioluminescence in the animal, the efficiency of the collection optics, and the sensitivity of the detector (95).

Reporter genes commonly used for BLI. To track cells in vivo, reporter genes and reporter probes must be able to reveal cellular and molecular processes throughout an entire study period, be highly sensitive to small changes in cell function and distribution over time, and must not alter the labeled biological process itself. A variety of reporter genes have been introduced and validated for different imaging modalities, including various luciferases for BLI (5, 67, 74). Luciferase (Luc) is the only one that produces light without requiring an external excitation source, and they offer inherently low background signals because animal tissues do not emit significant amounts of light. Since the first report of the cDNA encoding Luc in 1985 (23), DNA sequences have been optimized so that the Luc gene can be expressed at high levels and its product localized in the cytoplasm of the cells. For all reporter systems, the intensity of light is proportional to the amount of luciferase expressed in each individual cell or the number of cells in which a gene has been transferred.

Of the many available luciferase enzymes, only a subset have been developed and used as reporter genes. Luciferase from the North American firefly Photinus pyralis is the most common choice, but other luciferases such as the sea pansy Renilla reniformis, the click beetle Pyrophorus plagiophalum, and the copepod Gaussia princeps have also been investigated (48, 79). Gaussia and Renilla luciferase enzymes emit in the blue/green region of the UV visible spectrum, where light is strongly absorbed and scattered by tissues. Consequently, the imaging performance suffers from poor sensitivity and spatial resolution. On the contrary, Photinus pyralis and click beetle luciferase emit ~60% of their light at >600 nm, which enables great tissue penetration. Photinus pyralis has emission at 620 nm when collected at 37°C, making it among the longest emitting luciferases at mammalian body temperatures and the most sensitive for in vivo applications (100).

Bacterial luciferases (Lux) such as from Photobacterium luminescens emit blue light that has been used for BLI of bacterial infections. They are unique in that their lux operon cassette codes for the luciferase and also for enzymes that produce the substrate required for luminescence reaction, thereby eliminating the need for exogenous substrate. Transferring this operon to mammalian cells would be advantageous for in vivo imaging; however, limited research has been done to determine whether this is possible (16). Mutations in luciferases can change the wavelength of the luminescence emitted. A mutation of a single amino acid has been shown to cause red-shifted luminescence and improve in vivo performance. Recently, a red-shifted mutant of luciferase from Photinus pyralis was created and described to have an emission maximum of 612 nm at pH 7.0, a narrow emission bandwidth, and to be thermostable (with a half-life of 8.8 h at 37°C vs. 0.26 h for the wild-type luciferase). This Photinus pyralis mutant, called Ppy RE-TS reporter, has been successfully used in small animals to visualize cancer progression and shown to have superior in vivo imaging performance compared with the wild-type Photinus pyralis luciferase (6, 7).

The choice of reporter gene reporter probe pair to be used should ultimately be based on the specific biological process to be monitored, the duration and intensity of the signal needed, and the tissue to be imaged (21, 100) (Fig. 3). As longer wavelengths of light penetrate mammalian tissues with less absorbance, luciferase from Photinus pyralis (>600 nm) is more readily detected (17). Moreover, its substrate α-luciferin remains in circulation longer than other substrates because it is poorly catalyzed by mammalian tissues (101). However, Photinus pyralis luciferase has the disadvantage of having a longer coding sequence (1,653 bp) compared with marine luciferases such as Renilla and Gaussia. Renilla and Gaussia luciferases have coding sequences of 936 and 558 bp, respectively, making them more suitable for studies that require compact transgene sequences, such as gene transfer and gene expression studies. Another advantage of Renilla and Gaussia luciferases over Photinus pyralis is the fact that they do not require ATP as a cofactor during bioluminescent reaction and thus can be used for imaging cells independent of their metabolic state (5, 66). One particular characteristic of Gaussia luciferase is that it is naturally secreted (96) and therefore can be used as a reporter for quantitative assessment of cells in vivo by measuring its concentration in blood (61). However, Renilla and Gaussia luciferases produce shorter wavelengths of light (peaking at 480 nm), which are not transmitted through tissues as effectively as Photinus pyralis (100). Overall, the utility of Renilla and Gaussia for in vivo BLI can benefit from improvements in sensitivity (46–48).

Codon-optimized humanized Gaussia (2, 79) has improved its sensitivity in mammalian cells, but the pharmacokinetics of its reporter probe coelenterazine are somewhat limiting in vivo. Coelenterazine is prone to quick inactivation, including degradation through autoxidation. This substrate is also costly with low solubility. Furthermore, coelenterazine binds to serum proteins, is cleared rapidly from the bloodstream (101), and decays rapidly with time (5). Therefore, when imaging in vivo with coelenterazine, the signal needs to be acquired immediately after substrate administration. On the other hand, this short half-life can be advantageous in cases in which sequential imaging of two luciferases, such as Gaussia princeps and Photinus pyralis, is required to monitor two biological processes in tandem in the same animal (5).

In summary, the development of reporter gene variants with better emission spectra, brightness, and stability can ultimately improve sensitivity and the overall performance of luciferases in BLI imaging studies. Moreover, cloning new luciferases from different organisms, such as those from Luciola italica and Cratophorus distinctus, can certainly improve current applications and make novel ones possible (8, 88).
Vector-mediated expression of reporter genes. A fundamental requirement for BLI is the expression of a reporter gene (e.g., luciferase) by the cells or tissues to be imaged, which requires the introduction of genetically encoded imaging reporters into cells cultured in vitro (50). Alternatively, luciferase-expressing cells can be obtained from luciferase transgenic mice (Fig. 1). This can be achieved by using a reporter vector that incorporates a luciferase gene driven by a promoter that allows luciferase to be constitutively expressed by all cells in the animal’s body (11, 83).

Delivery of bioluminescent reporter genes to cells has been achieved through several means, but lentiviral-based gene transfer has been the method of choice because of its effective gene delivery and high expression levels of transgenes in mammalian cells in culture as well as in vivo (22). Lentiviruses have the capability to deliver target genes to both dividing and nondividing cells and are capable of inserting genetic information into the host genome, ensuring prolonged gene expression with a more limited host immune response (80). The safety of the lentiviral vectors has been further improved with the generation of self-inactivating vectors and the use of minimal packaging systems. The efficiency of gene expression has been improved by the introduction of a relatively strong internal promoter such as cytomegalovirus. This promoter drives the expression of the reporter gene and guarantees that the reporter expression is always “on” under all conditions, in all tissue types. Moreover, lentiviral vectors can be used to simultaneously induce the expression of multiple genes in a cell. Coupling the expression of a gene with a luciferase reporter gene provides a simple yet effective mechanism for studying the regulation of gene expression and monitoring it by BLI. This provides exciting opportunities for transcriptional targeting, double reporter labeling for BLI monitoring, as well as gene therapy. For example, by linking the expression of luciferase to the cardiac-specific promoter myosin light chain 2v, it is possible to monitor cells undergoing cardiac differentiation over time via the detection of reporter gene expression by BLI (27, 35).

Applications of BLI in Cardiovascular Research

Because physiological processes are dynamic in time and space, end-point assays do not always provide a comprehensive understanding of biology in vivo. In cardiovascular research and many other scientific areas, BLI has been used for noninvasive visualization of a variety of biological processes in real time. In this section, we will review the many applications of BLI in cardiovascular research.

Monitoring expression of therapeutic genes in the heart. Gene therapy is a rapidly evolving field in cardiovascular medicine. This technology allows for the correction of functional gene loss and enables the expression of a therapeutic gene in a target tissue (52). Nevertheless, gene therapy studies continue to be plagued by suboptimal delivery of genes, poor survival of cells carrying the therapeutic gene(s), and the inability to evaluate the levels of gene expression in vivo (28). Thus the information gathered from BLI studies in small animals can be used to design solid clinical trials that will help improve gene therapy for cardiac repair. In cardiovascular research, BLI has been used to address a variety of questions that range from determining the effects of transgene expression (e.g., BCL2) on cardiomyoblast survival and cardiac repair (40) to testing the efficiency of anti-inflammatory drugs on the expression of specific genes (e.g., inducible nitric oxide synthase) (99). Additionally, BLI has been used to optimize vector systems (33) and treatment regimens (71) for delivery of therapeutic genes (e.g., hypoxia-inducible factor-1α) to the heart.

The applications of BLI in research are constantly expanding with the development of new therapeutic modalities. For instance, BLI enabled tracking the activity of a short hairpin RNA plasmid in knocking down inhibitory factors of angio-

![Fig. 3. Emission wavelengths for the most commonly used reporter luciferase enzymes in BLI and their advantages and disadvantages. V, violet; B, blue; Y, yellow; O, orange; R, red.](http://apjheart.physiology.org/doi/10.220.32.247/4)
BLI has demonstrated the poor survival of bone marrow mononuclear cells (72), cardiac resident stem cells (43), mesenchymal stem cells (83), adipose stromal cells (82), ESC-derived cardiac cells (10), and ESC-derived endothelial cells (45) by 8 wk after cell injection. Conversely, the tumorigenic potential of ESCs can also be uncovered by BLI. Lee et al. (42) demonstrated that a minimum of 100,000 human ESCs are necessary to form teratoma in the heart. Overall, BLI studies have provided significant insights into the biology of stem cells in vivo and how issues such as poor survival and potential tumorigenicity must be overcome before translation into the clinic.

Monitoring immune rejection of heart transplant and cell grafts. Allogeneic heart transplantation is the most commonly used therapy for end-stage heart disease. Transplant loss due to immune rejection remains a significant problem in clinical heart transplantation despite current immunosuppressive therapies (73). Acute rejection of heart transplants is an immune response mediated by the coordinated infiltration and function of host alloantigen-specific T cells in the allograft (57). In this context, BLI has allowed monitoring of cardiac allograft over time and revealed its rejection by day 12 after transplantation. A decrease in the intensity of bioluminescence signals was detected as early as day 4, suggesting acute graft rejection (78). The correlation of bioluminescence intensity to other measures of heart function such as beating score, fractional shortening, and lymphocyte infiltration has provided additional means to assess function and to determine the possible mechanisms responsible for rejection.

Acute rejection can also be a major issue in human ESC-based therapy (25, 84). BLI has provided significant information for the development of therapeutic strategies to prevent the rejection of stem cells. An immunosuppressant cocktail consisting of tacrolimus and sirolimus has been shown by BLI to mitigate the rejection of human ESCs (77). Recently, Pearl et al. (63) used BLI to demonstrate that a cocktail of costimulatory blockade agents (anti-CD40 ligand, cytotoxic T-lymphocyte antigen 4-immunoglobulin, and anti-lymphocyte function-associated antigen-1) induced long-term allogeneic and xenogeneic human ESCs and induced pluripotent stem cell engraftment. Overall, these studies elucidate the importance of in vivo imaging for the development of therapies that may one day enable stem cell therapy to become feasible.

### Table 1. Cells types that have been investigated for their potential to promote cardiac repair using BLI

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BLI, bioluminescence imaging; MI, myocardial infarction.
Limitations

BLI has been successfully used to obtain semiquantitative measurements of biological processes because of a strong correlation between the number of cells and the bioluminescence signal detected both in vitro (75) and in vivo (68). However, a simple quantification of light emission may not provide a true representation of biological processes. This is because the firefly luciferase reaction is a complex interaction of a variety of molecules (e.g., ATP, Mg$^{2+}$, oxygen, and luciferin) and because the intensity of the bioluminescence signal depends on multiple factors. In particular, the number of metabolically active luciferase-transfected cells, the concentration of luciferin, ATP and oxygen levels, the spectral emission of bioluminescence probes, and the depth and optical properties of tissues are known to alter the intensity of bioluminescence signal (69). Another issue that should be considered during quantitative BLI is the limited and wavelength-dependent transmission of light through animal tissues. Light sources closer to the surface of the animal appear brighter compared with deeper sources because of tissue attenuation properties (91). It is estimated that for every centimeter of depth, there is a 10-fold decrease in bioluminescence signal intensity (18). Mathematical models can be used to predict in vivo imaging signal levels and spatial resolution as a function of depth and to help define the requirements for imaging instrumentation. However, the overall low spatial resolution (3–5 mm range) and limited tissue penetrance restrict the use of BLI to small animal studies (69).

Changes in tissue oxygenation can also alter bioluminescence signal. In rat gliosarcoma for instance, bioluminescence signal has been shown to decrease by $\sim 50\%$ at 0.2% oxygen (58). Thus, for reliable BLI measurements, it is important to understand the effects of local niche in which the luciferase-expressing cells of interest reside. This is especially the case in cardiovascular studies involving hypoxia (e.g., myocardial infarction). Similarly, BLI quantification has to be carefully interpreted in studies that involve surgical procedures. Changes in tissue thickness because of the presence of inflammation, edema, sutures, and animal growth can alter light absorption and scattering as well as the bioluminescence signal.

Conclusions and Future Directions

Many different organisms, ranging from bacteria and fungi to fireflies and fish, are endowed with the ability to emit light. The discovery of new luminescence reporters and the use of genetically modified reporters may further strengthen reporter gene expression in mammalian cells, thus improving the sensitivity and expanding the applications of this imaging...
modality (7, 14). Additionally, alternative methods for transduction of reporter gene may reduce the risk of anomalous and inappropriate transcription of unwanted sequences in mammalian cells (37).

BLI allows real-time monitoring of survival and homing of various therapeutic cells that are currently being investigated to promote cardiac repair. By the use of cardiac-specific promoters linked to reporter genes, BLI can be used to monitor cell differentiation in vivo. It also provides excellent opportunities to evaluate strategies designed to improve the survival of therapeutic cells or transplanted hearts. The ability to monitor physiological processes in vivo in real time is extremely beneficial to evaluate the success or failure of the various therapies designed to promote cardiac repair. As most basic cardiovascular research is performed in rodent models, BLI can efficiently reveal problems and provide insight into solutions for validating and optimizing novel therapies for the treatment of heart disease. Overall, this technology can furnish great insights that can drive the development of better clinical trials in cardiovascular medicine.

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Because of space limitations, we were unable to cite all the important papers relevant to bioluminescence imaging and cardiovascular research. We apologize to investigators not mentioned here who have made significant contributions to this field.

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