REVIEW ARTICLE

Noninvasive Molecular Neuroimaging Using Reporter Genes: Part I, Principles Revisited

T.F. Massoud
A. Singh
S.S. Gambhir

SUMMARY: In this first article, we review the basic principles of using reporter genes for molecular imaging of the brain in living subjects. This approach is emerging as a valuable tool for monitoring gene expression in diverse applications in laboratory animals, including the study of gene-targeted and trafficking cells, gene therapies, transgenic animals, and more complex molecular interactions within the central nervous system. Further development of more sensitive and selective reporters, combined with improvements in detection technology, will consolidate the position of in vivo reporter gene imaging as a versatile method for greater understanding of intracellular biologic processes and underlying molecular neuropathology and will potentially establish a future role in the clinical management of patients with neurologic diseases.

Molecular imaging is the latest addition in an astounding evolution of imaging during the past few decades, bringing in vivo observations to a new and more meaningful dimension. Its novelty lies in the fact that unlike the traditional means of imaging living subjects, which rely on nonspecific macroscopic physical, physiologic, or metabolic changes to differentiate pathologic from normal tissue, molecular imaging seeks to shed new light on both structure and function by creating images that directly or indirectly reflect specific cellular and molecular events (eg, gene expression), which can reveal pathways and mechanisms responsible for disease within the context of physiologically authentic and intact living subject environments.[1]

This change in emphasis from a nonspecific to a more specific imaging approach represents a significant paradigm shift in neuroradiology. The impact of this shift in philosophy means that molecular neuroimaging could now provide the potential for the following: 1) understanding a patient's abnormal biology in a quick noninvasive manner, and with less labor, than that achievable by conventional pathology or clinical chemistry-based assays; 2) earlier detection and characterization of disease and its pathogenesis; and 3) assessment of the therapeutic effectiveness at a molecular level, long before phenotypic change. Many of the attributes of this new imaging discipline are already being exploited in the laboratory, where molecular neuroimaging techniques are currently used in research animals to develop and validate these novel imaging strategies, with a view to future extrapolation to the clinical setting.[2,3] We and others have previously reviewed the factors contributing to the emergence of molecular imaging, the particular advantages of these approaches, and the general goals potentially achievable in biomedical research and clinical practice by adopting molecular imaging strategies.[1,4-7]

One of the subdisciplines in molecular imaging that is least familiar to clinical imaging specialists, arguably one that holds future promise in neuroimaging, is that of reporter gene expression imaging.[8,9] Here we review the basic principles and recent technologic developments in reporter gene expression imaging in living subjects. The fact that current applications of these new techniques are mostly confined to experimental small animal models should not diminish their relevance or interest to clinical neuroradiologists. Knowledge of the fundamentals of reporter gene expression imaging is essential for at least 2 reasons: 1) these novel imaging methods would constitute the same techniques potentially extrapolated into future clinical practice and applicable to future molecular imaging of patients with neurologic disorders, and 2) these novel analytic research techniques, adapted from standard in vitro assays used in laboratory biologic research, are likely to be used with increasing frequency within academic research centers by a new breed of neuroradiology clinician-scientist specialists, who, through their research endeavors, will continue building the foundations for future improved molecular neuroimaging in clinical practice.

From the Department of Radiology (T.F.M., A.S.), Section of Neuroradiology, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Cambridge, UK; and the Molecular Imaging Program at Stanford (T.F.M., S.S.G.) and Departments of Radiology and Bioengineering (S.S.G.), Bio-X Program, Stanford University School of Medicine, Stanford, Calif.

Please address correspondence to Tarik F. Massoud, MD, PhD, Department of Radiology, Section of Neuroradiology, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Box 219, Level 5, Hills Rd, Cambridge CB2 2QQ, United Kingdom; e-mail: tfm23@radiol.cam.ac.uk

DOI 10.3174/ajnr.A0864

Reporter Gene Expression Imaging: A Subfield of Molecular Imaging

Although the term "imaging" may be clearly defined as the creation of a visual representation of the measurable property of a person, object, or phenomenon, confusion exists regarding the term "molecular imaging." This is not surprising because this term is borrowed from "molecular biology," which itself is "an elusive term whose definition depends on who is doing the defining."[10] One definition of "molecular biology" is the study of gene structure and function at the molecular level.[10] A "gene" may be defined as a locus of cotranscribed deoxyribonucleic acid (DNA) exons that ultimately results in the production of a peptide or protein. Genes are hereditary units controlling identifiable traits of an organism. Rigorous exploration is taking place in the biologic sciences to determine the patterns of gene expression that encode normal biologic processes. There is also a growing belief that diseases result from alterations in normal regulation of gene expression that transition cells to phenotypes of disease. These alterations in gene expression can result from interactions with the environment, hereditary deficits, developmental errors, and the aging process.[11] Molecular imaging of gene expression is the process by which a gene product, made via the 2 steps of transcription and translation, can be visualized, quantified, and located in intact living subjects. The achievement of molecular imaging of gene expression with the use of particular genes, termed imaging reporter genes, forms the basis of reporter gene expres-

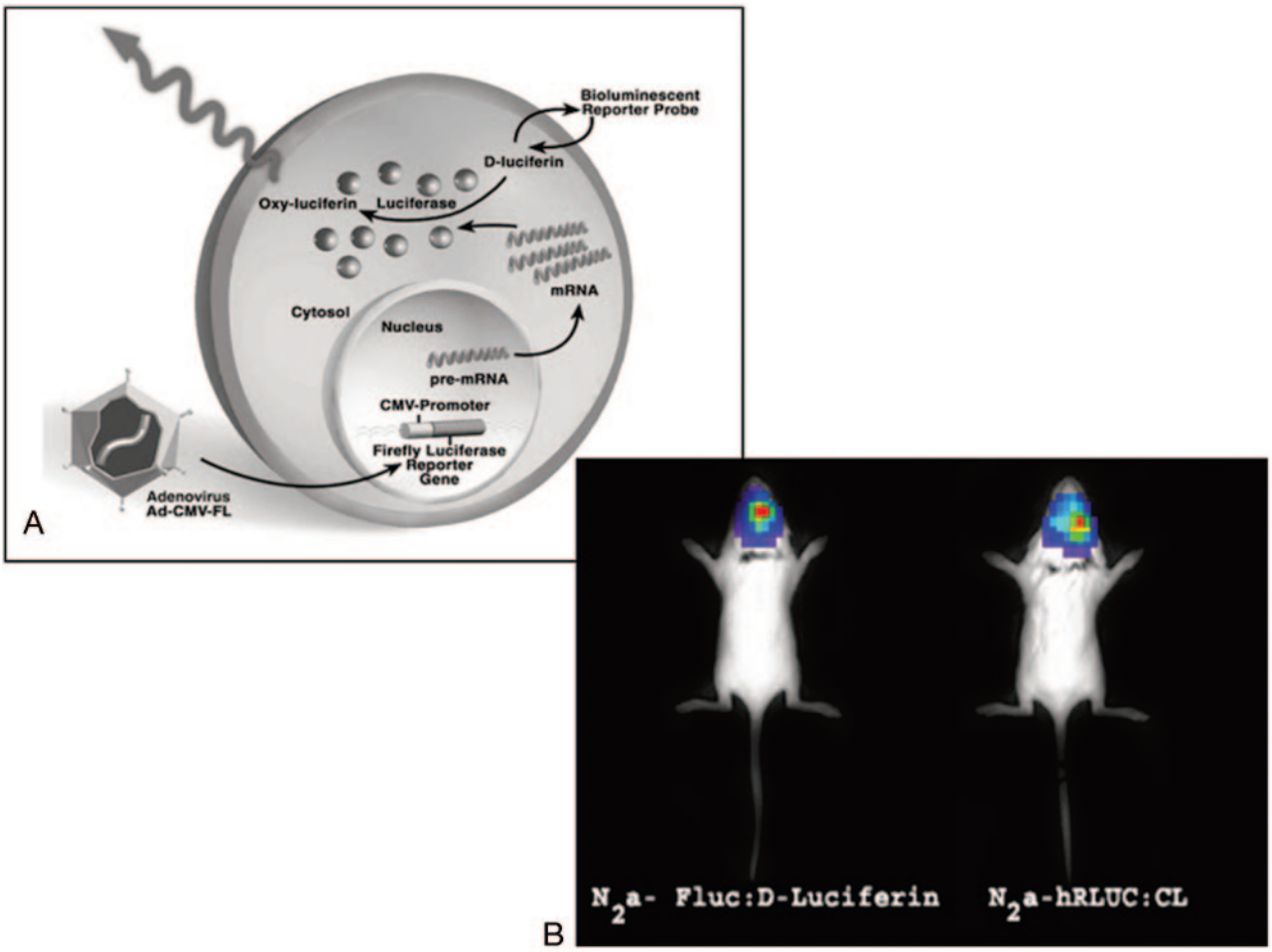

Fig 1. Reporter gene imaging. *A,* Schematic diagram of the principle of reporter gene imaging by using the enzyme firefly luciferase. Once the cell is transduced with a viral vector containing the imaging gene cassette, a promoter of choice drives the transcription of the imaging reporter gene (*Fluc*). If the promoter leads to transcription of *Fluc,* then translation of the imaging reporter gene mRNA leads to a protein product (the enzyme firefly luciferase) that can interact with the imaging reporter probe (D-Luciferin). This interaction is a chemiluminescent reaction that catalyzes the transformation of the substrate D-Luciferin into oxyluciferin in a process dependent on ATP, Mg^{++}, and O_2, leading to the emission of light, which can be detected by using low-light sensing instruments. Other gene/substrate combinations may be used as well (eg, *hRluc* and its substrate CL, see text). *B,* Bioluminescence neuroimaging in mice. Balb/c mice with 10^5 intracranially injected N_2a cells transfected 24 hours previously with CMV-*hRluc* (right) and CMV-*Fluc* (left). Intracranial injections were performed immediately before substrate administration. The mice received intraperitoneal injections of the substrates D-Luciferin (1.5 mg) or CL (5μg) respectively. The charge-coupled device camera images were taken approximately 5–7 minutes post-substrate injections. Maximum signal intensity detected in photons per second per square centimeter per steradian is the following: *Fluc,* 2.1×10^6; *hRluc,* 9.2×10^4. CMV indicates cytomegalovirus; CMV-FL, adenoviral vector containing an imaging cassette with the CMV promoter driving the transcription of firefly luciferase gene.

sion imaging in living subjects. Previously, the only means to obtain anything akin to this information had been near impossible (aside from whole-body imaging of small transparent animals) other than by in vitro or cell culture methods.

Principles of Reporter Gene Expression Imaging

A dictionary might define a "reporter" as one who broadcasts news or carries a message. This colloquial meaning of the word is quite apt when characterizing reporter systems in molecular biology and imaging. Reporter molecules broadcast their presence by producing a signal intensity that can be measured in the laboratory by benchtop or cell culture techniques or with imaging instrumentation in the case of molecular imaging. More precisely, a reporter gene is one with a readily measurable phenotype that can be distinguished easily from a background of endogenous proteins.[12] Molecular and cell biologists might typically use reporter genes (DNA that codes for reporter protein molecules) to determine how the expression of other genes of interest is regulated under various conditions. Thus, reporter genes are used to study the following: 1) promoter/enhancer elements involved in gene expression ("promoters" are short stretches of DNA that signal ribonucleic acid [RNA] polymerase to begin transcription of a gene, and "enhancers" are DNA binding sites for protein factors that boost gene transcription), 2) inducible promoters to look at the induction (the gradual or complete switching on or off) of gene expression, and 3) endogenous gene expression through the use of transgenes containing endogenous promoters fused to the reporter.[13] In all these cases, the expression of a gene of interest can be studied if it is linked to the expression of a reporter gene (the transcription of which can be tracked)—for example, by their sharing of the same promoter/enhancer elements. Researchers always look for reporter systems that are sensitive and convenient to use and have assays that yield rapid, quantitative, and reproducible results with a wide dynamic range under a variety of conditions.[8,14]

Customary methods in biomedical research to monitor reporter gene expression include the following: 1) tissue biopsy

Features of molecular imaging techniques used in reporter gene imaging

Imaging Technique	Advantages	Disadvantages
Bioluminescence optical imaging	Very high sensitivity	Very low spatial resolution
	High throughput	Only planar imaging, not tomographic
	Very versatile	Surface-weighted images owing to light scatter and absorption
	Cheap	Semiquantitative imaging data
		Mass amount of probe required (? toxic)
		Not an established clinical technique
MR imaging	Very high spatial resolution	Low sensitivity
	Tomographic imaging	Mass amount of probe required (? toxic)
	Widely available established clinical technique	
PET, SPECT	High sensitivity	Low spatial resolution
	Fully quantitative imaging data	Probes for using HSV1-*tk* gene do not cross the blood-brain barrier
	Tomographic imaging	
	Nanogram amount of probe required (nontoxic and safe)	*D2R* gene normally expressed in basal ganglia interferes with image interpretation when using this reporter system
	Established clinical techniques	

Note:—? toxic indicates a question concerning potential toxicity.

or gross pathologic observation, with or without histochemical or immunohistochemical staining for reporter gene proteins; 2) in situ hybridization with probes targeted at reporter gene messenger RNA (mRNA); and 3) blood sampling when the reporter gene product is a secretable protein (eg, alkaline phosphatase).[8] These conventional methods to detect reporter proteins are hindered by their inability to noninvasively determine the location, magnitude, and extent of gene expression in a living subject. To image gene expression in living subjects, one must target either genes externally transferred into cells of organ systems (transgenes) or endogenous genes. Most current applications of reporter gene imaging are of the former variety, and this will be stressed in the examples provided in this article. The expression of endogenous genes can also be imaged indirectly if a promoter that is endogenous to the cell drives the reporter gene. Thus, whenever the endogenous gene is upregulated, the reporter gene is also upregulated.[15,16]

There are 2 different strategies for interrogating the myriad biologic processes that could be targeted for molecular imaging. The first strategy is a direct one that uses de novo synthesis of unique molecular probes targeted to specific molecular markers/targets, such as a receptor, transporter, or enzyme. For reasons outlined elsewhere,[1] the development and validation of these specific imaging agents are time-consuming and require significant effort. The second general strategy to image specific molecular and cellular events is an indirect one, and imaging reporter systems can be grouped under this approach. This strategy (Fig 1) entails the use of a pretargeting molecule (a reporter gene under the control of one of many possible promoters) that is subsequently activated (to yield a reporter protein) on occurrence of a specific molecular event (promoter switch-on and reporter gene expression). Following this, a molecular probe (the reporter probe) specific for the activated pretargeting molecule (reporter protein) is used to image its activation.

This approach provides a generally applicable system that may be used to image many different biologic processes with the same reporter probe and different pretargeting molecules (on account of the diversity of possible promoters that can form part of these pretargeting molecules). This is an attractive means to indirectly visualize transcriptional and post-transcriptional regulation of gene expression, protein-protein interactions, or trafficking of proteins or cells in living subjects. The downside, however, is the necessity to introduce 1 or more foreign proteins/genes into a cell, and the delivery of the reporter gene may be a limitation of this strategy in living subjects. Conceptually, the latter is less of an issue in the context of gene therapy approaches in which a therapeutic gene needs to be delivered in any case or for molecular imaging of transgenic animals expressing the reporter gene already as part of their construct. When adopting this strategy, one must determine how accurately the reporter protein reproduces regulation and function of the corresponding endogenous pathway, proving that the reporter does not perturb the underlying biologic process being examined.

Accordingly, an imaging reporter gene driven by a promoter of choice must first be introduced into the cells of interest. This is a common feature for all delivery vectors in a reporter gene imaging paradigm (ie, a complementary DNA expression cassette [an imaging cassette] containing the reporter gene of interest must be used). The promoter can be constitutive (ie, switched on all the time) or inducible; it can also be cell-specific (endogenous) or foreign to the cell (eg, promoters of viral origin). If the imaging reporter gene is transcribed, an enzyme or receptor product is made, capable of trapping or interacting with an imaging reporter probe, which may be a substrate for an enzyme or a ligand for a receptor. The trapping/interaction with the probe leads to an imaging signal intensity, whether it is from a radioisotope, a photochemical reaction, or an MR metal cation, depending on the exact nature of the probe itself (see below). Unlike most conventional reporter gene laboratory methods (eg, chloramphenicol acetyl transferase, LacZ/β-galactosidase, alkaline phosphatase, Bla/β-lactamase, etc[17]), reporter gene molecular imaging techniques offer the possibility of monitoring the locations, magnitude, and persistence of reporter gene expression in intact living animals or humans.

Characteristics of an ideal imaging reporter gene/probe are reviewed elsewhere.[13] No single imaging reporter gene/probe system currently meets all these criteria. Therefore, the development of multiple systems provides a choice based on the application of interest. The availability of multiple reporter gene/reporter probes also allows monitoring the expression of more than 1 reporter gene in the same living animal (multiplex imaging).

Categories of Reporter Gene Imaging Systems

A broad classification of imaging reporter systems consists of those in which the gene product is intracellular or is associated with the cell membrane (reviewed recently by Gross and Piwnica-Worms[18]). Examples of the former include thymidine kinase, green fluorescent protein (GFP), the luciferases, xanthine phosphoribotransferase, cytosine deaminase, and tyrosinase. Examples of reporter proteins on or in the cell surface include the receptors for somatostatin or transferrin and the sodium iodide symporter. The dopamine-2 receptor may be located in the cytoplasm and on the cell surface, and its probe (3-[2′-^{18}F fluoroethyl]-spiperone) probably binds to both forms. The major advantages of intracellular protein expression are the relatively uncomplicated expression strategy and the likely lack of recognition of the expression product by the immune system. The relative theoretic disadvantage is the presence of potentially unfavorable kinetics, requiring the need for the substrate or ligand to penetrate a cell. The major advantages of surface-expressed receptors and acceptors are favorable kinetics (sometimes avoiding the need for the tracer to penetrate into a cell) and the fact that synthetic reporters can be engineered to recognize already-approved imaging drugs. More detailed descriptions of some positron-emission tomography (PET)–based (eg, the herpes simplex virus type 1 thymidine kinase (HSV1-*tk*) enzyme with probes based on radiolabeled uracil nucleosides or acycloguanosine derivatives), single-photon emission CT (SPECT)–based, MR imaging–based, and fluorescence-based (eg, GFP) reporter systems are available elsewhere.[1,19] The main features of the common molecular imaging techniques used in reporter gene imaging are summarized in the Table.

Principles of Optical Bioluminescence Reporter Gene Neuroimaging

One addition to the list of reporter gene expression imaging techniques described previously is bioluminescence imaging, a noninvasive optical imaging technique that allows sensitive and semi-quantitative detection of bioluminescence reporter genes in intact living small research animals.[1,20] Bioluminescence refers to the enzymatic generation of visible light by living organisms. In this review, particular emphasis is placed on the topic of bioluminescence imaging because it is perhaps the molecular imaging technique least familiar to clinical neuroradiologists, and yet, there has been a recent veritable explosion in the assortment and extent of its experimental use in molecular neuroimaging research laboratories. The main advantage of optical bioluminescence imaging is that it can be used to detect very low levels of signal intensity because the light emitted is virtually background-free. Although not precisely characterized to date, the sensitivity of bioluminescence imaging is thought to be in the 10^{-15} to 10^{-17} mol/L range at limited depths of no more than 1–2 cm, the highest for any available molecular imaging technique (sensitivity of PET is $\sim 10^{-11}$ to 10^{-12} mol/L and that of MR imaging is $\sim 10^{-3}$ to 10^{-5} mol/L).[1] It is quick and easy to perform and allows relatively inexpensive and rapid testing of biologic hypotheses and proofs of principle in living experimental models. It is also uniquely suited for high-throughput imaging because of its ease of operation, short acquisition times (typically 10–60 seconds), and the possibility of simultaneous measurement of several anesthetized living mice. Bioluminescence imaging is well suited for use with small animal models, is relatively easily accessible to researchers in their laboratory setting, and offers particular flexibility in experimental investigations of molecular mechanisms of disease. Despite many technologic advances in this field, the clinical uses of this imaging technique are likely to be limited owing to inherent limitations of photon scatter and absorption in living tissues. Nevertheless, particularly relevant to clinicians is the fact that many of the current applications of bioluminescence imaging would likely provide the theoretic groundwork and experimental proof of principle necessary for potential extrapolation and translation into future clinical practice by using other more clinically applicable techniques (eg, PET) for molecular neuroimaging of patients.

The light emission in bioluminescence follows a chemiluminescent reaction that can take place under physiologic conditions within living cells when adenosine triphosphate (ATP) is required, or it can be extracellular (eg, *Renilla* luciferase, see below) when the reaction is independent of ATP. The most commonly used bioluminescence reporter gene for research purposes has been the luciferase from the North American firefly (*Photinus Pyralis*, *Fluc*). Luciferase genes have also been cloned from a variety of other organisms, including corals (*Tenilla*), jellyfish (*Aequorea*), sea pansy (*Renilla, Rluc*), several bacterial species (*Vibrio fischeri, Vibrio harveyi*), and dinoflagellates (*Gonyaulax*).[21] Several of these genes, including *Fluc*, have been modified for optimal expression in mammalian cells, and these have been used for many years in bioassays for ATP quantification and to study gene expression in transfected cells in culture. Firefly luciferase (*Fluc*, 61 kDa) catalyzes the transformation of its substrate D-Luciferin into oxyluciferin in a process dependent on ATP, magnesium (Mg^{++}), and oxygen (O_2), leading to the emission of light that can be detected by using low-light sensing instruments including standard luminometers. These biochemical assays are typically conducted on cell lysates, though there are several reports of live cell assays that use *Fluc*, as reviewed in Edinger et al.[22]

Although the most common luciferin-luciferase system used in molecular imaging is that derived from the firefly *P Pyralis*, the sea pansy *Renilla* luciferase (*Rluc*), which uses a different substrate (coelenterazine [CL]) and is not ATP- or Mg^{++}-dependent, has also been validated for applications in living subjects.[23] *Rluc* enzyme (36 kDa) is capable of generating a flash of blue light (460–490 nm, peak emission at 482 nm) on reaction with its substrate. The synthetic humanized *Rluc* gene (*hRluc*) is a systematically redesigned *Rluc* gene, encoding the same 311-residue protein as wild-type *Rluc* but yielding only codon changes for higher expression in mammalian cells. Both colorimetric (eg, rhodamine red) and fluorescent (eg, GFP) reporter proteins require an external source of light for excitation and emit light at a different wavelength for detection, thus, making them more susceptible to background noise (autofluorescence). In contrast, the bioluminescence luciferase enzymes and substrate systems described previously have several characteristics that make them useful reporter proteins. For example, *Fluc* does not need external light excitation and self-emits light from green-to-yellow wavelengths (560–610 nm, peak emission at 562 nm) in the presence of D-Luciferin, ATP, Mg^{++}, and O_2 (Fig 1). Secondly, the fast rate of enzyme turnover ($T_{1/2}$ = 3 hours) in the presence of substrate D-Luciferin allows real-time measurements because the enzyme does not accumulate intracellularly to the extent of

other reporters. Third, the relationship between the enzyme concentration and peak height of emitted light in vitro is linear, up to 7–8 orders of magnitude. Therefore, these properties potentially allow sensitive noninvasive imaging of *Fluc* (and *Rluc*) reporter gene expression in living subjects.

Broadening the use of *Fluc* as a bioluminescence reporter from biochemical and cell culture assays to living subjects depended on the development of low-light imaging systems (see below) and 2 other crucial observations.[22] The first observation was the demonstration that D-Luciferin would seem to circulate within minutes throughout many body compartments (also readily crossing the blood-brain barrier) after intravenous or intraperitoneal administration and rapidly enters many cells.[24] Previous studies have quantified the uptake kinetics and biodistribution of the *Fluc* substrate D-Luciferin[25] and recent newer D-Luciferin derivatives for enhanced in vitro and in vivo bioluminescence assays.[26] The second discovery was that the level and spectrum of emitted light from *Fluc*-expressing mammalian cells is adequate to penetrate tissues of small research animals such as mice and rats and can be detected externally with low-light imaging cameras.[27]

Several factors governing the interaction of emitted light with tissues deserve particular consideration. The absorption coefficient of light depends on its wavelength (more light is absorbed as the wavelength decreases below 600 nm) and results from absorbers such as hemoglobin (the main absorber), lipids, and water. Because the emission spectrum of *Fluc* is very broad, the lower end of the spectrum is absorbed to a greater extent within tissues, resulting in relatively more red-shifted light emitted from the surface, particularly when the source of light is in a deep location. The blue spectrum emitted from *Rluc* is absorbed to an even greater extent than that of *Fluc*, but this is counteracted by the much greater initial quantum yield from *Rluc*. Bhaumik et al[28] have found the measurable signal intensity from C6 cells transfected with *hRluc* to be 30- to 40-fold higher than that from C6 cells transfected with *Fluc* when implanted subcutaneously in the same mouse, though this depends on the amount of substrate used. The difference in light emission in cell culture is even greater at 120-fold. For deeper tissues (eg, the lung), the measurable light when using higher doses of CL is also higher for cells transfected with *hRluc*, even after absorption by deeper tissues. Additional studies are still needed to directly compare *Fluc* and *hRluc* in living subjects.

The signal intensity of measurable light is further determined, to a large extent, by attenuation of light, owing to the effects of scattering. Scattering results from changes in the refractive index at cell membranes and organelles. The signal intensity from a depth of 1 cm is attenuated by a factor of $\sim 10^{-2}$ for wavelengths at ~650 nm.[29] This scatter results in the relatively poor spatial resolution of bioluminescence imaging when compared with other techniques that rely on more penetrating electromagnetic radiation to generate images (eg, PET, SPECT, CT). The spatial resolution of bioluminescence images is depth-dependent, being slightly worse or equal to the depth of the object—that is, an object 3–5 mm deep has an ~3- to 5-mm spatial resolution.

Bioluminescence imaging of living subjects requires that the gene encoding the bioluminescence reporter protein be transferred to cells or tissues of interest. This transfer can be accomplished by using 1 of 3 gene-transfer methods: ex vivo, in vivo, or as part of a transgenic construct. When cells transfected ex vivo and transiently or stably expressing the bioluminescence reporter gene are injected into the research animal, the light emitted from the gene-marked cells can be monitored externally. To generate such an image, one anesthetizes and places the animals in a light-tight chamber equipped with the charge-coupled device (CCD) camera (Fig 2). A gray-scale reference image (digital photograph) is acquired under weak illumination; and then in complete darkness, the photons emitted from within the body of the animal are detected externally by using a range of integration times from 1 second to several minutes. The data are transferred to a computer equipped with image acquisition, overlay, and analysis software for quantification. A bioluminescence image is most often shown as a color image representing light intensity (usually from blue for least intense to red for most intense) that is superimposed on the gray-scale photographic image to display the anatomic origin of the photon emission. Usually a region of interest is manually selected over an area of signal intensity, and the maximum or average intensity is recorded as photons per second per square centimeter per steradian (steradian is a unit of solid angle) (Fig 1*B*). Whenever the exposure conditions (including time, f/stop, height of sample shelf, binning ratio, and time after injection with optical substrate) are kept identical, the measurements are highly reproducible.

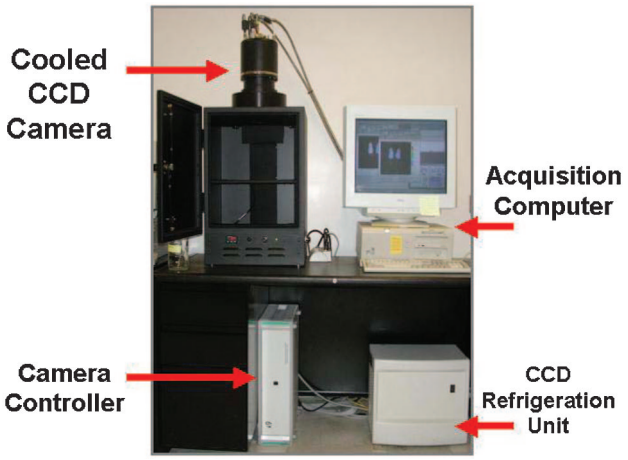

Fig 2. The Xenogen In Vivo Imaging System (Xenogen Corporation, Hopkinton, Mass) consists of a cooled CCD camera mounted on a light-tight imaging chamber, a cryogenic refrigeration unit, a camera controller, and a computer system for data analysis

In this review, the principles and recent technologic advances in molecular imaging of reporter gene expression are discussed. This approach is emerging as a valuable tool for monitoring the expression of genes in animals and humans, especially in the former when using bioluminescence imaging. Further development of more sensitive and selective reporters, combined with improvements in detection technology, will consolidate the position of reporter gene imaging technology as a versatile method for understanding of intracellular biologic processes and the molecular basis of neurologic diseases. In the second article of this series, we review the various strategies and applications that make use of reporter genes for molecular imaging of the brain in living subjects.[30]

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