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In Vivo Reporter Gene Imaging: Recent Progress of PET and Optical Imaging Approaches

Jung-Joon Min[1,2]

[1]**Department Nuclear Medicine, Chonnam National University Medical School, Gwangju, Korea**
[2]**Laboratory of In Vivo Molecular Imaging, Institute for Molecular Photonic Imaging Research, Chonnam National University Hwasun Hospital, Hwasun, Jeonnam, Korea**

Abstract

Recent progress in the development of non-invasive imaging technologies continues to strengthen the role of molecular imaging biological research. These tools have been validated recently in variety of research models, and have been shown to provide continuous quantitative monitoring of the location(s), magnitude, and time-variation of gene delivery and/or expression. This article reviews the use of radionuclide, magnetic resonance, and optical imaging technologies as they have been used in imaging gene delivery and gene expression for molecular imaging applications. The studies published to date demonstrate that noninvasive imaging tools will help to accelerate pre-clinical model validation as well as allow for clinical monitoring of human diseases.

Keywords: Molecular imaging, Positron Emission Tomography, Magnetic Resonance Imaging, Optical Imaging, Reporter gene, Gene expression

Introduction

Recent advances in molecular/cell biology, the ability to decode entire genomes, the continuous search for new targets, the unraveling of the molecular pathways of many disease and high sensitivity multimodality imaging instrumentation have allowed interactions between these fields that extends morphological observations in living subjects to a more meaningful dimension within the context of "Molecular Medicine." The term 'molecular imaging' implies the convergence of multiple imaging techniques, molecular/cell biology, chemistry, medicine, pharmacology, medical physics, biomathematics, and bioinformatics into a new imaging paradigm.

Present imaging technologies rely mostly on non-specific morphological, physiological, or metabolic changes that differentiate pathological from normal tissue rather than identifying specific molecular events (e.g. gene expression) responsible for disease. Molecular imaging usually exploits specific molecular probes as the source of image contrast. This change in emphasis from a non-specific to a specific approach represents a significant paradigm shift, the impact of which is that imaging can now provide the potential for understanding of integrative biology, earlier detection and characterization of disease, and evaluation of treatment. Massoud et al. (Massoud et al., 2003) suggested several important goals in molecular imaging research, namely: (1) To develop non-invasive in vivo imaging methods that reflect specific molecular processes such as gene expression, or more complex molecular interactions such as protein-protein interactions; (2) To monitor multiple molecular events near-simultaneously; (3) To follow trafficking and targeting of cells; (4) To optimize drug and gene therapy; (5) To image drug effects at a molecular and cellular level; (6) To assess disease progression at a molecular pathological level; and (7) To create the possibility of achieving all of the above goals of imaging in a rapid, reproducible, and quantitative manner, so as to be able to monitor time-dependent experimental, developmental, environmental, and therapeutic influences on gene products in the same animal or patient.

Molecular imaging has its roots in nuclear medicine. Nuclear medicine is focused on characterizing enzyme activity, receptor/transporter status and biodistribution of various radiolabeled substrates (tracers). The underlying principles of molecular imaging can now be tailored to other imaging modalities such as optical imaging and magnetic resonance imaging (MRI). The development, validation, and application of these novel imaging techniques in living subjects should further enhance our understanding of disease mechanisms and go hand in hand with the development of molecular medicine (Massoud et al., 2003; Min et al., 2004; Min, 2004).

Molecular Imaging Technologies

To image small animals, the size of the subject, the total

volume that must be evaluated, the spatial resolution (voxel size) necessary for meaningful anatomical/functional data, and the total time spent on acquiring a set of image are quite different for a 20-g mouse than for a 70-kg person. Instrumentation requirements reflects these differences and can be exploited to maximize the information that can be obtained from small animal moldels of disease states. Further reviews of issues centered on molecular imaging techniques can be found elsewhere (Min et al., 2004; Cherry et al., 2001; Weissleder, 2001; Weissleder, 2002; Chatziioannou, 2002).

Radionuclide Imaging

In vivo techniques like SPECT and PET involve the use of isotopes that emit high energy particles like gamma rays and positrons (which result in the generation of gamma rays), respectively, to label probes (Cherry et al., 2001; Gambhir et al., 2000). These high energy particles are necessary to readily penetrate tissue and be detected outside of the body. Gamma emitting isotopes include Technetium-99m (^{99m}Tc), Indium-111 (^{111}In) and Iodine-131 (^{131}I), while the common positron emitting isotopes include Fluorine-18 (^{18}F), Carbon-11 (^{11}C), Nitrogen-13 (^{13}N), Iodine-124 (^{124}I), and Copper-64 (^{64}Cu). To clarify, in PET, positrons are not directly detected. Positrons (particles with the same mass as an electron, but having the opposite charge) emitted from the atom travel up to a few millimeters from the source. Eventually they undergo an annihilation reaction with an electron in the tissue resulting in the simultaneous emission of two gamma rays of 511 keV (the mass-energy of an electrion/positron) at approximately 180° apart. These two gamma rays are what is detected and used to determine the tomographic information collected in PET. SPECT also supplies tomographic information, by rotating position-sensitive "gamma camera" detectors around the specimen and only collecting gamma particles that hit the detectors through a collimator (an aperture that allows only particles traveling directly through the hole to be detected). Both of these modalities use only femto-grams of radiolabeled tracer, offering high levels of sensitivity, and can be used with autoradiographic methods to correlate in vivo data. In general, PET has better spatial resolution and sensitivity than SPECT, but both are used in the clinical setting. PET and SPECT have also been adapted from the clinic for use with small animal research. These micro-PET and -SPECT scanners have increased spatial resolution (1-2 mm in each axial direction) to allow the imaging of small animals (Cherry et al., 2001).

Optical Imaging

Visible light can be generated in living cells using two methods: fluorescence (Yang et al., 2000) and bioluminescence (Contag et al., 1998). For fluorescence methods, cells are labeled with dyes (for trafficking) or proteins that emit light of a limited spectrum when excited by different wavelengths of light (Zhang et al., 2002). Bioluminescence methods use an enzymatic reaction between a luciferase enzyme and its substrate, a luciferin, to produce visible light. The most common bioluminescence reporter proteins for use in living small animals are firefly luciferase (Fluc) and renilla luciferase (Rluc). For in vitro and ex vivo studies when considering smaller cell populations, fluorescence imaging is employed. Fluorescent proteins, like green fluorescent protein (GFP) and red fluorescent protein (RFP), are used often with microscopy or flow cytometry to observe the localization of proteins within a cell, to label specific proteins, or to monitor the production of a gene product. Both fluorescence imaging and bioluminescence imaging can be used to detect cells within the living animal because mammalian tissues allow for light to be transmitted through relatively thin layers tissue (Rice et al., 2001). Fluorescence imaging in vivo usually results in fairly intense light signal, due to the high photon output of the fluorophor. In contrast in bioluminescence imaging the light generated is of a much lower intensity and is detected and quantified using highly sensitive cooled charge coupled device (CCD) cameras (Rice et al., 2001; Spibey et al., 2001).

Charged coupled device (CCD) detectors are made of silicon crystals sliced into thin sheets for fabrication into integrated circuits using similar technologies to those used in making computer silicon chips. One of the properties of silicon-based detectors is their high sensitivity to light, allowing them to detect light in the visible to near-infrared range. CCD cameras operate by converting light photons at wavelengths between 400 and 1000 nm that strike a CCD pixel with an energy of just 2-3 eV into electrons. A CCD contains semiconductors that are connected so that the output of one serves as the input of the next. In this way, an electrical charge pattern, corresponding to the intensity of incoming photons, is read out of the CCD into an output register and amplifier at the edge of the CCD for digitization. Thermal noise is dramatically reduced if the chip is cooled; dark current falls by a factor of 10 for every 20°C decrease in temperature(Spibey et al., 2001). For bioluminescence imaging, CCD cameras are usually mounted in a light-tight specimen chamber, and are attached to a cryogenic refrigeration unit (for camera cooling to minus 120-150°C).

A key advantage of bioluminescence imaging is that no light is produced or is detectable until the substrate/enzyme interaction of a luciferase and luciferin occurs. Consequently, there is very low background luminescence levels in most animals. In contrast, the wavelengths of light used for excitation in fluorescence imaging can also excite other fluo-

rescent molecules within tissues, and often results in high levels of background auto-fluorescence which can sometimes hinder detection of the reporter protein or dye (Billinton et al., 2001). Using either optical imaging modality, there are significant drawbacks to imaging visible light in living animals: i) there is a limited range of wavelengths (approx. 600-900nm) that can be transmitted through tissue due to light absorption by hemoglobin and water, ii) light scatter in the tissue further affects the ability to image by preventing accurate tomographic or anatomic analysis, and also requires that to image a signal from deeper within the animal, more cells must be present to produce an optical signal detectable at the surface (Rice et al., 2001). Newer strategies are being developed in order to achieve tomography with fluorescence-based

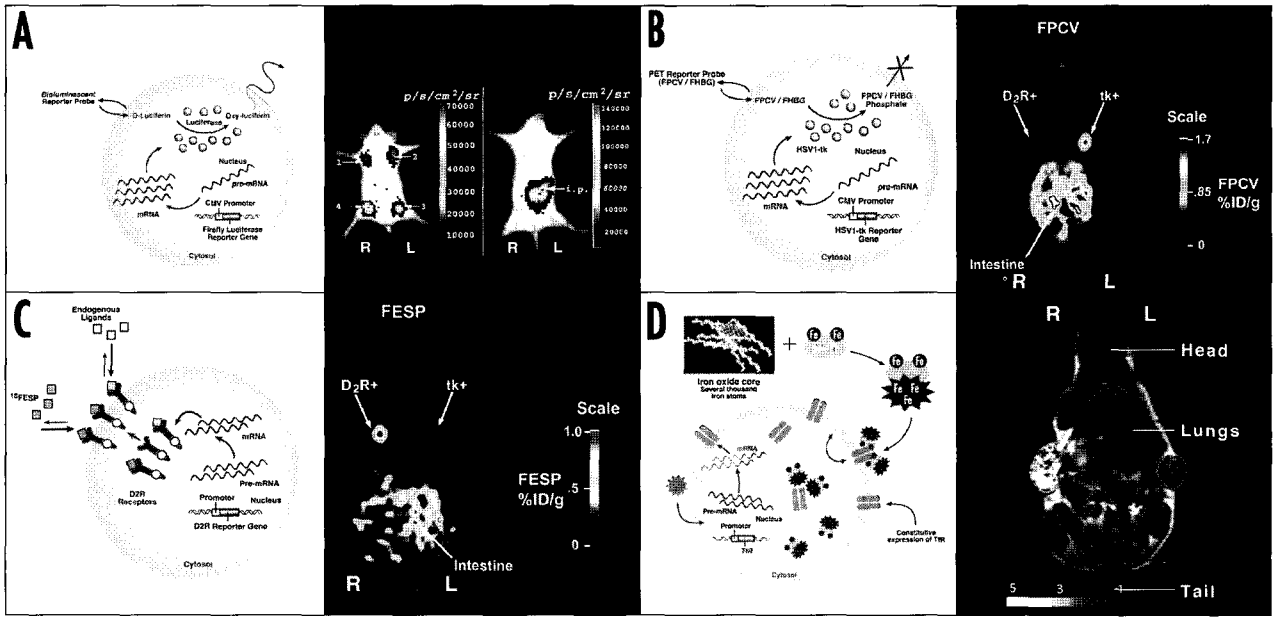

Figure 1. Four different types of imaging reporter gene/probe strategies. **A** : Enzyme-based bioluminescence imaging: D-Luciferin is a substrate molecular probe that is acted upon by the enzyme firefly luciferase (Fluc) to result in bioluminescence via a chemiluminescent reaction under physiological conditions only within living cells expressing the Fluc gene. Images show examples of mice implanted with subcutaneous xenograft tumors expressing Fluc (left mouse) or intraperitoneal tumor cells expressing Fluc (right mouse) after administration of D-Luciferin (either IV or IP). **B:** Enzyme-based PET imaging: ^{18}F-fluoropenciclovir (^{18}F-FPCV) or other acycloguanosines are substrate molecular probes phosphorylated by the Herpes Simplex Virus Type 1 thymidine kinase (HSV1-TK) enzyme to result in intracellular trapping of the probe in cells expressing the HSV1-tk gene. Images of same mouse are shown in (B) and (C). Images show two reporter genes (HSV1-tk and D2R) in the same mouse and imaged with two different reporter probes (^{18}F-FPCV for HSV1-TK and ^{18}F-FESP for D2R). These images show specific accumulation of probes in a mouse carrying a tumor stably expressing HSV1-tk on the left shoulder and a separate tumor stably expressing D2R on the right shoulder. The accumulation of ^{18}F-FPCV and ^{18}F-FESP in each tumor reflects trapping due to HSV1-tk and D2R expression, respectively. **C:** Receptor-based PET imaging: ^{18}F-FESP is a ligand molecular probe interacting with the dopamine-2-receptor (D2R) to result in trapping of the probe on/in cells expressing the D2R gene. Images of same mouse are shown in (B) and (C). This image was taken a day after that shown in (B) to allow for decay of ^{18}F. Color scale of %ID/g indicates percentage of injected dose that accumulates per gram of tumor. Nonspecific activity in the intestines is due to hepato-biliary excretion of molecular probe. **D:** Receptor-based MRI imaging: Over-expression of engineered transferrin receptors (TfR) results in approximately 500% more cell uptake of the transferrin-monocrystalline iron oxide nanparticle (Tf-MION) probes per hour than would occur otherwise. During each internalisation event, several thousand iron atoms (rather than just 2 atoms in holo-transferrin [holo-Tf]) accumulate in the cytosol and within endosomes. Also, the cellular internalisation of iron does not downregulate the level of receptor over-expression. These changes result in a detectable contrast change on MRI. Image shows coronal MRI in a living mouse with a right TfR(+) flank tumor and a left TfR(-) flank tumor. The image is a co-registered color-mapped one of a T1-weighted spin echo image obtained for anatomical detail with superimposed gradient-echo image after Tf-MION administration, showing the increased uptake of iron in the right TfR(+) tumor. Color scale is an arbitrary one with relative units, where white/yellow indicates highest uptake and blue the lowest. Reproduced with permission from Massoud et al. (2003)

approaches (Ntziachristos et al., 2002). There is potential for human applications with red and near-infra red light, which better penetrate tissues (Sevick-Muraca et al., 2002). For instance, breast imaging, as well as intra-operative devices, may eventually be possible with optical technologies (Bremer et al., 2002). If bioluminescence is to be utilized in humans it will require further testing of the safety of the substrates needed at high mass levels before being introduced into human subjects.

Imaging Reporter Gene

Imaging of gene expression in living subjects can be directed either at genes externally transferred into cells of organ systems (transgenes) or at endogenous genes. Most current applications of reporter gene imaging are of the former variety. By adopting state-of-the-art molecular biology techniques, it is now possible to better image cellular/molecular events. One can also engineer cells that will accumulate imaging probes of choice, either to act as generic gene 'markers' for localizing and tracking these cells, or to target a specific biological process or pathway. In the last few years there has been a veritable explosion in the field of reporter gene imaging, with the aim of determining location, duration, and extent of gene expression within living subjects (Jacobs et al., 2002; Gambhir et al., 1999).

Reporter genes are used to study (1) promoter/enhancer elements involved in gene expression, (2) induction of gene expression using inducible promoters, and (3) endogenous gene expression through the use of transgenes containing endogenous promoters fused to the reporter (Wu et al., 2003). In all these cases, transcription of the reporter gene can be tracked and therefore gene expression can be studied. Unlike most conventional reporter gene methods (e.g. chloramphenicol acetyl transferase, LacZ/β-galactosidase, alkaline phosphatase, Bla/β-lactamase, etc. Spergel et al., 2002) molecular imaging techniques offer the possibility of monitoring the location, magnitude, and persistence of reporter gene expression in intact living animals or humans. The 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, i.e. a complementary DNA expression cassette (an imaging cassette) containing the reporter gene of interest must be used. The promoter can be constituitive or inducible; it can also be cell-specific. If the reporter gene is transcribed, an enzyme or receptor product is made, thus trapping the imaging reporter probe, which may be a substrate for an enzyme or a ligand for a receptor. The trapping of the probe leads to an imaging signal, be it from a radioisotope, a photochemical reaction, or a magnetic resonance metal cation, depending on the exact nature of the probe itself.

Massoud et al. (2003) and Ray et al. (2001) have previously reviewed the many examples of these imaging reporter systems.

Classification of Imaging Reporter Gene Systems

A broad classification of reporter systems consists of those where the gene product is intracellular (Figures 1a and 1b), or is associated with the cell membrane (Figures 1c and 1d). Examples of intracellular reporters include thymidine kinase, GFP, luciferase [Contag et al., 2997; Benaron et al., 1997), cytosine deaminase, and tyrosinase (Weissleder et al., 1997) to name a few. Examples of reporters on or in the cell surface in the form of receptors include the dopamine 2 receptor (D2R), and receptors for somatostatin, transferring (Hogemann et al., 2000; Moore et al., 1998), or the sodium iodide symporter (Boland et al., 2000; Haberkorn et al., 2001; Chung et al., 2002; Min et al., 2002). The major advantages of intracellular protein expression are the relatively uncomplicated expression strategy and lack of recognition of the expression product by the immune system. 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 (Weissleder et al., 2001).

A representative isotope-based intracellular reporter system is the reporter probe for the Herpes Simplex Virus Type 1 thymidine kinase (HSV1-TK) enzyme. Substrates that have been studied to date as PET reporter probes for HSV1-TK can be classified into two main categories - pyrimidine nucleoside derivatives (e.g. FIAU) (Tjuvajev et al., 1995), and acycloguanosine derivatives (e.g. ^{18}F-FPCV, ^{18}F-FHBG), and have been investigated in terms of sensitivity and specificity (Min et al., 2003). These radiolabeled reporter probes are transported into cells, and are trapped as a result of phosphorylation by HSV1-TK. When used in non-pharmacological tracer doses, these substrates can serve as PET or SPECT targeted reporter probes by their accumulation in just the cells expressing the HSV1-tk gene. Recently, we have attempted to improve the sensitivity by using a mutant version of this gene, HSV1-sr39tk which was derived using site-directed mutagenesis to obtain an enzyme more effective at phosphorylating ganciclovir (GCV) (and also less efficient at phosphorylating thymidine) with consequent gain in imaging signal(Gambhir et al., 2000). More recently, Iyer et al. (2001) and Min et al. (2003) reported the ^{18}F-penciclovir (PCV) to be an improved probe over ^{18}F-GCV and HSV1-sr39tk reporter gene system with the ^{18}F-PCV or ^{18}F-FHBG is a better combination over the HSV1-tk reporter gene system with FIAU in C6 cell

mouse xenografts.

The *D2R* reporter gene has also been validated for PET reporter on or in the cell surface in the form of receptors while using ^{18}F-fluoroethylspiperone (FESP) as the reporter probe ligand (MacLaren et al., 1999). More recently, a mutant D2R that uncouples signal transduction while maintaining affinity for FESP has also been reported (Liang et al., 2001).

Bioluminescent photoproteins such as luciferase have been used as reporter proteins for optical imaging in living animals (Contag et al., 2002). Firefly luciferase (*Fluc*) catalyzes D-Luciferin to produce oxyluciferin in the presence of oxygen, cofactors, Mg^{+2}, and ATP to produce light with peak at 562 nm. Recently validated renilla luciferase (*Rluc*) catalyzes the oxidation of coelenterazine in presence of oxygen to generate a flash of blue luminescence with a peak wavelength at 482 nm. Bacterial luciferase gene (lux) operon encodes five gene clusters, lux C, D, A, B, E. Lux A, B control luciferase enzyme expression, while lux C, D, E control fatty aldehyde enzyme complex production which synthesize substrates. With expression of lux operon in prokaryotic cells, substrate injection is not necessary because lux expressing cells produce bacterial luciferase as well as its substrates.

Four categories of applications for molecular imaging will be reviewed: imaging of cell trafficking, imaging of immunotherapies, imaging of gene therapies, and imaging of molecular interactions such as protein-protein interactions.

Applications of Reporter Gene Imaging: Imaging Cell Trafficking

An important application of these imaging modalities and reporter constructs is to noninvasively monitor cell trafficking. Imaging can be used to look at different properties of cellular trafficking including metastasis, stem cell transplantation, and lymphocyte response to inflammation. Cells have been labeled ex vivo or in vivo with various reporters including those compatible with MR, PET, and optical imaging. Optical imaging has been used to follow metastasis in a prostate cancer and in a lymphoma animal model (Edinger et al., 2003). CD4+ T cells marked with *Fluc* have been used in an adoptive immunotherapy protocol whereby lymphocytes deliver a cytokine antagonist to the brains of mice with experimental autoimmune encephalomyelitis (costa et al., 2001; Hardy et al., 2001). CD4+ T cells marked with *Fluc* were also used as vehicles to deliver an immunoregulatory protein for treatment of collagen-induced arthritis in a mouse model of rheumatoid arthritis (Nakajima et al., 2001). Recently, cancer cells marked with *Fluc* and injected into the left ventricle have helped in the study of micrometastatic spread to bone marrow (Wetterwald et al., 2002). PET radiotracers and reporter genes have been used to study cell migration and anti-tumor responses. ^{18}F-FDG, a common PET tracer used to look at cellular metabolism of glucose as a good indicator of neoplasia, is highly retained in lymphocytes. This aspect of FDG has been exploited to follow monocyte trafficking (Paik et al., 2002). ^{64}Cu-pyruvaldehyde-bis(N^4-methylthiosemicarbazone) (Cu-PTSM), has also been assessed in a glioma cell line and in splenocytes (Adonai et al., 2002). The cells were labeled *ex vivo* with tracer and subsequently injected and monitored in the living animal with microPET. Le et al. (2002) assessed the role of the BCR-ABL oncogene in lymphoid leukemogenesis

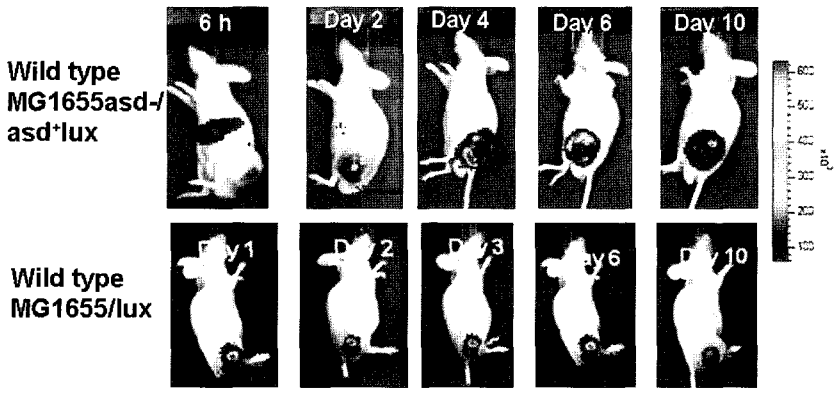

Figure 2. Imaging cancer with genetically enginnered light-emitting E. coli. pUC19 plasmid encloning Lux was transformed into wild type (MG1655). For stably expressing lux, lux was cloned with asd (aspartate β-semialdehyde dehydrogenase) gene and transformed into asd defective E. coli (MG1655asd-/asd$^+$lux). The imaging signal from MG1655lux was detected initially at liver (20min), thereafter at tumors for at least 1 week in both nude and Balb/c mice. MG1655asd-/asd$^+$lux produced stronger and longer (for 2 weeks) signal from tumor than does MG1655lux.

using bone marrow cells gene marked with *HSV1-tk*. Utilizing HSV1-sr39tk as a reporter gene in adoptively transferred lymphocytes, Dubey *et al.* (2003) were able to show that T cell anti-tumor responses could be quantified using microPET. Similarly, Koehne *et al.* (Koehne et al., 2003) showed that Epstein-Barr virus (EBV)- specific T lymphocytes, marked with HSV1-tk, can be shown to traffic and accumulate in EBV+ tumors in mice using microPET. These approaches can be used to assess the effects of immunomodulatory agents intended to potentiate the immune response to cancer, and can also be useful for the study of other cell-mediated immune responses, including autoimmunity. Our group express bacterial luciferase reporter gene in E. coli or Salmonella typhimurium to investigate its migration inside the body. We currently explore that bacteria have a capacity to specifically target tumor tissue using in vivo optical imaging system (fig. 2) (Min et al., 2005). Monitoring cell trafficking is feasible in many disease conditions, although careful consideration must be taken in choosing the labeling agent (possible toxicity) and/or the reporter gene (possible immunogenicity). These types of studies would allow for better understanding of the disease process, and the response of neoplastic and immune cells to therapeutics.

Applications of Reporter Gene Imaging: Imaging Gene Therapies

In molecular biology, reporter genes like green fluorescent protein (GFP) and firefly luciferase (*Fluc*) (note, in general a lowercase abbreviation refers to the gene while an uppercase abbreviation refers to the protein) have been used to monitor the transfer and regulation of genes in cells (Welsh et al., 1997). Previously, to assay the expression of a gene, invasive techniques were used, but reporter genes have been validated that can be used in radionuclide and optical imaging modalities, to study gene expression *in vivo*. The reporter gene can itself be the therapeutic gene or can be coupled to the therapeutic gene (Gambhir et al., 2000). In the former approach, the reporter gene and therapeutic gene are one in the same. For example, anti-cancer gene therapy using HSV1-tk and GCV can be coupled with imaging of the accumulation of radiolabeled probes (^{18}F-FHBG or ^{124}I-FIAU). Jacobs et al. (2001) used ^{124}I-FIAU PET imaging of humans in a prospective gene-therapy trial of intratumorally infused liposome-gene complex (LIPO-HSV1-tk) followed by GCV administration in 5 recurrent glioblastoma patients. These preliminary findings showed that [^{124}I]FIAU PET is feasible and that vector-mediated gene expression may predict a therapeutic effect. Recently, sodium/iodide symporter (NIS) which facilitate the uptake of iodide by thyroid follicular cells is also being applied in radioiodide gene therapy (Chung et al., 2002). The conventional radioiodide or ^{99m}Tc-pertechnetate scintigraphy has been used to directly monitor NIS expression. Since the iodine is not trapped, issue of efflux have to be optimized but initial studies show significant promise.

A second approach involves *indirect* imaging of therapeutic transgene expression using expression of a reporter gene which is coupled to a therapeutic transgene of choice. This strategy requires proportional and constant co-expression of both the reporter gene and the therapeutic gene over a wide range of transgene expression levels. An advantage of this approach is that it provides for a much wider application of therapeutic transgene imaging, because various imaging reporter genes can be coupled to various therapeutic transgenes while utilizing the same imaging probe each time. Linking the expression of a therapeutic gene to a reporter gene has been validated using PET and optical imaging through a variety of different molecular constructs. Examples include fusion approaches (Ray et al., 2003; Richard et al., 2002; Ray et al., 2004), bicistronic approaches using internal ribosomal entry site (IRES) (Liang et al., 2002; Yu et al., 2000), dual-promoter approaches (Zinn et al., 2002; Hemminki et al., 2002), a bidirectional transcriptional approach (Sun et al., 2001), and a two vector administration approach (Min et al., 2004). A fusion gene approach can be used in which two or more different genes are joined in such a way that their coding sequences are in the same reading frame, and thus a single protein with properties of both the original proteins is produced. An advantage of the fusion gene approach is that the expression of the linked genes is absolutely coupled (unless the spacer between the two proteins is cleaved). However, the fusion protein does not always yield functional activity for both of the individual proteins and/or may not localize in an appropriate sub-cellular compartment. Another approach is to insert an IRES sequence between the two genes so that they are transcribed into a single mRNA from the same promoter but translated into two separate proteins. Although the IRES sequence leads to proper translation of the downstream cistron from a bicistronic vector, translation from the IRES can be cell type specific and the magnitude of expression of the gene placed distal to the IRES is often attenuated (Yu et al., 2000). Two different genes expressed from distinct promoters within a single vector (dual-promoter approach) may avoid some of the attenuation and tissue variation problems of an IRES-based approach (Zinn et al., 2002). Bi-directional transcriptional approach utilizes a vector in which the therapeutic and the reporter genes are driven by each minimal CMV promoter induced by tetracycline-responsive element (TRE), transcribing separated mRNA from each gene which would then be translated into separate protein products (Sun et al., 2001). This

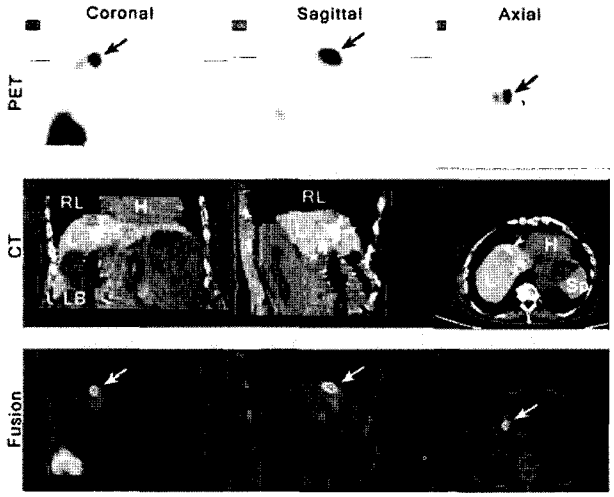

Figure 3. PET imaging of adenoviral-mediated transgene expression in liver cancer patients. PET-CT imaging of HSV1-tk transgene expression in humans. Columns 1 to 3 show the 5-mm-thick coronal, sagittal, and transaxial slices, respectively, from a [^{18}F]FHBG-PET-CT study in patient 5. All sections are centered on the treated tumor lesion (dotted lines in the CT images) and show [^{18}F]FHBG accumulation at the tumor site (arrows). Anatomic - metabolic correlation can be obtained by fused PET-CT imaging. The white spots on the liver seen on the CT images correspond to lipiodol (arrowheads) retention after transarterial embolization of the tumor and a transjugular intrahepatic portosystemic shunt (★). Tracer signal can be seen in the treated lesion (arrows), whereas no specific accumulation of the tracer can be seen in the necrotic, lipiodol-retaining regions around it. H, heart; L, liver; LB, large bowel; RL, right lung; Sp, spleen.

system also avoids the attenuation and tissue variation problems of the IRES based approach and may prove to be one of the most robust approaches developed to date. Another way to image both the therapeutic and reporter genes can be through administration of two separate vectors, by cloning of the therapeutic and reporter genes in two different vectors but driven by same promoter. Further issues centered on imaging gene therapy have been reviewed by Min et al. (2004)

Applications of Reporter Gene Imaging: Imaging of Molecular Interactions

To image protein-protein interaction in living mice, Ray et al. (2002) have used the well studied yeast two-hybrid system adapted for mammalian cells and modified it to be inducible. They used the *NF-κB* promoter to drive expression of two fusion proteins (VP16-MyoD and GAL4-ID), and modulated the *NF-κB* promoter through TNFα. *Fluc* reporter gene expression was driven by the interaction of MyoD and ID through a transcriptional activation strategy. They demonstrated the ability to detect this induced protein-protein interaction in cell culture and image it in living mice by using transiently transfected cells. More recently, Paulmurugan et al. (2002) have also validated the use of split reporter technology to show that both complementation and intein-mediated reconstitution of Firefly luciferase can be used to also image protein-protein interactions in living mice. This approach has the advantage of potentially imaging interactions anywhere in the cell, whereas the yeast two-hybrid approaches are limited to interactions in the nucleus. Imaging interacting protein partners in living subjects could pave the way to functional proteomics in whole animals and provide a tool for evaluation of new pharmaceuticals targeted to modulate protein-protein interactions.

An important likely future possibility is the ability to image multiple molecular events in one population of cells. This may be attainable by combining two or more of the above described strategies for gene marking and imaging the trafficking of cells with those entailing linked expression of an imaging gene to an endogenous promoter, or to an exogenous therapeutic gene. As such, in these experiments it is foreseeable that one reporter may reveal the spatial distribution of cells and whether they have reached a specific target, and another reporter may indicate whether a certain gene becomes upregulated at this site or if a more complex interaction occurs. Efforts are underway to demonstrate the feasibility of this concept of simultaneous multiplexing of molecular imaging strategies, with a view to a better understanding the complexities of molecular pathways and networks. Further issues centered on imaging gene therapy have been reviewed by Massoud et al. (2003) and Haberkorn et al. (2003)

Applications of Reporter Gene Imaging: Imaging of Stem Cell Implantation

Therapeutic effect of stem cell may be related to the secretion of multiple arteriogenic cytokines by stem cells, which would contribute to the formation of a mechanical scaffold or to the recruitment of other beneficial cells to the legion. However, most techniques used for the analysis of stem cell survival in animal models has relied on postmortem histology to determine the fate and migratory behavior of the stem cells. This approach, however, precludes any sort of longitudinal monitoring. An approach which would allow for the monitoring of stem cell activities within the context of the intact whole-body system, rather than with histological slides, would allow us to gain further insights into the underlying biological and physiological properties of stem cells.

In recent years, several investigators have attempted to address this issue, using optical reporter gene labeling. This approach was initially intended to allow for the serial tracking and quantification of transplanted stem cells, in a non-invasive and highly sensitive manner.

Several imaging strategies are currently under active investigation, including radionuclide labeling, ferromagnetic labeling, and reporter gene labeling (Wu et al., 2004). In a study of radionuclide labeling, Aicher *et al.* (2003) injected indium-111 oxine-labeled endothelial progenitor cells into the infarcted myocardia of nude rats, and imaged them at 24 to 96 hours, using a gamma camera. The main limitation associated with this approach is that radionuclides have physical half-lives, making it possible to monitor cell distribution only for a limited number of days.

In a study of ferromagnetic labeling, Kraitchman *et al* (2003) injected mesenchymal swine stem cells, which contained ferrumoxide particles, into the hearts of pigs. After 24 hours, these injected sites became ovoid hypoenhancing lesions, with sharp borders. One to 3 weeks after the cells had been injected, the borders became less clearly delineated, due to the degradation of the ferrumoxide particles. As the ferrumoxide particles continue to register magnetic resonance signals, even when the injected cells have undergone apoptosis or cell death, it becomes more difficult to correlate the magnetic resonance signal with the actual number of viable cells.

During the process of reporter gene labeling, the cells are transfected with reporter genes before being implanted into the myocardium (Wu et al., 2003). In cases in which the cells remain alive, the reporter gene will be expressed. In cases in which the cells are dead, the reporter gene will not be expressed. Employing this approach, Wu *et al.* (2003) recently used embryonic cardiomyoblasts which express herpes simplex virus type I thymidine kinase (HSV1-tk) or firefly luciferase (Fluc) reporter genes, which they then noninvasively tracked using either micro-PET or bioluminescence optical imaging. Drastic reductions were noted in signal intensity within the first 1 to 4 days, and this was tentatively attributed to acute donor cell death as the result of inflammation, adenoviral toxicity, ischemia, or apoptosis.

Clinical Application and Future Prospects

Currently, Penuelas, et al. (Penuelas et al., 2005) monitored thymidine kinase gene expression after intratumoral injection of a first-generation recombinant adenovirus in patients with hepatocellular carcinoma using ^{18}F-FHBG PET (Fig. 3). In this study, transgene expression in the tumor was dependent on the injected dose of the adenovirus and was detectable in all patients who received > 10^{12} viral particles. This study is the pioneering trials on clinical application of human gene therapy imaging along with colon cancer (Penuelas et al. 2005) and glioblastoma (Jacobs et al., 2001) patients trials.

Molecular imaging strategies will likely expand significantly over the next few years as imagingestigators may readily move between the various technologies should help to also test various pre-clinical models. This potential power of molecular imaging to see fundamental biological processes in a new light will not only help to enhance our knowledge and understanding but should also accelerate considerably the rate of discovery in the biological sciences.

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