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REVIEW ARTICLE

In vivo Noninvasive Small Animal Molecular Imaging

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Abstract
The remarkable efforts that are made on molecular imaging technologies demonstrate its potential importance and range of applications. The generation of disease-specific animal models, and the developments of target-specific probes and genetically encoded reporters are another important component. Continued improvements in the instrumentation, the identification of novel targets and genes, and the availability of improved imaging probes should be made. Multimodal imaging probes should provide easier transitions between laboratory studies, including small animal studies and clinical applications. Here, we reviewed basic strategies of noninvasive *in vivo* imaging methods in small animals to introducing the concept of molecular imaging.

1. Introduction

Recent advances in molecular imaging allow us to visualize both cellular and subcellular processes within living subjects at the molecular level as well as at the anatomic level [1]. Molecular imaging is molecular-genetic imaging for visualizing cellular processes by combination of molecular biology and biomedical imaging. This marvelous technique provides research attention not only in molecular cell biology but also in related fields. Remarkable improvement of molecular imaging was achieved in visualization, characterization, and quantification of biologic processes by integration of many different fields such as genetics, pharmacology, chemistry, physics, engineering, and medicine. In particular, the development of controlled gene delivery and gene expression vector systems promotes generation of various types of reporter genes for visualization, for

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example, chloramphenicol acetyltransferase, b-galactosidase, luciferases, and fluorescent proteins. Conventionally, a recombinant plasmid, which contains a target gene and a reporter gene, has been used to monitor target gene expression by assaying reporter gene expression. However, this method cannot be used directly in living animals because the invariable light intensity from reporter proteins was not enough to be visualized in animals for non-invasive imaging. Different strategies are required for monitoring gene expression *in vivo* imaging. Accumulation of specific imaging signal for amplifying its intensity makes it possible to visualize localization, quantification, and repetitive determination of gene expression *in vivo* noninvasive imaging [2,3]. More effective strategies have been tried to overcome the obstacles for monitoring gene expression *in vivo* by recruiting methods from radio-pharmaceutics and physics. Radiolabeled small compounds and paramagnetic probes were developed for imaging specific proteins and magnetic signals, accelerating non-invasive molecular imaging technology [4,5]. In recent publications, these strategies have been reviewed by researchers for introducing the concept of molecular imaging [6,7].

The development of molecular imaging technologies has been facilitated by associated development of imaging instruments as well as imaging materials such as enhancement agents, probes, ligands, and reporter constructs. Small animal models have a great advantage in disease studies that are difficult or impossible to be performed in humans. Repetitive observation is a virtue of noninvasive small animal imaging, which provides information about a spatial and temporal dimension in disease development and progression. Multiple imaging modalities, including micro-computed tomography (CT), micro-single photon emission computed tomography (SPECT), micro-positron emission tomography (PET), micro-magnetic resonance imaging (MRI), micro-ultrasonography (US), and various optical techniques using fluorescence and bioluminescence, are available for small animal imaging (Figure 1). Recently, the resolution of some imaging modality is approaching cellular level [8], and the advances in imaging technology have resulted in developing combined imaging modalities, such as PET/CT, SPECT/CT, and PET/MRI [9,10]. Using the newly developed instrumental merging techniques, more precise localization information of both anatomic and molecular activity can be acquired in a single imaging session [11]. Advantages of multimodal approaches to molecular imaging provide better images for visualizing cellular, functional, and morphologic changes. Molecular and genetic changes usually precede biochemical, physiologic, and anatomic changes. Anatomic morphology changes can be visualized by conventional imaging modalities such as CT, MRI, US, and radiography. Biochemical and physiologic changes can be monitored through the use of PET, SPECT, and MRI efforts. Molecular genetic imaging offers several different options in visualizing molecular genetic changes, which is occurring at the beginning of most diseases (Figure 2). The strategies for monitoring gene expression in small animal molecular imaging are broadly defined as direct and indirect imaging (Table 1).

Direct imaging strategies usually consist of a specific target and a target-specific probe, and the interaction between a target and a probe is directly related with the intensity of imaging signal. Synthetic radiolabeled antisense oligonucleotide can be used as a probe for direct imaging to visualize endogenous gene expression at the transcription level. For indirect imaging, reporter-gene—based techniques, which have been identified and widely used to study cell biology, are most frequently performed for monitoring gene expression *in vivo*. Reporter genes are genetic markers that easily encode detectable proteins or involve in metabolism of labeled probe. These markers are great tools to determine activities of specific promoters and the factors when they are located at the downstream of a specific promoter/enhancer sequence. Many genes contain more than one promoter, and promoter activities may be specific for a disease process. By placing an imaging reporter gene under the control of such promoter, promoter activity can be dynamically visualized and gene expression can be monitored. The reporter proteins are accumulated in the cells with a promoter-reporter construct, and the measurements of specific imaging signals from accumulated reporter proteins provide indirect information that reflects the level of reporter gene expression. A variety of molecular imaging techniques, including optical, nuclear, magnetic resonance modalities, can be used for reporter imaging. Since reporter-based imaging system represents a part of molecular signature in cellular process, it may be useful in gene therapy as well as an imaging tool.

2. Direct Imaging for Small Animal Molecular Imaging

The strategies of direct imaging for small animal molecular imaging have been established using nuclear medicine, optical, and MRI modalities with a specific target and a target-specific probe for gene expression. The localization and the concentration of a probe are directly related to its interaction with a target. In biomarker imaging, the metabolic trapping of specific probe molecule reflects the molecular events of a disease. Specific ligands for receptors, antibodies, or antibody fragments (e.g., minibody, affibody) for a specific antigen and synthetic small molecular tracers are other examples of direct imaging probes. Recently, synthetic small molecular tracers, such as antisense oligonucleotide or aptamer probes for targeting specific

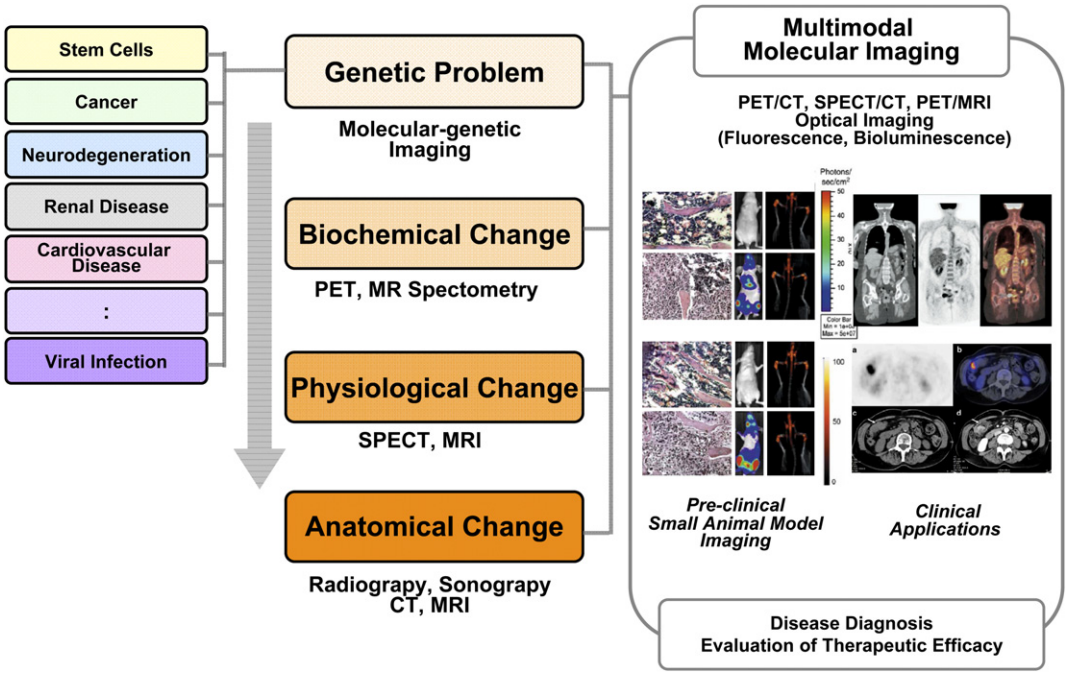

Figure 1. Multimodal imaging modalities for small animal imaging.

mRNA or protein, have been developed to visualize gene expression (Figure 3).

2.1. Biomarker imaging

For visualizing downstream effects of changes in specific molecular events, biomarker imaging can be useful. One good example of biomarker imaging is based on the fact that malignant tumors frequently show elevated level of glucose utilization and glycolysis [12]. This imaging strategy utilizes a radiolabeled glucose analogue [2'-fluoro-2'-deoxyglucose-(F-18) FDG] and PET, which reflects increased glucose transport and hexokinase activity. [F-18] FDG PET has been widely used in clinic to access tumor diagnosis and monitor therapeutic effect. However, biomarker imaging may reflect more than a single protein or signaling pathway. For [F-18] FDG PET, glucose metabolism is regulated by many different kinds of extracellular signals such as

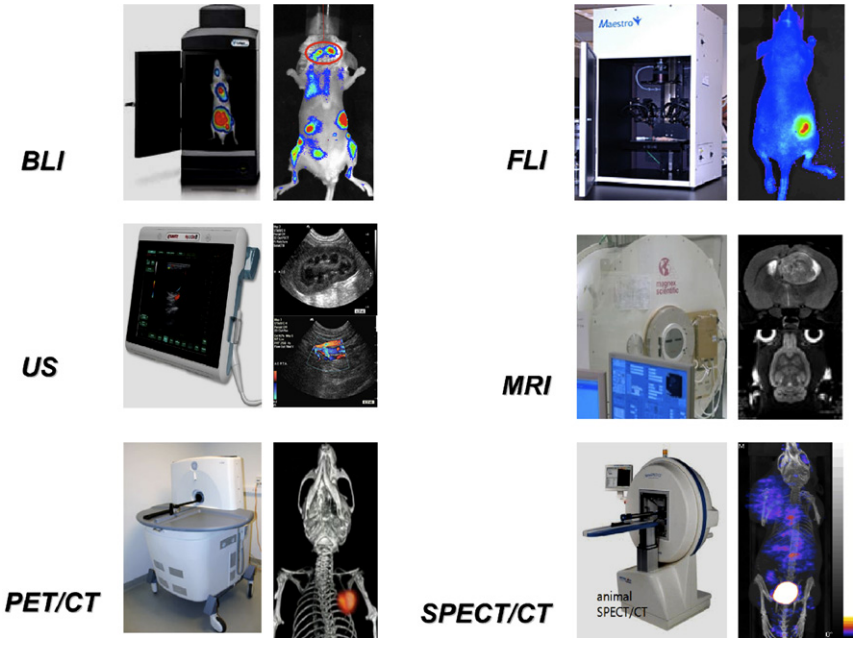

Figure 2. Multimodal imaging modalities for preclinical and clinical research.

Table 1. Applicable molecular imaging methods for visualizing gene expression in small animals

Direct imaging		
Target	Probes	Imaging modality
Biomarker		
Glucose transporter	[F-18] FDG	Nuclear
Receptor	Antibody	Nuclear, MR
	Minibody, affibody	Nuclear
	Peptide ligand	Nuclear, MR, optical
mRNA, protein	Synthetic small molecular tracer	
	Antisense oligonucleotide	Nuclear
	Aptamer	Nuclear, optical
Receptor, transporter, biomarker	Paramagnetic iron oxide	
	SPIO, MION	MR
Indirect imaging (reporter gene imaging)		
Reporter	Probes/contrasting agents	Imaging modality
Fluorescent protein	GFPs, RFPs	Optical
Luciferase		Optical
Enzyme type reporter		
HSV1-TK,	[F-18] FEAU, FHBG, [I-124] FIAU, FMAU, etc	Nuclear
Tyrosinase	Paramagnetic iron oxide	MR
Receptor type		
D2R	[F-18] FESP	Nuclear
SSTr	[I-123,124,131,Tc-99 m] octreotide	Nuclear
hNET	[I-123, 124,131] MIBG,	Nuclear
Estrogen	[C-11]ephedrine	Nuclear
Transferrin	[F-18] FES	MR
Ferritin	Paramagnetic iron oxide	MR
LRP	Paramagnetic iron oxide	MR
	H+	
Transporter type		
NIS	[I-123,124,131], [Tc-99 m]O4	Nuclear

D2R = dopamine 2 receptor; FDG = fluoro deoxy glucose; FEAU = 2'-fluoro-2'-deoxy-5-ethyl-1-β-D-arabino furanosyl uracil; FES = F-18 labeled estradiol; FESP = fluoro ethyl spiperone; FHBG = 9-(4-fluoro-3-hydroxymethylbutyl)-guanine; FIAU = fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil; FMAU = 2'-fluoro-2'-deoxy-5-methyl-1-β-D-arabinofuranosyl-uracil; hNET = human norepinephrine transporter; HSV1-TK = herpes simplex virus-1 thymidine kinase; LRP = lysine rich protein; MIBG = metaiodobenzylguanidine; MION = micrometer sized particles harboring iron oxide; MR = magnetic resonance; NIS = sodium/iodide symporter; SPIO = superparamagnetic ion oxide; SSTr = soma statin receptor.

the PI3 kinase/Akt pathway, mammalian Target of Rapamycin (mTOR), c-kit, and Hif-1a activation [6]. Nonetheless, [F-18] FDG PET whole body imaging is most widely used in clinic for tumor diagnosis and monitoring the efficacy of anticancer therapy that take advantages of the predeveloped radiolabeled probe. In particular, the application of biomarker imaging for monitoring the response of treatment is commonly used for evaluating the efficacy of newly developed drugs.

2.2. Receptor imaging

Since the enhanced intensity of probe signal is directly related to its interaction with a specific target, imaging specific receptors has been evolved to improve probe sensitivity and specificity. For this reason, radiolabeled antibodies for a specific receptor have been used over for the plast 20 years to visualize the localization of the receptor. However, the use of conventional radiolabeled antibody has a major problem of higher background signal from non-specific binding, slow penetration, and prolonged clearance by a larger size. More recently, genetically engineered small fragments of antibody, such as minibodies or affibodies, have been introduced as imaging probes for reducing size disturbance and increasing affinity [13]. In addition, small small-sized radiolabeled peptide ligands have been also used for specific receptor imaging. Moreover, recent advances have increased the detection sensitivity, which provides more options for selecting probes including fluorescent or paramagnetic nanoparticle based probes.

Tyrosine kinase HER2 is overexpressed in most breast cancers, and a radiolabeled monoclonal HER2 antibody for targeting HER2 is a good example of target-specific receptor imaging. A gadolinium-chelated HER2 specific antibody was also successfully used for magnetic-resonance—based molecular imaging of the HER2 receptor. A series of small fragmented antibodies was derived from a parental HER2 monoclonal antibody. A variant of anti-p185 HER2 minibodies showed high affinity to p185 HER2 as well as rapid clearance.

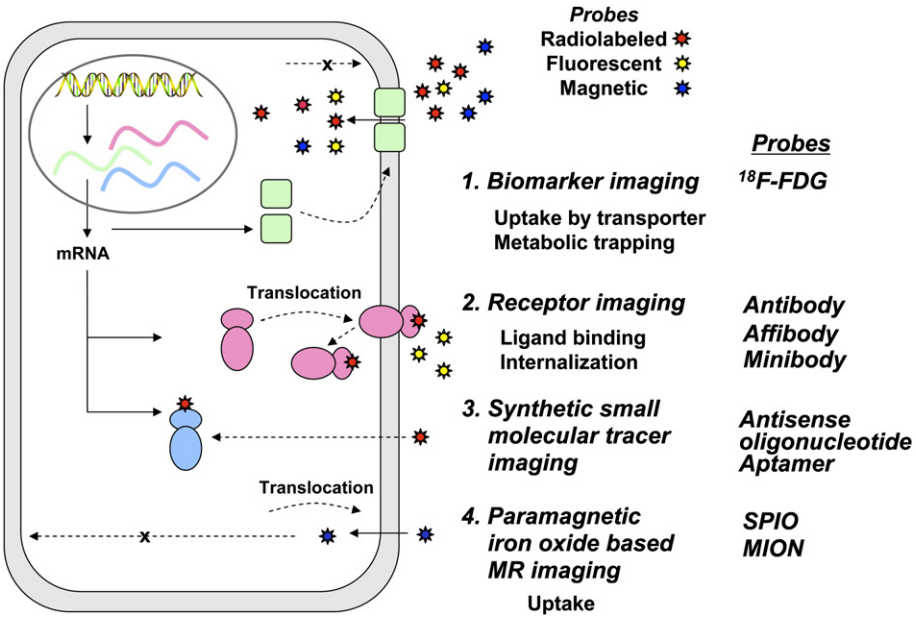

Figure 3. Schematic illustration of direct imaging with a target-specific probe. Stars are radioisotope labeled, fluorescent, or magnetic probes.

However, the results demonstrated that the tumor targeting properties *in vivo* mouse studies were less effective than *in vitro* [14]. Instead of antibody libraries, affibody molecules are selected from phage display. A new type of molecule named $(Z_{HER2:4})_2$ showed high affinity to HER2 and the tumor was easily visualized with a gamma camera in a xenografted mouse [15].

Radiolabeled glycosylated Arg-Gly-Asp (RGD) peptides are also developed for targeting alpha(v) beta3 integrin, which is highly expressed on tumor vasculature and plays an important role in tumor metastasis and angiogenesis. Recent researches focus on the wider use of a different imaging modality or multimodality using RGD. Kiessling and colleagues [16] reported that the RGD-labeled ultra-small super paramagnetic iron oxide for MRI successfully accumulated in the tumor vasculature of xenograft mouse model. Multimodal imaging approach using RGD peptide was also tried, and tumor selective localization was observed by bioluminescence, fluorescence, gamma scintigraphy, and SPECT imaging [17].

2.3. Synthetic small molecular tracer imaging

The development of synthetic small molecular tracer that specifically hybridizes to target mRNAs or proteins provided another strategy for direct imaging, including radiolabeled antisense (or aptamer) oligonucleotide probes (RASONs). Efficacy of RASONs was demonstrated for endogenous gene expression using a gamma camera and PET imaging [18]. A newly developed oligonucleotide-based molecule called aptamer, which can bind to almost any targets including proteins, peptides, antibodies, and small molecules such as DNAs and RNAs, provides a wide range of possible probes for specific targeting. An efficient targeting example using a MUC-1 specific aptamer has been demonstrated in most tumors [19,20]. However, RASON imaging still has several serious limitations, such as high background activity, limited tracer delivery, and poor stability.

A further development of direct radiotracer imaging strategies is required for a specific probe for each molecular target. In recent studies, the application of cell penetrating peptide with oligonucleotide probe is suggested to improve the delivery efficiency of tracing probe across the cell membrane, and the use of modified oligonucleotide is also recommended for longer stability of the tracing probe. Positively charged cell-penetrating peptides, such as transactivator of transcription protein transduction domain and arginine/lysine−rich peptide domain, have been used as conjugating peptides for intracellular delivery of a variety of small molecules, including oligonucleotides [21,22]. Many types of oligonucleotide analogues, including locked nucleic acid or peptide nucleic acid, have been reported [23], and the structural modification prevents enzymatic degradation of oligonucleotides from nucleases.

2.4. Paramagnetic iron-oxide−based MRI

Recently, the application of super paramagnetic nanoparticle based probe has increase for high resolution of *in vivo* MRI. This strategy uses MRI based on a T2 effect by superparamagnetic ion oxide (SPIO) nanoparticle for visualization [24]. The clinically approved SPIO containing Fe^{2+} and Fe^{3+} ion-oxide core is coated with carboxy dextran. A larger amount of ion oxide can be loaded into the nanoparticle, and micrometer-sized

particles harboring iron oxides are available for this purpose [25]. In particular, paramagnetic iron oxide has been successfully used with a better spatial resolution in monocyte-macrophage based liver imaging and stem cell trafficking [26]. For imaging the gene expression, the conjugation with a targeting peptide or oligonucleotide with paramagnetic ion oxide has been widely used. Although the possible toxicity of iron oxide nanoparticle has always been a challenge, recent findings have shown that SPIO can be safely used in mesenchymal stem cells trafficking without changing the viability, proliferation, and differentiation capability [27].

3. Reporter Gene Imaging for Small Animal Molecular Imaging

Although the originally devised reporter genes have been widely utilized to study *in vitro* cell biology, recent technical developments allow direct *in vivo* visualization to analyze gene expression and regulation. Reporter genes, located at the downstream of a specific promoter, are genetic markers that encode easily detectable proteins, and these markers become extraordinary tools to determine the activities of specific promoters. Imaging reporter genes uses genetic markers that involve in metabolism of labeled probes, which are great tools for determining activities of specific promoters and factors when they are located at the downstream of a specific promoter/enhancer sequence. By placing an imaging reporter gene under the control of such a promoter, dynamic visualization of promoter activity can be achieved.

3.1. Optical reporter imaging

The major advantages of optical imaging modalities, such as fluorescence and bioluminescence imaging, include that they are simpler, cheaper, more convenient, and more user friendly than other imaging modalities. Another advantage, especially for bioluminescence imaging, is their highly sensitivity for detecting low levels of gene expression. Various optical reporter genes constructs, which have been already used *in vitro*, are available for testing the same biologic hypotheses in living animal models (Figure 4). Combining the fluorescence and bioluminescence reporter genes into a single gene product could provide a better resolution for the analysis of gene expression by taking advantages of fluorescence *in vitro* as well as bioluminescence *in vivo*. Although one of the critical components of the optical imaging modalities is the sensitivity of detecting devices, recent advances have greatly increased their sensitivity. The charged coupled device (CCD) camera is the detection device that captures photons by photocathode, converting photons to electrons for amplification. For final detection using phosphor screen, another conversion of the amplified electrons to photons is required. For reducing thermal noise, this device can be cooled down to $-120^{\circ}C$. The sensitive range of this system is across the entire visible and near-infrared wavelengths. However, the blue, green, and yellow range of the light spectrum can be easily absorbed by mammalian tissue; the red, or longer wavelength of light, is preferable for *in vivo* optical imaging.

Recently, a fluorescent protein based reporter system has become very popular for monitoring gene expression, localization, movement, and protein-protein interaction *in vitro* [28]. Imaging fluorescent proteins is measured by the light emission from the excitation of external source of light. Various factors are involved in the brightness of fluorescent proteins, including folding, maturation, extinction coefficient, quantum yield, and the photostability of proteins. For this reason, many types of genetically engineered variants from natural fluorescent proteins are also developed for better imaging. Green fluorescent protein (GFP) has been widely used in molecular cell biology, and most GFP variants have come from spectral shifted variants. Synthetic variants including an enhanced GFP (eGFP) have been developed for improving stability and brightness of fluorescence. A number of red fluorescent proteins (RFP) have also been developed by genetic modification to overcome its limitations, such as tetrameric toxicity and incomplete maturation. Since many wild type fluorescent proteins have a tetrameric structure that causes aggregation and toxicity, a variety of genetically engineered mutant RFPs show longer emission wavelength. Bright, less toxic and more suitable RFPs for mammalian cell studies have been generated by Roger Tsien's group [28]. Monomers or tandem dimers of tetrameric fluorescent proteins with very bright fluorescence and higher photostability have been developed and terms such as mPlum, mCherry, and tdTomato have been used [28]. However, fluorescent reporter imaging for *in vivo* small animal imaging has major limitations, such as the requirement of an external light source and the exponentially decreasing intensity of light with increasing depth of the target localization. Moreover, the sensitivity and specificity of fluorescence imaging are frequently disturbed by endogenous tissue autofluorescence, which results in substantial background emissions. For this reason, the proper use of selective filters or the application of spectral analysis is required to reduce the interference of autofluorescence to the acquired images.

Among the various kinds of reporter genes, luciferases are the only ones that produce light, and they do not require an external excitation source. Because mammalian tissue does not emit a significant amount of light, luciferase imaging offers lower background signal compared with fluorescence imaging. The family of luciferase enzymes presents in certain bacteria, marine crustaceans, fish, and insects. The Firefly luciferase (FLuc) and Renilla luciferase (RLuc) are most commonly

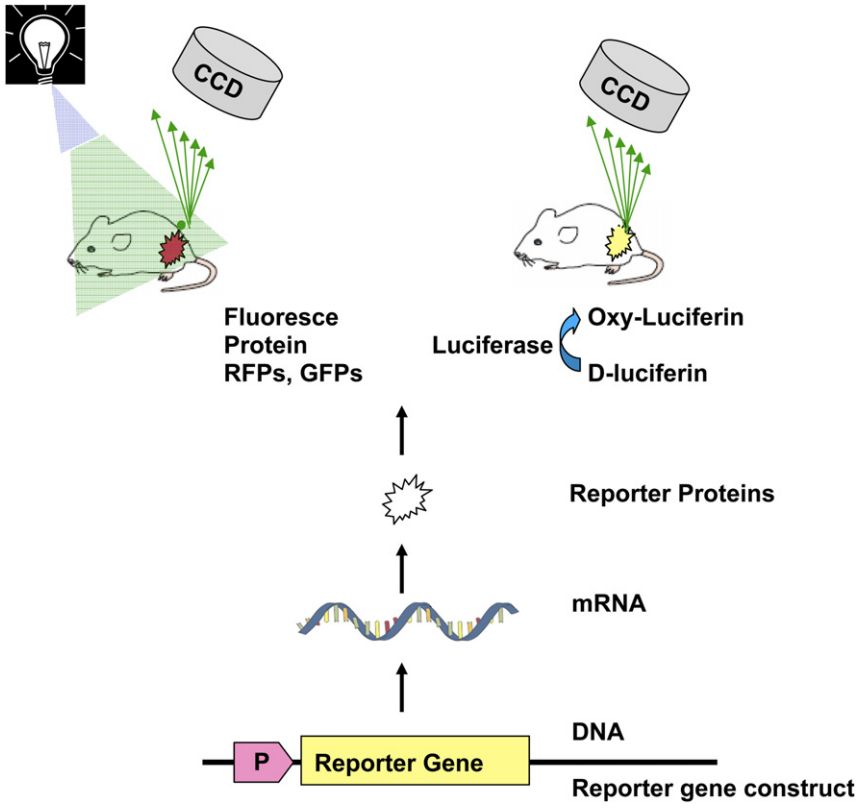

Figure 4. Strategy of the promoter-reporter gene construct for monitoring gene expression using optical imaging. P is a promoter/enhancer sequence.

used luciferase genes, and their corresponding substrates are luciferin and coelenterazine. Native luciferase of American firefly, *Photinus pyralis*, produces light with broad emission that peaks at 560 nm and above 600 nm fraction, making it suitable for *in vivo* imaging. Since the first report of Luc gene [29], this reporter gene has been modified for improving expression in mammalian cells by codon optimization. The peroxisomal targeting sequence was also deleted for higher expression in cytosol, and some of the amino acids were substituted for shifting emission wavelength toward the red region above 600 nm [29]. More recently, a synthetically derived luciferase Fluc2 has been developed with humanized codon optimization that is designed for high expression and reduced anomalous transcription [30]. The native substrate, D-luciferin [D-(-)-2-(6'-hydroxy-2'-benzothiazolyl) thiazone-4-carboxylic acid], is converted into oxyluciferin in an magnesium:ATP complex (Mg-ATP) dependent process. These luciferases generate a visible light through the oxidation of an enzyme-specific substrate in the presence of oxygen, and ATP is also required as an additional cofactor for luciferase imaging. For *in vivo* administration, it was reported that luciferin was found to be nontoxic and well distributed in the whole body of mouse after exogenous application (usually intraperitoneal injection, also can use intravenous route), even crossed the blood-brain or placental barrier. In a mouse model, luciferase reaction peaked at 10−20 minutes after the injection. Another type of commonly used luciferase from Renilla emits blue light with a 480-nm peak, limiting its use *in vivo*. FLuc and RLuc are distinguishable because they use different substrates and emit different light spectra. For this reason, RLuc has been used for the normalization of firefly luciferase expression. Fast induction of luminescence and the short half-life of luciferin and luciferase in bioluminescence system provide a suitable method for monitoring transcriptional activation using these two luciferases [30].

In vivo bioluminescence imaging was originally developed using a bacterial infection model [31], and a set of genes from soil bacterium *Photohabus luminescens* was first introduced into Salmonella bacteria. Bacterial luciferase Lux operon consists of five polycistronic genes under the same promoter, named Lux A, B, C, D, and E. Lux A and B encode heterodimeric bacterial luciferase and additional genes encode substrate synthesizing enzymes such as fatty-acid reductase enzyme complex. The lux operon has also been reorganized and optimized for expression. Labeled bacteria can be detected in the mouse model and represented as the location of an infection. A reduced form of flavin mononucleotide can be used as a substrate for bacterial luciferase, and emission peak is 490 nm.

For whole-body imaging of small animals, bioluminescence reporter genes have been more widely used

than fluorescence imaging due to the higher sensitivity and the lower background luminescence or autoluminescence. However, the basic problem of optical imaging system is the attenuation of light photons. About 90% of bioluminescence signal flux is lost per centimeter of tissue; thus, photon intensities detected by CCD cameras may not proportionately or sufficiently reflect endogenous reporter gene expression in the inner organs of even small animals [29].

3.2. Nuclear medicine reporter imaging

Many radionuclides emitting positrons and gamma rays have been used for a diagnosis and therapeutic purposes. PET scanners produce the image of positron emitters such as F-18, C-11, and I-124. To generate planar images and tomography of gamma ray emitters, a conventional gamma camera and SPECT have been routinely used. I-131, I-123, In-111 and Tc-99 m are the source of gamma emitters in these cases. For small animal studies, several small animal imaging instruments for nuclear imaging have been developed to meet the level of spatial resolution for the basic research requirement. Recently commercialized micro-PET scanners have resolution of around 2 mm^3, and newly developed micro-SPECT systems have pinhole collimators for high resolution [3]. Various positron and gamma ray emitting probes and reporter genes for nuclear imaging have been developed (Figure 5), but choices have to be made for particular situations and unique advantages and disadvantages of each strategy should be considered [6,7].

The herpes simplex virus type1 thymidine kinase (HSV1-tk) has been most widely used as a reporter gene for radionuclide-based molecular imaging and as a therapeutic suicidal gene for targeted gene therapy. The expressed viral thymidine kinase phosphorylates thymidine to thymidine-monophosphate, which is then di-phosphorylated and/or tri-phosphorylated by many cellular kinases. These phosphorylated compounds can serve as inhibitors of DNA replication, blocking DNA polymerization, which leads to cell death. Unlike mammalian TK, HSV1-tk can phosphorylates modified thymidine analogues, e.g., F-18 labeled 2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil (FIAU). Phosphorylated FIAU cannot traverse the cellular membrane and it is retained in the cell. PET can detect HSV1-tk gene expression by visualizing positron emission from a reporter probe—F-18 labeled phosphorylated FIAU in this case. The magnitude of the radioactive reporter probe accumulation reflects HSV1-tk enzyme activity, which represents HSV1-tk gene expression. Two types of substrates for HSV1-tk have been reported, which are pyrimidine nucleoside derivatives and acycloguanosine derivatives. Pyrimidine nucleoside derivatives are similar to natural thymidine in their structure, and they can be more sensitive probes than acycloguanosine derivatives for HSV1-tk imaging. Pyrimidine nucleoside derivatives include FIAU, 2'-fluoro-2'-deoxy-5-methyl-1-β-D-arabinofuranosyl-uracil (FMAU), and 2'-fluoro-2'-deoxy-5-ethyl-1-β-D-arabino furanosyl uracil (FEAU). A tracer dose of these drugs with radioisotopes can be successfully utilized as a probe for monitoring HSV1-tk expression by taking advantage of high sensitivities of PET and SPECT. Since HSV1-tk is less substrat-specific, it can phosphorylate acycloguanosine derivatives. Acycloguanosine derivatives have been used, and newly developed antiherpetic drugs, such as F-18 labeled acyclovir, ganciclovir, penciclovir, and

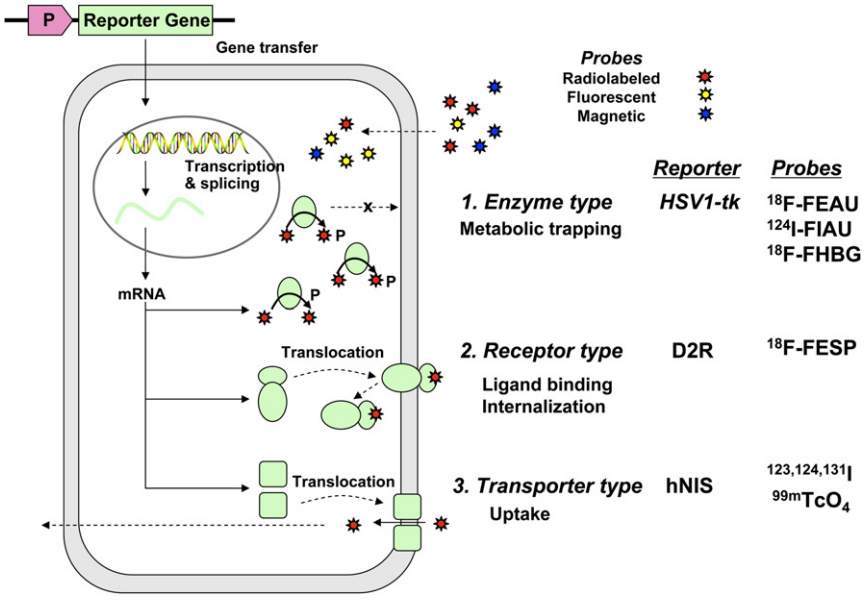

Figure 5. Schematic illustration of three-type reporter gene expression used in nuclear imaging modalities. Stars are radioisotope labeled substrates (enzyme) or ligands (receptor) or the radioisotope itself (transporter type).

9-(4-fluoro-3-hydroxymethylbutyl)-guanine, have been found to accumulate better than older drugs in HSV1-tk transfected cells. Since most thymidine kinase substrates do not cross the blood−brain barrier very rapidly, the development of a new kind of reporter gene/probe has been requested. Dopamine D2 receptor (D2R) system has been introduced for this reason, which uses D2R as an imaging reporter gene and F-18-fluoroethyl spiperone as a radiolabeled probe.

Since conventional PET reporter gene imaging requires the complicated substrates and expensive PET equipment, the development of simpler and less expensive system is requested. The sodium iodide symporter (NIS) gene system, which utilizes iodide uptake driven by the sodium ion concentration gradient across the membrane, has been shown to be the simplest and most applicable reporter system. In thyroid cells, sodium ion gradient is generated and maintained by the sodium-potassium pump (Na+-K+ATPase). An iodide enters with two sodiums through a specific transporter, NIS. In addition to the iodide, several other anions are transported by NIS, i.e., $ClO_4^- > ReO_4^- > I^- \geq SCN^- > ClO_3^- > NO_3^-$, in order of transport rates. Pertechnetate (TcO_4^-) and perrhenate (ReO_4^-) are also transported by NIS, and radioactive forms (Tc-99 m or Re-188) of such anions are important in terms of nuclear medicine imaging and radionuclide therapy. NIS has many advantages as an imaging reporter gene because various probes are available, such as radioiodines and Tc-99 m, and their metabolism are well understood. Unlike the D2R or HSV1-tk system using radiolabeled ligand, NIS has no problem with associated labeling stability because NIS directly uses radioiodine or Tc-99 m. In addition, NIS seems to perturb cells less because the iodide is not metabolized in most tissues and no adverse effects have been observed except for sodium influx. Availability of a less immunoactive human origin gene is another important merit of NIS. Since NIS genes are expressed on cell surfaces, reporter probes can reach to the cells easily. Importantly, reporter gene imaging with NIS may be more convenient, because most nuclear medicine departments have easily access to a gamma camera, SPECT, radioiodines, and Tc-99 m. Nevertheless, NIS also has several limitations. NIS occurs naturally at high concentrations in the thyroid, stomach, and urinary tract, causing difficulties in interpreting the image. NIS system is also hampered by the rapid efflux of radionuclides from cells, but co-transfection with the thyroid peroxidase gene may improve radioiodine retention in target cells.

3.3. Magnetic resonance reporter imaging

Recently developed micro-MRI units with higher tesla have made it possible to image small animals at higher resolution (~50 mm) [7]. Although MRI has not been developed for reporter imaging, many trials have been made on the development of new MRI reporters for imaging molecular events of interaction, including the binding of specific MRI contrasting agents with specific surface receptors, proton exchange−mediated by enzymatic cleavage of functional group, and the binding of metalloprotein or MRI contrasting agents [32,33]. The transferrin proteins are easy to bind with iron, and the iron-loaded transferrin proteins rapidly bind to transferrin receptors. The transferrin receptor-transferrin-Fe complex is internalized by receptor-mediated endocytosis. The iron is then released from the endosome by the acidic environment, and the released iron decreases T2 MRI signals [34]. As a reporter gene, the transferrin gene was cloned and transfected into the target cells, showing an elevated level of transferring receptor gene expression. Successful MRI was demonstrated using superparamagnetic iron oxide for imaging the expression of transferrin receptor. The human tyrosinase enzyme coded gene was successfully introduced and imaged in mouse fibroblast by iron induced T1 hyperintensities *in vitro*. As seen in an iron reservoir, ferritin also has roles in reporter MRI [35]. Nonmetallic, biodegradable MRI reporter gene encoding lysine-rich protein (LRP) is the prototype of a potential family of genetic engineered reporter, expressing artificial proteins with frequency-selective contrast [36]. The endogenous contrast is based on the transfer from amide protons or LRP to water protons, and this proton exchange reduces MRI signal intensity.

4. Multimodality Imaging for Small Animal Molecular Imaging

Because each imaging technology has unique advantages and disadvantages, it is useful to develop multimodal reporter gene system and detectors compatible with several imaging modalities. PET/bioluminescent imaging appears to be the most amenable technology because PET can provide three-dimensional images and allow quantitative analyses of reporter expression, and optical bioluminescent imaging can easily and rapidly produce bidimensional images with high sensitivity. The development of instruments for combined modality, such as microPET/microCT and microSPECT/microCT, has been increased, and instruments that permit concurrent, coregistered optical imaging as well as nuclear medicine imaging are under development. These multimodal instruments should provide convenient and sensitive means of bioluminescent noninvasive reporter gene imaging with the advantage of different modalities.

For generating multimodal reporter gene system, several strategies are being used to link the expressions of multiple reporter genes (Figure 6). Most genomic DNA is involved in the regulation of gene expression, which can be exercised at the transcriptional level or at the post-translational level. Many genes contain more than one promoter, and the activity of a particular

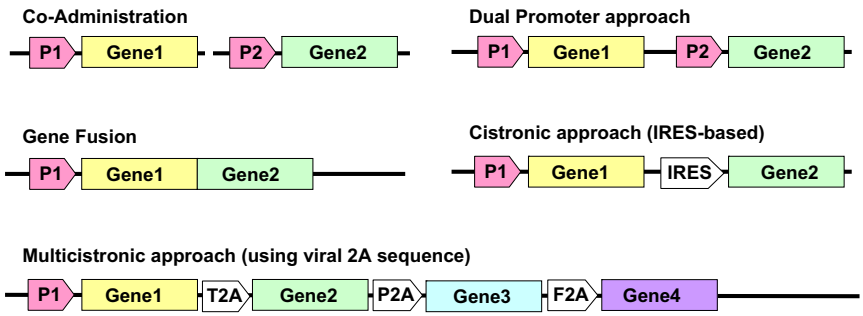

Figure 6. Strategies of promoter-reporter gene constructs for monitoring gene expression. P1, P2 are promoter/enhancer sequences. Gene1, Gene2, Gene3 and Gene4 can be reporter genes; internal ribosomal entry site (IRES); ribosomal skipping 2A sequence from foot-and-mouth disease virus (F2A), porcine teschovirus-1 (P2A), and Thosea asigna virus (T2A).

promoter may be specific to a diseases process. These types of promoter are of particular interest. First, they are a part of the molecular signature of the pathologic process concerned, and, second, they are potentially useful as specific promoters for gene therapy. Dual promoter or a coadministration approach may be used according to the purpose of research, but the level of expression is relatively low and uncontrollable. The most widely used strategy is a biscistronic approach using an internal ribosomal entry site (IRES) sequence between the two genes. Both genes are then transcribed into a single mRNA and later translated into two different proteins. However, the biscistronic approach, including IRES system, has demonstrated that a biased expression of the two transgenes with the second gene is underexpressed. As an alternative option for multiple reporter gene expressions is the use of ribosomal skipping via 2A peptides. Several viruses use 2A peptides to mediate protein cleavage, including foot-and-mouth disease virus (F2A), equine Rhinitis A virus, porcine teschovirus-1 (P2A), and Thosea asigna virus (T2A). The 2A peptide consensus motif is extremely rare and is associated with cleavage-like activity through a ribosomal skip mechanism; the 2A peptide impairs normal peptide bond formation without affecting the translation of other gene. 2A peptides have been shown to initiate the production of up to four proteins both *in vitro* and *in vivo*. Another strategy involves the use of a fusion gene vector, whereby two genes are connected, and their coding sequences are in the same reading frame to generate a single protein. Fusion between the two reporter genes, such as FLuc and GFP or its color shift variants, allow a dual mode of optical imaging. This has been conventionally used to monitor the biologic process *in vitro*. Since the fusion of engineered eGFP with other reporter gene, such as FLuc or the NIS gene, has provided successful employment without changing their functional properties. Chimeric fusion genes or biscistronic vectors have been used for the noninvasive imaging of reporter gene expression, monitored by bioluminescence and fluorescence, microPET and fluorescence, microPET and bioluminescence, and by microPET, fluorescence, and bioluminescence [37–39].

5. Application of Small-animal Molecular Imaging

With the advantage of noninvasive *in vivo* imaging, small-animal molecular imaging can be applied to understand biologic events in preclinical studies. Linking molecular imaging to gene therapy could allow real-time assessments of therapeutic efficacy, and linking an imaging reporter gene with a therapeutic gene could become a general approach to the monitoring of the *in vivo* expression of the therapeutic gene. For monitoring endogenous gene expression, several investigators have designed specific reporter gene constructs named, "The Cis-Promoter/Enhancer Reporter Gene System" under the control of upstream promoter/enhancer elements, possessing binding sites for specific transcription factors. Once a promoter/enhancer element has been activated due to the expression or activation of an endogenous gene product, imaging reporter gene expression occurs, thus enabling visualization. Using cis-promoter/enhancer imaging reporter genes, some intracellular biologic events, such as the activation of specific signal transduction pathways and nuclear receptors, can be visualized. The expression of a noninvasive reporter-imaging gene in small animals offers excellent opportunities to understand cancer progression, metastasis, and therapy. Individual animals can be visually monitored for tumor burden at primary sites, and the differences in tumor progression rates can be distinguished by reporter imaging. The possibility of metastases can be investigated, and individual responses to alternative therapies can be repeatedly monitored. Moreover, therapies can be altered and the consequences of these alterations observed.

Small animal molecular imaging can also be applied to monitoring *in vivo* distributions of immune and stem cells. The imaging of targeted T-cell trafficking using optical luciferase bioluminescence imaging has been

demonstrated in several models of autoimmunity, including collagen-induced arthritis [40] and experimental autoimmune encephalomyelitis [41]. In addition, the transplantation of cells, such as stem cells or progenitor cells, into damaged tissues has tremendous potential for the treatment of a number of disorders. Once stem cells have been administered systemically or locally, they may be able to migrate and repopulate pathologic sites. Three important aspects of cellular implants, i.e., cell tracking, cell viability, and cell numbers, can be monitored by molecular imaging [42]. Recently, the translation into a large animal model was successfully performed for evaluating the possibility of clinical application. Although *in vivo* imaging of transplanting mesenchyma stem cells in a porcine heart showed signs of inflammation [43,44], this kind of pilot study could provide precious information toward future clinical trials.

Small animal gene expression imaging techniques provide a new means of identifying drug targets and of preclinical testing. The ability to noninvasively image endogenous gene expression and various intracellular biologic phenomena, such as signal transduction, nuclear receptor activation, and protein−protein interactions, has important implications for drug discovery. Current imaging strategies, based on a suitable reporter or an imaging probe, can provide new information on the level, timing, and duration of action of many biologically active gene products. The selection of optimal probes or promoters for reporter gene expression control should be carefully considered when monitoring the effects of drugs on cells. Reporter gene expression imaging has emerged as a useful means of monitoring tumor growth and regression in preclinical models at subcutaneous, orthotopic, or intraperitoneal sites [45,46].

6. Conclusion

Molecular imaging embraces proteomic, metabolic, cellular biologic processes, and genetic imaging. Several imaging probes and reporter genes have been developed, and successful transitions from bench to bedside have already occurred [46,47]. Unique roles of small animal molecular imaging allow us to increase our knowledge on the critical biologic pathways involved in disease progression by characterizing biologic processes or tumor properties, and provide bridges to clinical application, i.e., in diagnosis, staging, determination of therapeutic targets, monitoring therapy, and in the evaluation of prognoses.

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