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Molecular imaging of in vivo gene expression

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Abstract

Background—Advances in imaging technologies have taken a prominent role in experimental and translational research and provide essential information on how changes in gene expression are related to downstream developmental and disease states.

Discussion—Magnetic resonance imaging contrast agents and optical probes developed to enhance signal intensity in the presence of a specific enzyme, genetic marker, second messenger or metabolite can prove a facile method of advancing the understanding of molecular events in disease progression.

Conclusion—The ability to detect changes in gene expression at the early stages of disease will lead to a greater understanding of disease progression, the use of early therapeutic intervention to increase patient survival, and tailored therapies to the detected genetic alterations in individual patients.

Keywords

gene expression; MRI; gadolinium; near-infrared; fluorescent protein; reporter gene

Introduction

Significant advances have been made in the understanding of the role of genetics in human diseases as a result of the human genome project [1]. Genomics allow for the manipulation of genes that are involved in developmental processes, cell physiology, and cell fate. In this post-genomic era, the focus has been on assigning functionality to genes involved in cellular processes and disease. The ability to track changes in gene expression and the subsequent downstream events in vivo at the genetic and molecular level using non-invasive imaging techniques is advancing basic, translational and clinical research.

Molecular imaging enables the visualization of normal and pathologic processes in living systems at the molecular level as opposed to simply imaging anatomy. Genetic and molecular processes in early stages of disease progression and development including changes in gene expression and activation of enzymatic activity precede anatomical changes that are detected by clinical imaging technologies. Molecular imaging techniques to visualize changes in cellular activity that occur in a pre-pathologic state will allow for earlier

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disease detection and intervention and the evaluation of specific molecular markers for therapy assessment.

The development of molecular imaging probes in optical and magnetic resonance imaging has led to the in vivo detection of reporter genes to track individual cell types, organelles or proteins and the identification of endogenous gene expression and enzymatic activity such as proteases in vivo. The ability to monitor these changes in living animals in real time continues to advance our understanding of cancer progression, infectious diseases and their treatments, protein-protein interactions, and numerous other signaling events and developmental states. Molecular imaging is becoming increasingly common in experimental and clinical settings with advances in probe and technology development demonstrating the impact of molecular imaging in vivo.

Advances in molecular imaging

Molecular imaging defined as “the spatially localized and/or temporally resolved sensing of molecular and cellular processes in vivo” [2] combines multiple disciplines including engineering, biomedical physics, chemistry, molecular biology, genetics and medical imaging that have direct impact on basic, translational and clinical research. This combination made possible the level of advancement currently seen in all areas of molecular imaging. Dramatic advances in imaging instrumentation and technologies especially in small animal imaging have provided scientists and clinicians with the ability to acquire images of anatomy and physiology well below the surface [3].

Each of the in vivo molecular imaging techniques including magnetic resonance imaging (MR) imaging, ultrasound (US), microscopic single photon emission tomography (SPECT) and positron emission tomography (PET) possesses unique advantages and disadvantages with varying limits. MRI and optical imaging, in experimentation and the clinical setting alike, have varying spatial resolution, temporal resolution, sensitivity and cost (Figure 1) [4–6]. Reporter probe development for cell tracking, gene expression and protease activity enhances the application possibilities of these imaging tools. For example, optical fluorescence techniques, which were previously limited to the microscopic regime using fluorescent dyes and proteins have expanded into the small animal realm using tissue-penetrating near-infrared light, bioluminescence and tomographic techniques [7, 8]. As a result, cellular events, biochemical processes and tissue morphogenesis can be monitored in vivo.

Advances in optical imaging technologies

Optical imaging is the most widely used imaging technique because of its accessibility, high resolution and sensitivity. Optical imaging has long been used as a molecular imaging technique to image expression patterns of fluorescent proteins [9]. Advances in near-infrared technologies and its application in whole animals draws attention to the development of fluorescent contrast agents to detect biochemical and molecular markers. Fluorescence in the near-infrared (NIRF) lies in the range of 700–950 nm, corresponding to the absorption minima of water, lipids, hemoglobin and deoxyhemoglobin [10].

In vivo small animal imaging can be carried out using reflectance imaging systems and conventional CCD cameras. Images are enhanced with the use of low-light imaging equipment (e.g., back-illuminated CCD cameras) and cooling to enhance instrument sensitivity [11]. In reflectance imaging, light of a defined bandwidth excites a fluorescent probe whose emission spectrum is different than the absorption spectra. This difference is resolved by the application of an emission filter that allows detection by a CCD camera. These white light images of the animals are acquired to correlate anatomical features with the fluorescence signal [12].

Tomographic techniques are capable of detecting and quantifying deep tissue fluorescence at centimeter depth [13, 14]. In contrast, reflectance imaging is limited to millimeter depth [15]. Fluorescence molecular tomography (FMT) allows researchers to acquire three-dimensional images of fluorescence by measuring molecular probes at the excitation and emission wavelengths to determine local probe concentration or activation [15, 16]. To perform fluorescence tomography, the tissue is illuminated from different projections, and multiple measurements are collected from the tissue boundaries. FMT requires only fluorescence and intrinsic light measurements using a constant wave source such as a laser [12]. Practical application of FMT is made possible with the use of a constant wave course, multiple detectors and CCD cameras [7]. Even with these advances, the depth of penetration using optical techniques is limited because of tissue scattering and absorption [17].

Advances in MR imaging technologies

MRI has excellent spatial and temporal resolution and at low field strengths (< 3 T) provides images of anatomical features at submillimeter resolution [18]. Using high-field magnets (> 11 T), single cells have been visualized with reported resolution of $10\ \mu\text{m}$ [19–22]. Clinical MR imaging systems typically use 1.5 and 3 T while many research-oriented small animal systems operate at high field strengths of 4.7, 9.4, 11.7, and 14 T [23–26]. High field strengths allows for the development of high resolution and high sensitivity pulse sequences using small animals prior to translation to clinical applications. High resolution images can be acquired because the signal-to-noise (SNR) is proportional to field strength, and the observed signal is proportional to the volume of tissue within any voxel [27]. Due to the reduction of observed signal at high-field strength, images with a high resolution require longer acquisition times.

MRI contrast agents

Endogenous tissue contrast can be enhanced with the use of paramagnetic and superparamagnetic contrast agents that increase signal in an area of interest [28]. MRI contrast agents are composed of transition metals [Mn(II) and Fe(II/III)] or lanthanides [Gd(III) and Eu(III)], that modulate water relaxation in the target area [29]. However, with the reduced signal of high-resolution MR imaging due to the decreased number of signal-generating nuclei, the amount of contrast agent must be increased [30]. Strategies used in molecular imaging to obtain high-resolution MR images include signal amplification based on multiplexed Gd(III) chelates, attachment of targeting ligands to contrast agents and development of enzymatically activated contrast agents [31–35]. The combination of high spatial resolution of MR for imaging anatomy with contrast enhancement of isolated

molecular events provides a tool that is quickly becoming an imaging modality to track molecular and genetic events such as reporter genes non-invasively in animals [36].

Molecular-genetic imaging of reporter gene expression

Reporter genes provide information on the location, duration and levels of gene expression of any gene of interest within a living system [37]. With the recent advances in small animal imaging technologies, there has been a significant increase in reporter gene imaging to explore the cellular function of genes in real-time and at high resolution [9]. Molecular imaging using reporter genes is an indirect method of detecting gene expression [9].

The reporter gene is genetically encoded either as a fusion protein tag or in promoter function studies to allow for expression of the reporter gene after a specific molecular event [9, 38]. The expression of the reporter gene is visualized after the addition of the activating substrate (i.e., light for fluorescent proteins or a chemical substrate for enzymes). The advantage of reporter genes is their proven versatility in usage, including the analysis of the timing of changes in gene expression during development, the evaluation of subcellular localization, the use of split-protein complementation assays and visualizing protein--protein interactions [9, 39].

Fluorescent reporter proteins

Since the first report of the cloning of GFP in the 1990's, fluorescent proteins have been used as a tool in molecular biology to examine subcellular localization of proteins, gene expression and track cells [40]. GFP and its mutagenic variants have been widely used owing to its stability over a broad range of pH and temperature, and low cytotoxicity [9, 41]. GFP emits green light (507 nm) when excited by UV or blue light (488 nm). The desire for broader spectral characteristics led to the mutation of GFP to yield blue FP (BFP), cyan FP (CFP) and yellow FP (YFP) which have since been tuned for enhanced stability and spectral properties [42–44]. The ability to use fluorescent proteins with different spectral properties permits multi-protein labeling with different colors to examine the changes in localization and interactions over time [45].

The spectral shift of fluorescent proteins into red and near-infrared wavelengths increases the depth of tissue penetration for in vivo studies [46]. DsRed, along with many other yellow and red fluorescent proteins are tetrameric, and have been shown to be toxic to cells, which limits their use as fusion proteins [47, 48]. Genetic manipulations have allowed for spectral tuning to generate the “fruit series” including mRFP1 (ex: 625 nm), mCherry (ex: 625 nm) and mPlum (ex: 650 nm) that are non-toxic monomers and have increased light output in the NIR range in mice and have been used as fusion proteins in vivo [49, 50]. Due to recent improvements in fluorescent protein stability, toxicity and spectral tuning, these probes are integral in standard protein and cell labeling techniques [48].

Genetically encoded FRET biosensors based on fluorescent proteins

A significant contribution of fluorescent proteins toward advancing the functional understanding of proteins in vivo is the use of fluorescence resonance energy transfer

(FRET) to visualize the interaction of two molecules [51, 52]. FRET is a quantum mechanical process that involves the non-radiative transfer of energy between a donor and an acceptor molecule through the Förster mechanism [53, 54]. The energy transfer requires the overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor molecule and the distance between the acceptor and donor molecules ($< 100 \text{ \AA}$) [54]. When the donor and acceptor molecular are in close proximity, the excitation of the donor excites the acceptor molecule, which subsequently emits light of a wavelengths that is further red-shifted than the donor emission (Figure 2) [55].

A requirement for efficient FRET quantification is a one-to-one ratio of donor to acceptor. If the donor-acceptor ratio is not 1:1 the observed fluorescent signal may not be accurate due to spectral contamination of the donor when spectral overlap is close. FRET has been used to visualize changes in protein conformation and quantify protein-protein interactions in vivo [39, 56].

FRET biosensors based on fluorescent proteins often utilize tethered modified GFP pairs as the donor and acceptor molecules [57, 58]. Many FRET biosensors, including a Ca(II) probe based on calmodulin utilize the modified GFPs cyan FP as the donor and yellow FP as FRET acceptor (Figure 2) [59, 60]. Ca(II) FRET indicators have used cyan FP and yellow FP tethered to the N and C-termini of calmodulin and the calmodulin-binding peptide M13. In the presence of Ca(II), calmodulin wraps around the M13 peptide increasing FRET between the donor and acceptor [59]. Mutations in calmodulin allow for facile tuning of Ca(II) affinities to allow for the detection of different pathological processes. Transgenic animals have been generated expressing the Ca(II) FRET indicator to monitor Ca(II) changes in vivo in real time [60–62].

Detection of caspase-3 activation with a genetically encoded FRET biosensor utilizes a cyan FP as a donor and a yellow FP as an acceptor. These are coupled by a sequence containing a caspase-3 cleavage site DEVD [63]. In this example, FRET efficiency is decreased upon cleavage by caspase-3. Caspase-3 biosensors have been used in vivo to detect the efficacy of apoptosis induced in tumors by photodynamic therapy and cisplatin [64]. Genetically encoded FRET biosensors can be used to monitor interactions in vivo to evaluate the efficacy of cancer therapies in real-time providing further functional information about molecular events using reporter genes.

Bioluminescence

Bioluminescence utilizes the transmission of an internal source of light through tissues emitted from luciferase enzymes to track cells and genetic changes [65]. As with other reporter genes, luciferase genes can be encoded in cells and in transgenic animals through genetic manipulations [65]. Luciferase enzymes catalyze the oxidation of a substrate to produce photons of light. The North American firefly (*Photinus pyralis*) enzyme is the most commonly used [66]. The firefly luciferase enzyme converts the substrate D-luciferin to oxyluciferin resulting in the emission of green light at 562 nm [67]. Other enzymes commonly used include Renilla luciferase, that emits blue light with an emission maximum at 480 nm [68, 69]. Therefore, numerous cell types or proteins can be differentially labeled

using multiple luciferase enzymes as with fluorescent proteins. Luciferase has been used to quantify promoter activity in vitro and in vivo by genetically encoding the luciferase gene upstream of a promoter of interest [70, 71].

Luciferase chemiluminescence has considerable advantages over fluorescent proteins as a method for detecting reporter genes in vivo because i) mammalian tissues do not naturally emit light, and therefore in vivo bioluminescence has an inherently low background [72]. ii) Luciferases have a broad spectrum of emission and some have significant emission components of wavelengths longer than 600 nm increasing the tissue penetrating ability [69]. iii) Since the light-producing ability of luciferase is a chemiluminescent reaction and not fluorescence, there is no photobleaching of these molecules. iv) Neither the expression of luciferase or the administration of substrate has been shown to be toxic, increasing its application for repeated exposure in vivo. v) Luciferin and other luciferase substrates have been shown to be detected in all tissues, including the central nervous system [73] and are quickly cleared by renal excretion [74].

Bioluminescence was first developed in vivo using a bacterial infection model [75, 76]. The lux operon from *Photobacterium luminescens* has been introduced into pathogenic bacteria either as a plasmid or through integration into the chromosome for increased stability [75]. Labeling infectious bacteria with a light-producing signal allows for the label to be detected in the tissue of mice for evaluation of the location and the extent of infection. This model has been used to evaluate the efficacy of antibiotic therapies [77, 78].

Bioluminescence has been used to visualize viral infection [79, 80]. The detection of viral infection has been used to study the efficacy of anti-viral treatments such as the effects of valacyclovir on herpes simplex virus type 1 in mice [81]. Additionally, bioluminescence can be used to detect the efficiency of vector delivery for gene therapy [73, 82, 83]. The location and efficiency of gene delivery can be evaluated visually and quantified in vivo.

Tracking and quantifying tumor growth and migration non-invasively in real-time has been made possible through the use of bioluminescent reporter genes [84]. Tumor size can be measured using bioluminescence. This was validated by caliper measurements of subcutaneous tumors and demonstrated that bioluminescence can be used to monitor tumor burden [85]. Bioluminescence can be used to track small tumors and metastases and has been shown to have better sensitivity than x-ray in detecting micrometastases in the bone with foci as small as 0.5 mm³ [86]. To track and quantify metastasis of breast cancer to the bone, a mouse syngeneic model of metastasis has been developed. In this system, TM40D-MB cells expressing luciferase implanted in the mammary fat pad selectively metastasize to the bone [87]. This system demonstrated that the tumor suppressor maspin inhibits mammary invasion and motility [87, 88].

Bioluminescence resonance energy transfer (BRET) indicators

Bioluminescence resonance energy transfer (BRET) is analogous to FRET and is based on the non-radiative energy transfer between the electromagnetic dipoles of a luminescent donor molecule and a fluorescent protein acceptor [89]. The close proximity of the BRET

donor and acceptor has been used to study binding events that result in conformational changes of proteins and protein-protein interactions (Figure 2) [39].

Three main classes of BRET reporters have been reported (based on Renilla luciferase (RLuc)). In BRET1, RLuc is coupled to enhanced yellow fluorescent protein (EYFP) as the acceptor. The RLuc substrate coelenterazine (CLZ) produces a donor emission with a peak centered at 480 nm resulting in the acceptor emission at 530 nm [89]. Commercially available BRET (BRET2) uses GFP2 as the acceptor (excitation 400 nm, emission 511 nm) and the RLuc substrate is a modified coelenterazine DeepBlueC (bisdeoxycoelenterazine, 395 nm) [90].

Recently, the most red-shifted BRET reporter, BRET3, has been developed.[91] In this combination, RLuc8 is coupled to the DsRed variant mOrange with emission in the orange region at 564 nm (excitation 548 nm).[91] RLuc8 (480 nm) has increased stability and has been shown to enhance light output, while BRET3 has the highest light output of reported BRET sensors [91]. Single cells and small tumor metastases in vivo have been visualized using the BRET3 reporter system because it has the greatest total light output and deepest tissue penetration capability of reported BRET probes [91].

BRET offers significant advantages over FRET owing to the high sensitivity and decreased cell toxicity [89]. Unlike FRET, BRET does not require excitation of the donor molecule by an external light source. Therefore, BRET assays do not result in photobleaching or photoisomerization of the donor molecule and therefore no phototoxicity to cells or tissues [38]. Without using external light for donor excitation there is no cellular scattering of excitation light or cellular autofluorescence [38].

One of the major advantages of BRET is the lack of direct excitation of the acceptor molecule during excitation of the donor molecule. This decrease in background signal permits increased sensitivity and the detection of events occurring at lower levels than has been previously possible with FRET [39].

In vivo BRET has been used to detect ligand-induced changes in protein conformation and promoter activation [92]. BRET reporters have been generated that contain FKBP12 and FRB domains as interacting partners upon rapamycin binding [91, 92]. These BRET constructs are used to probe intermolecular protein interactions for drug detection in vivo [91, 92]. This BRET construct was shown to be an effective model system when the GFP²-RLuc8 (GFP²-FRB-FKBP12-RLuc8) was used to demonstrate that rapamycin could be detected at picomolar concentrations in vivo [92]. Using the BRET3 (mOrange-FRB-FKBP12-RLuc8) reporter increased the sensitivity of drug detection because of the increased tissue penetration and light output of BRET3 acceptor emission [91].

Transcriptional activity and second messenger signaling events can be visualized in vivo because of the sensitivity of BRET [93]. Sensing Ca(II) ion concentration changes allows for the recording of spontaneous, transient events in real-time [93]. Upon binding of Ca(II) to aequorin with coelenterazine bound, BRET emission is recorded from the tethered GFP [93]. Transgenic fruit-flies expressing the Ca(II) BRET sensor in neurons were monitored over time for luminescence using an electron-multiplying CCD (EMCCD) [93]. As videos

were recorded, spontaneous and induced changes in Ca(II) concentration were visualized [93]. This work demonstrates the ability to monitor short, transient events in signaling pathways that have been difficult to accurately record. Transcriptional activity has been visualized in tumor-bearing mice using a mCherry-Luc BRET fusion reporter under the control of a HIF1- α inducible promoter [94]. Specifically, mCherry fluorescence was detected only when tumor cells co-expressed HIF1- α demonstrating the use of a genetically engineered probe to visualize deep tissue events that vary greatly in the heterogeneous environment of the tumor [94].

MRI reporter genes based on metalloproteins

MRI offers excellent spatial and temporal resolution and is used to visualize the internal organs of whole animals [95]. MRI reporter genes based on metalloproteins are transgenes that express a reporter that is made superparamagnetic by the accumulation of iron from the host organism [96, 97]. As a result, there is no need for the introduction of an exogenous contrast agent for image enhancement to monitor gene expression simplifying intracellular delivery.

Ferritin is a metalloprotein that can contain up to 4000 Fe atoms in a ferric oxyhydroxide core [98]. Ferritin acts as the body's main form of iron storage by sequestering iron in a biochemically innocuous protein shell. The ferritin reporter gene has been introduced into the neurons and glia in the brain of C57Bl/6J mice by stereotactic injection of replication-defective adenovirus ferritin (AdV-FT) vector [99]. The virus-transduced area of the brain showed robust image contrast in a T₂-weighted MR image for up to 5 weeks. In contrast, a LacZ-expressing AdV control injected on the contralateral side showed no change in contrast [99]. Transgenic mice expressing the heavy chain of ferritin (h-ferritin) under tetracycline regulation (Tet-on) were generated under tissue specific promoters [100]. This allowed for the visualization of a small population of cells, the endothelial cells of the mouse brain, demonstrating the elevated sensitivity of ferritin contrast enhancement at high field strengths. MRI enabled in utero detection of endothelial cells expressing ferritin, as well as in tissues with high iron loading such as the liver [100]. This is due to the unusually high relaxivity of ferritin at low iron loading and the linear dependence of R₂ on the magnetic field which can be further enhanced by intracellular ferritin aggregation that is seen with ferritin but not other iron loading proteins [100, 101].

Improved cell viability and iron loading of ferritin has been postulated through the coexpression of transferrin and ferritin transgenes [102]. Free cellular iron can be toxic and can cause oxidative stress, while low levels of iron can compromise metabolic enzymes due to the vacant iron-binding sites [103]. The coexpression of the iron transporter, transferrin increases cellular uptake of iron to load the overexpressed ferritin transgene product, while maintaining cellular levels of iron closer to a wildtype cellular equilibrium [102].

Iron accumulation in cells to form a superparamagnetic iron oxide MRI contrast agent has been reported in vivo using the bacterial MagA iron transport protein [104]. The advantage of the use of a bacterial gene is the lack of endogenous gene expression. MagA expression in

293FT cells resulted in the accumulation of iron from endogenous sources and the subsequent formation of iron oxide nanoparticles in cells [104]. MagA expressing cells transplanted into the mouse brain show significant contrast enhancement, and are readily detected 5 days after implantation.

Optical and MRI reporter genes based on enzymatic activity

The bacterial LacZ gene encoding β -galactosidase (β -gal) is a common reporter gene used in transgenic mice [105]. Until recently, the lack of β -gal sensing fluorescent or MRI contrast agents for use in non-invasive, live animal imaging has limited its effectiveness in tracking gene expression [106]. β -gal has been used to detect gene expression in vitro on tissue sections, biopsies, and post-mortem samples using colorimetric substrates such as 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-gal), that are not suitable for long-term follow-up of genetic regulation [105]. NIR fluorescent, luminescent and MRI contrast agents that are activated upon cleavage by β -gal have provided researchers with the tools to enable the visualization of changes in LacZ gene expression over time in deep tissues [36].

β -galactosidase responsive MRI contrast agents

The first example of a probe for in vivo imaging of LacZ is EgadMe that consists of a Gd(III) chelate conjugated to a galactopyranose residue [25, 107–111]. This caged complex has all nine coordination sites of Gd(III) saturated, limiting access of water to the paramagnetic ion. Upon cleavage of the galactopyranose moiety from the chelate by β -gal, water access is restored resulting in an increase in relaxivity and signal enhancement on a T_1 -weighted image (Figure 3) [107, 108]. Enzymatic processing of the agent modulates the T_1 , and in *Xenopus laevis* the detection of LacZ was shown in vivo [25].

Further modifications of this class of β -galactosidase activated probes have allowed for the determination of the mechanism that modulates this change in the coordination environment of the Gd(III) ion [108]. A series of isomers were synthesized with a methyl group substituted at the α or β position of the linker (α -EgadMe and β -EgadMe respectively). The structure of the two isomers is dramatically different and employs two different mechanisms of activation by β -gal. With the cleavage of the galactopyranose by β -gal the hydroxyl group is sufficient to displace a coordinated carbonate ion (Figure 3) [108]. The galactopyranose in α -EgadMe is positioned over the Gd(III) ion and functions to sterically block water (Figure 3) [108].

Another example of a β -gal responsive MRI contrast agent [Gd(DOTA-FPG)(H₂O)] has detected the presence of β -gal in CT26-LacZ tumors in mice [112]. The relaxivity of this contrast agent sees an increase after the cleavage of the galactopyranose due to the subsequent interaction with a protein such as albumin (HSA) thereby increasing the rotational correlation time (τ_R) [112] and represents a different mechanism to modulate relaxivity after enzymatic cleavage of the contrast agent substrate.

NIR fluorescent β -galactosidase responsive optical contrast agents

NIR fluorescent probes designed to be activated upon cleavage by β -galactosidase allow for effective visualization gene expression in animals using 2-D reflectance imaging [113–115].

The compound 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β -D-galactopyranoside (DDAOG) is basically a far red fluorescent switch with different spectral properties before and after cleavage by β -galactosidase. DDAOG has been adapted for use in whole animal imaging [113–115]. In aqueous solution DDAOG excites at 465 nm, with emission at 608 nm. The cleavage product DDAO excites at 646 nm, and the maximum emission peak is centered at 658 nm [113]. The bathochromatic shift resulting from galactopyranose cleavage permits deeper tissue visualization and simultaneous detection of the uncleaved substrate (DDAOG) and the cleaved product (DDAO).

DDOAG cleavage by β -gal in implanted tumors (9L–LacZ) can be detected by imaging DDOA fluorescence while DDOA fluorescence was not detected in the contralateral 9L tumor [113]. This method has been validated in visualizing and quantifying the efficacy of gene transfer. When a β -gal reporter was delivered to the lung via PEI-mediated gene transfer, β -gal was detected in the lungs at 5 ng of enzyme per mg of lung protein [114]. Low levels of β -gal expression under the control of the VE-cadherin promoter in transgenic mice has been detected using DDOAG [114]. Endogenous levels of protein as well as overexpressed reporter constructs have been visualized using a far red contrast agent to visualize β -gal expression non-invasively in small animal models.

Bioluminogenic contrast agent for β -lactamase reporter gene imaging

Activatable bioluminogenic imaging of dual reporter gene systems has been used to detect the enzymatic activity of reporter genes such as β -galactosidase and β -lactamase in the presence of Luciferase [116, 117]. Bioluminogenic probes take advantage of the spectral properties of bioluminescence, while using an activatable system to detect the reporter activity of interest.

The bacterial enzyme β -lactamase has been used in infectious disease research as well as a reporter gene in mammalian cells [118]. Bacteria expressing β -lactamases (Bla) are resistant to β -lactam antibiotics such as penicillins and cephalosporins. Fluorescence and bioluminescent imaging of β -lactamase activity is valuable to detect bacterial infection, to assess new antibiotics, and as a reporter gene in mammalian cells [118, 119]. A bioluminogenic substrate for Bla has been developed that has been used to detect reporter gene expression in vivo with the coexpression of firefly luciferase (fLuc) [117]. This reporter system has been used to track cancer cells in a mouse tumor model, as well as ribozyme splicing activity. These experiments validate the use of β -lactamase as a reporter gene to visualize protein function and interactions in vivo. The 6-hydroxy group of D-luciferin is required for the oxidation reaction catalyzed by fLuc.

The Bla reporter contrast agent (Bluco) is synthesized through the conjugation at the 3' position of cephalosporin to the 6-hydroxy of D-luciferin through an ether bond [117]. Bla catalysis is required to open the β -lactam ring that triggers spontaneous fragmentation resulting in the release of D-luciferin. D-luciferin is then accessible to fLuc that catalyzes the light-producing oxidation reaction. Bla activity is visualized as light emission, as fLuc alone can not cleave the Bla substrate. In a COS-7 mouse xenograft model of cancer, cells transfected with both Bla and fLuc were detected after the addition of Bluco, however fLuc cells showed a vastly decreased signal intensity [117].

Imaging endogenous gene expression with contrast agents

MRI and CT are being exploited to obtain submillimeter resolution images of tumors [5]. Information obtained from these modalities monitoring tumor progression includes tumor size and location [4]. These anatomical data reflect late demonstrations of the progression of molecular events in cancer progression. Molecular imaging can be used to supplement this data with more specific parameters of tumor progression including the identification and the levels of expression of proteases,[120, 121] cell surface markers,[122] steroid and growth factor receptors,[123] and other signal transduction proteins. With this information in hand, more specific evaluation of therapies can be performed at the molecular level before gross physiological changes are manifested. Ultimately, these techniques will allow for more individualized treatments for patients.

Two general classes of molecular imaging probes are used to enhance visualization of endogenous gene expression [124]. The first class of molecular contrast agents are bioactivated probes. A signal is detected after interaction with the target protein such as the irreversible chemical modification by an enzyme (as seen in the β -gal activated contrast agents previously described). The second approach is to use targeting agents to increase the localization and accumulation of the contrast agent in the diseased versus normal tissue. A potential limitation of this approach is that the signal-to-noise ratio can be limited by the receptor protein density, the availability and/or level of expression, and non-specific binding, and kinetics of cellular uptake and clearance.

Protease detection in cancer

Many tumors have elevated levels of proteolytic enzymes and are associated with more aggressive carcinomas [125]. Proteases such as cathepsins and matrix metalloproteinases promote invasion, metastasis and angiogenesis which is why there is so much interest in developing protease inhibitors as anti-cancer therapeutics [126–128]. Optical NIRF and MRI probes have been synthesized to detect protease activity in vivo and have been used to further understand protease activity in tumor progression and evaluate therapeutics.

Effective amplification of fluorescent signal is obtained using a quenching-dequenching system [124]. First, a NIRF probe is tethered to a quencher by a peptide sequence. Upon cleavage of the peptide linker by the target protease the quencher no longer absorbs the emission of the NIRF probe, which is subsequently detected. The transition from an “off” to an “on” state after protease cleavage results in signal amplification.[120] Activity-based probes are comprised of a reactive group that covalently binds to the target protease after cleavage of the peptide linker [129, 130]. After cleavage, the quencher and fluorophore dissociate, resulting in conjugation of the activated fluorophore to the protein. This conjugate system is used to visualize cysteine cathepsin activity in tumors [130]. The covalent attachment to cathepsin allowed for further biochemical and fluorescent examination of the proteases in vivo. The covalent attachment to cathepsin provides a direct link between the imaging data and biochemical results, permitting an in vivo analysis of drug efficacy [130].

Nanoscale polymer-based probes are used to detect protease activity in tumors in vivo and have yielded several advantages over small-molecule contrast agents. Small-molecule probes tend to rapidly clear, whereas nanoscale particles have prolonged blood circulation half-time, allowing for increased tumor retention [131]. Additionally, the increased number of sites available for modification increases the number of new probes that can be developed. For example quenched dyes, targeting moieties and multimodal contrast agents improve targeting and reduce non-specific binding. The polymer backbone is composed of methoxy-PEG-protected poly-L-lysine (PLL) copolymer (PGC) to which NIRF probes were conjugated through a protease-cleavable peptide [132]. The close proximity of the dyes results in self-quenching by FRET. With the specific enzymatic cleavage of the peptide spacers, NIRF probes are separated from the backbone (and from each other) increasing their fluorescence [124, 132]. Variations of this probe in the peptide linker sequence have been used to visualize cathepsin [133, 134] and MMP-2 [135] activity in vivo.

Modulation of relaxivity in by the enzymatic activity of proteases has been achieved in Gd(III)-based MRI contrast agents [136, 137]. The PCA-2 switch agent consists of a Gd-DOTA chelate conjugated to a MMP-2 cleavable peptide, which is terminated with a PEG linker [137]. In the presence of MMP-2 the PEG linker is cleaved from the peptide sequence, decreasing the water solubility of PCA-2 resulting in slower clearance from tumors, and increasing the contrast in MMP-2 positive tumors. When a scrambled sequence is used, cleavage of the peptide does not occur and the contrast agent shows the same clearance rate in both MMP-2 positive and MMP-2 knock down tumors [137]. This result demonstrates that the cleavage is specific and that the change in solubility resulting in the image enhancement can be used to assess protease activity and identify which proteases are active.

Experimental and clinical use of molecular imaging of gene expression:

Future perspectives

Molecular imaging combines anatomical imaging with the sensitivity and resolution to visualize molecular interactions and signaling events in vivo. Technological improvement in imaging modalities has greatly enhanced the sensitivity, resolution and acquisition times to allow for in vivo imaging of molecular and genetic events in whole animals. Concurrent progress has been made in the development of imaging reporters through genetic manipulation or advanced materials science and chemistry. FRET and BRET reporters have been used to visualize protein-protein interaction, spontaneous signaling events such as transient ion release in single cells, and proteolytic events. Exogenous contrast agents have been used to visualize reporter gene enzymatic activity such as in the case of β -galactosidase and β -lactamase activity by optical imaging and MRI.

Researchers have capitalized on the use of genetic deletions and manipulations to determine their regulation and cellular function. Imaging reporter genes, such as LacZ and fluorescent proteins, have played a crucial role in facilitating the visualization of individual proteins in developmental events, disease progression and subcellular movement. The use of reporter genes has led to significant advances in the understanding of biological processes, and the

development of probes for detection of endogenous changes in gene expression associated with disease states.

Molecular imaging techniques to visualize these pre-pathological changes may allow for swift and patient-tailored intervention in disease. As advances in technologies that are available in the research setting become amenable to clinical use, molecular imaging will play a significant role in many diagnostic procedures. Biopsy, followed by histological examination to grading of tumors in an invasive procedure that is expensive and time consuming. With the use of molecular imaging agents, information on tumor grade can be evaluated through molecular imaging contrast agents (such as protease-cleavable or growth factor receptor-targeted probes) reducing the time to effective treatment.

The continued development of molecular imaging probes and expansion of applications makes in vivo molecular imaging a valuable experimental and clinical tool. A significant advantage of molecular imaging is the direct detection of a molecular event using an exogenous source of contrast. This allows for the immediate interpretation of data without the requirement further extraction of proteins, DNA, RNA or other components for analysis. Molecular imaging expedites both experimental and clinical data interpretation allowing for evaluation of both the position and intensity of the signal observed to make further decisions on experimental procedure or therapies. The use of reporter genes and experimental molecular imaging has become a laboratory standard. As the technologies and probes are validated in animals, molecular imaging will certainly have a significant impact in clinical evaluation.

References

1. Collins FS, Mckusick VA. Implications of the human genome project for medical science. *JAMA*. 2001; 285(5):540–544. [PubMed: 11176855]
2. Piwnica-Worms DR. Introduction to molecular imaging. *Journal of the American College of Radiology*. 2004; 1(1, Supplement 1):2–3. [PubMed: 17411748]
3. Meade TJ. Seeing is believing. *Academic Radiology*. 2001; 8(1):1–3. [PubMed: 11201450]
4. Weissleder R, Mahmood U. Molecular imaging. *Radiology*. 2001; 219(2):316–333. [PubMed: 11323453]
5. Weissleder R. Scaling down imaging: Molecular mapping of cancer in mice. *Nat Rev Cancer*. 2002; 2(1):11–18. [PubMed: 11902581]
6. Tsien RY. Imagining imaging's future. *Nat Rev Mol Cell Biol*. 2003; (Suppl):SS16–SS21. [PubMed: 14587522]
7. Ntziachristos V, Bremer C, Graves Ee, Ripoll J, Weissleder R. In vivo tomographic imaging of near-infrared fluorescent probes. *Mol Imaging*. 2002; 1(2):82–88. [PubMed: 12920848]
8. Ripoll J, Nieto-Vesperinas M, Weissleder R, Ntziachristos V. Fast analytical approximation for arbitrary geometries in diffuse optical tomography. *Opt Lett*. 2002; 27(7):527–529. [PubMed: 18007854]
9. Nowotschin S, Eakin GS, Hadjantonakis AK. Live-imaging fluorescent proteins in mouse embryos: Multi-dimensional, multi-spectral perspectives. *Trends Biotechnol*. 2009; 27(5):266–276. [PubMed: 19339068]
10. Sjm, Valentina Quaresima; Ferrari, Marco. Identification and quantification of intrinsic optical contrast for near-infrared mammography. *Photochemistry and Photobiology*. 1998; 67(1):4–14. [PubMed: 9477760]

11. Oshiro, M.; Moomaw, B. Methods in cell biology. Academic Press; 2003. Cooled vs. Intensified vs. Electron bombardment ccd cameras--applications and relative advantages; p. 133-156. (Ed.^(Eds).
12. Ntziachristos V, Bremer C, Weissleder R. Fluorescence imaging with near-infrared light: New technological advances that enable in vivo molecular imaging. Eur Radiol. 2003; 13(1):195–208. [PubMed: 12541130] * The use of NIR fluorescence tomography to detect tumors with a NIR probe in vivo.
13. Montet X, Ntziachristos V, Grimm J, Weissleder R. Tomographic fluorescence mapping of tumor targets. Cancer Res. 2005; 65(14):6330–6336. [PubMed: 16024635]
14. Graves, Ee; Ripoll, J.; Weissleder, R.; Ntziachristos, V. A submillimeter resolution fluorescence molecular imaging system for small animal imaging. Med Phys. 2003; 30(5):901–911. [PubMed: 12772999]
15. Ntziachristos V, Tung Ch, Bremer C, Weissleder R. Fluorescence molecular tomography resolves protease activity in vivo. Nat Med. 2002; 8(7):757–760. [PubMed: 12091907]
16. Ntziachristos V, Yodh AG, Schnall M, Chance B. Concurrent MRI and diffuse optical tomography of breast after indocyanine green enhancement. Proc Natl Acad Sci U S A. 2000; 97(6):2767–2772. [PubMed: 10706610]
17. Taroni P, Pifferi A, Torricelli A, Comelli D, Cubeddu R. In vivo absorption and scattering spectroscopy of biological tissues. Photochemical & Photobiological Sciences. 2003; 2(2):124–129. [PubMed: 12664972]
18. Schick F. Whole-body MRI at high field: Technical limits and clinical potential. Eur Radiol. 2005; 15(5):946–959. [PubMed: 15856252]
19. Tyszka, Jm; Fraser, Se; Jacobs, Re. Magnetic resonance microscopy: Recent advances and applications. Current Opinion in Biotechnology. 2005; 16(1):93–99. [PubMed: 15722021]
20. Pierre, Smirnov; Marie, Poirier-Quinot; Claire, Wilhelm, et al. In vivo single cell detection of tumor-infiltrating lymphocytes with a clinical 1.5 Tesla MRI system. Magnetic Resonance in Medicine. 2008; 60(6):1292–1297. [PubMed: 19030162]
21. Sigurdsson EM, Wadghiri YZ, Mosconi L, et al. A non-toxic ligand for voxel-based MRI analysis of plaques in ad transgenic mice. Neurobiology of Aging. 2008; 29(6):836–847. [PubMed: 17291630]
22. Lee S-C, Mietchen D, Cho J-H, et al. *In vivo* magnetic resonance microscopy of differentiation in *Xenopus laevis* embryos from the first cleavage onwards. Differentiation. 2007; 75(1):84–92. [PubMed: 17244024]
23. Jacoby C, B'ring YC, Beck A, et al. Dynamic changes in murine vessel geometry assessed by high-resolution magnetic resonance angiography: A 9.4T study. Journal of Magnetic Resonance Imaging. 2008; 28(3):637–645. [PubMed: 18777558]
24. Modo M, Mellodew K, Cash D, et al. Mapping transplanted stem cell migration after a stroke: A serial, in vivo magnetic resonance imaging study. NeuroImage. 2004; 21(1):311–317. [PubMed: 14741669]
25. Louie AY, Huber MM, Ahrens ET, et al. In vivo visualization of gene expression using magnetic resonance imaging. Nat Biotech. 2000; 18(3):321–325. * This is the first report of a q-modulated MRI contrast agent that has been visualized in vivo.
26. Belayev L, Obenaus A, Zhao W, et al. Experimental intracerebral hematoma in the rat: Characterization by sequential magnetic resonance imaging, behavior, and histopathology. Effect of albumin therapy. Brain Research. 2007; 1157:146–155. [PubMed: 17543290]
27. Cherry SR. In vivo molecular and genomic imaging: New challenges for imaging physics. Phys Med Biol. 2004; 49(3):R13–R48. [PubMed: 15012005]
28. Dodd SJ, Williams M, Suhan JP, Williams DS, Koretsky AP, Ho C. Detection of single mammalian cells by high-resolution magnetic resonance imaging. Biophysical Journal. 1999; 76(1):103–109. [PubMed: 9876127]
29. Caravan P, Ellison JJ, McMurry TJ, Lauffer RB. Gadolinium(III) chelates as MRI contrast agents: Structure, dynamics, and applications. Chemical Reviews. 1999; 99(9):2293–2352. [PubMed: 11749483]

30. Peter, Caravan; Christian, T. Farrar; Luca, Frullano; Ritika, Uppal. Influence of molecular parameters and increasing magnetic field strength on relaxivity of gadolinium- and manganese-based T₁ contrast agents. *Contrast Media & Molecular Imaging*. 2009; 4(2):89–100. [PubMed: 19177472]
31. Major JL, Meade TJ. Bioresponsive, cell-penetrating, and multimeric MR contrast agents. *Accounts of Chemical Research*. 2009; 42(7):893–903. [PubMed: 19537782]
32. Song Y, Kohlmeir EK, Meade TJ. Synthesis of multimeric MR contrast agents for cellular imaging. *Journal of the American Chemical Society*. 2008; 130(21):6662–6663. [PubMed: 18452288]
33. De Sousa PL, Livramento JB, Helm L, et al. *In vivo* MRI assessment of a novel Gd(III)-based contrast agent designed for high magnetic field applications. *Contrast Media & Molecular Imaging*. 2008; 3(2):78–85. [PubMed: 18412106]
34. Caravan P, Cloutier NJ, Greenfield MT, et al. The interaction of MS-325 with human serum albumin and its effect on proton relaxation rates. *Journal of the American Chemical Society*. 2002; 124(12):3152–3162. [PubMed: 11902904]
35. Sousa PLD, Livramento JOB, Helm L, et al. *In vivo* MRI assessment of a novel Gd(III)-based contrast agent designed for high magnetic field applications. *Contrast Media & Molecular Imaging*. 2008; 3(2):78–85. [PubMed: 18412106]
36. Kang JH, Chung J-K. Molecular-genetic imaging based on reporter gene expression. *J Nucl Med*. 2008; 49(Suppl_2):164S–179S. [PubMed: 18523072]
37. Gross S, Piwnica-Worms D. Spying on cancer: Molecular imaging in vivo with genetically encoded reporters. *Cancer Cell*. 2005; 7(1):5–15. [PubMed: 15652745]
38. Massoud TF, Paulmurugan R, De A, Ray P, Gambhir SS. Reporter gene imaging of protein-protein interactions in living subjects. *Curr Opin Biotechnol*. 2007; 18(1):31–37. [PubMed: 17254764]
39. Villalobos V, Naik S, Piwnica-Worms D. Current state of imaging protein-protein interactions in vivo with genetically encoded reporters. *Annu Rev Biomed Eng*. 2007; 9:321–349. [PubMed: 17461729]
40. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier Mj. Primary structure of the *aequorea victoria* green-fluorescent protein. *Gene*. 1992; 111(2):229–233. [PubMed: 1347277] *The first cloning of GFP.
41. Griesbeck O, Baird GS, Campbell RE, Zacharias Da, Tsien RY. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J Biol Chem*. 2001; 276(31):29188–29194. [PubMed: 11387331]
42. Heim R, Prasher DC, Tsien RY. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc Natl Acad Sci U S A*. 1994; 91(26):12501–12504. [PubMed: 7809066]
43. Heim R, Tsien RY. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr Biol*. 1996; 6(2):178–182. [PubMed: 8673464]
44. Rizzo MA, Springer GH, Granada B, Piston DW. An improved cyan fluorescent protein variant useful for fret. *Nat Biotechnol*. 2004; 22(4):445–449. [PubMed: 14990965]
45. Nowotschin S, Eakin GS, Hadjantonakis AK. Dual transgene strategy for live visualization of chromatin and plasma membrane dynamics in murine embryonic stem cells and embryonic tissues. *genesis*. 2009; 47(5):330–336. [PubMed: 19358158]
46. Chudakov DM, Lukyanov S, Lukyanov KA. Fluorescent proteins as a toolkit for in vivo imaging. *Trends in Biotechnology*. 2005; 23(12):605–613. [PubMed: 16269193]
47. Mizuno H, Sawano A, Eli P, Hama H, Miyawaki A. Red fluorescent protein from *discosoma* as a fusion tag and a partner for fluorescence resonance energy transfer. *Biochemistry*. 2001; 40(8):2502–2510. [PubMed: 11327872]
48. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Meth*. 2005; 2(12):905–909.
49. Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. Red fluorescent protein. *Nat Biotech*. 2004; 22(12):1567–1572. * The modification of GFP to generate

the fluorescent protein spectral variants of the “fruit series” consisting of new proteins with fluorescent emission ranging from blue to red.

50. Deliolanis NC, Kasmieh R, Wurdinger T, Tannous BA, Shah K, Ntziachristos V. Performance of the red-shifted fluorescent proteins in deep-tissue molecular imaging applications. *Journal of Biomedical Optics*. 2008; 13(4):044008–044009. [PubMed: 19021336]
51. Truong K, Ikura M. The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo. *Current Opinion in Structural Biology*. 2001; 11(5): 573–578. [PubMed: 11785758]
52. Didenko VV. DNA probes using fluorescence resonance energy transfer (FRET): Designs and applications. *Biotechniques*. 2001; 31(5):1106–1116. 1118, 1120-1101. [PubMed: 11730017]
53. Forster T. Transfer mechanisms of electronic excitation energy. *Radiation Research Supplement*. 1960; 2:326–339.
54. Wu PG, Brand L. Resonance energy transfer: Methods and applications. *Analytical Biochemistry*. 1994; 218(1):1–13. [PubMed: 8053542]
55. Mitra RD, Silva CM, Youvan DC. Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein. *Gene*. 1996; 173(1):13–17. [PubMed: 8707050]
56. Kamiyama D, Chiba A. Endogenous activation patterns of cdc42 gtpase within drosophila embryos. *Science*. 2009; 324(5932):1338–1340. [PubMed: 19498173]
57. Chan FK, Siegel RM, Zacharias D, et al. Fluorescence resonance energy transfer analysis of cell surface receptor interactions and signaling using spectral variants of the green fluorescent protein. *Cytometry*. 2001; 44(4):361–368. [PubMed: 11500853]
58. Pollok BA, Heim R. Using gfp in fret-based applications. *Trends in Cell Biology*. 1999; 9(2):57–60. [PubMed: 10087619]
59. Miyawaki A, Llopis J, Heim R, et al. Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature*. 1997; 388(6645):882–887. [PubMed: 9278050]
60. Hara M, Bindokas V, Lopez Jp, et al. Imaging endoplasmic reticulum calcium with a fluorescent biosensor in transgenic mice. *Am J Physiol Cell Physiol*. 2004; 287(4):C932–C938. [PubMed: 15163621]
61. Higashijima S-I, Masino MA, Mandel G, Fetho. Imaging neuronal activity during zebrafish behavior with a genetically encoded calcium indicator. *J Neurophysiol*. 2003; 90(6):3986–3997. [PubMed: 12930818]
62. Kerr R, Lev-Ram V, Baird G, Vincent P, Tsien RY, Schafer WR. Optical imaging of calcium transients in neurons and pharyngeal muscle of c. *Elegans*. *Neuron*. 2000; 26(3):583–594. [PubMed: 10896155]
63. Tyas L, Brophy VA, Pope A, Rivett AJ, Tavaré JM. Rapid caspase-3 activation during apoptosis revealed using fluorescence-resonance energy transfer. *EMBO Rep*. 2000; 1(3):266–270. [PubMed: 11256610]
64. Zhou F, Xing D, Wu S, Chen WR. Intravital imaging of tumor apoptosis with fret probes during tumor therapy. *Mol Imaging Biol*. 2009
65. Sato A, Klaunberg B, Tolwani R. In vivo bioluminescence imaging. *Comp Med*. 2004; 54(6):631–634. [PubMed: 15679260]
66. Fraga H. Firefly luminescence: A historical perspective and recent developments. *Photochem Photobiol Sci*. 2008; 7(2):146–158. [PubMed: 18264582]
67. McElroy WD, Seliger HH, White EH. Mechanism of bioluminescence, chemiluminescence and enzyme function in the oxidation of firefly luciferin. *Photochem Photobiol*. 1969; 10(3):153–170. [PubMed: 5824751]
68. Lorenz WW, Cormier MJ, O’kane DJ, Hua D, Escher AA, Szalay AA. Expression of the Renilla reniformis luciferase gene in mammalian cells. *J Biolumin Chemilumin*. 1996; 11(1):31–37. [PubMed: 8686494]
69. Bhaumik S, Gambhir SS. Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc Natl Acad Sci U S A*. 2002; 99(1):377–382. [PubMed: 11752410] * The first use of Renilla luciferase as a bioluminescent reporter gene in vivo.

70. Contag CH, Bachmann MH. Advances in in vivo bioluminescence imaging of gene expression. *Annu Rev Biomed Eng.* 2002; 4:235–260. [PubMed: 12117758]
71. Ma L, Xiang Z, Sherrill TP, et al. Bioluminescence imaging visualizes activation of nuclear factor-kappaB in mouse cardiac transplantation. *Transplantation.* 2008; 85(6):903–910. [PubMed: 18360274]
72. De Wet, Wood KV, Deluca M, Helinski Dr, Subramani S. Firefly luciferase gene: Structure and expression in mammalian cells. *Mol. Cell. Biol.* 1987; 7(2):725–737. [PubMed: 3821727]
73. Leo BM, Li X, Balian G, Anderson DG. In vivo bioluminescent imaging of virus-mediated gene transfer and transduced cell transplantation in the intervertebral disc. *Spine (Phila Pa 1976).* 2004; 29(8):838–844. [PubMed: 15082981]
74. Berger F, Paulmurugan R, Bhaumik S, Gambhir SS. Uptake kinetics and biodistribution of 14c-d-luciferin—a radiolabeled substrate for the firefly luciferase catalyzed bioluminescence reaction: Impact on bioluminescence based reporter gene imaging. *Eur J Nucl Med Mol Imaging.* 2008; 35(12):2275–2285. [PubMed: 18661130]
75. Contag CH, Contag PR, Mullins JJ, Spilman SD, Stevenson DK, Benaron DA. Photonic detection of bacterial pathogens in living hosts. *Mol Microbiol.* 1995; 18(4):593–603. [PubMed: 8817482]
76. Hutchens M, Luker GD. Applications of bioluminescence imaging to the study of infectious diseases. *Cellular Microbiology.* 2007; 9(10):2315–2322. [PubMed: 17587328]
77. Mook-Kanamori B, Rouse M, Kang C-I, Van De Beek D, Steckelberg J, Patel R. Daptomycin in experimental murine pneumococcal meningitis. *BMC Infectious Diseases.* 2009; 9(1):50. [PubMed: 19405978]
78. Thorn RMS, Nelson SM, Greenman J. Use of a bioluminescent pseudomonas aeruginosa strain within an in vitro microbiological system, as a model of wound infection, to assess the antimicrobial efficacy of wound dressings by monitoring light production. *Antimicrob. Agents Chemother.* 2007; 51(9):3217–3224. [PubMed: 17638701]
79. Dothager RS, Flentie K, Moss B, Pan M-H, Kesarwala A, Piwnica-Worms D. Advances in bioluminescence imaging of live animal models. *Current Opinion in Biotechnology.* 2009; 20(1):45–53. [PubMed: 19233638]
80. Luker KE, Luker GD. Applications of bioluminescence imaging to antiviral research and therapy: Multiple luciferase enzymes and quantitation. *Antiviral Research.* 2008; 78(3):179–187. [PubMed: 18358543]
81. Luker GD, Bardill JP, Prior JL, Pica CM, Piwnica-Worms D, Leib DA. Noninvasive bioluminescence imaging of herpes simplex virus type 1 infection and therapy in living mice. *J. Virol.* 2002; 76(23):12149–12161. [PubMed: 12414955]
82. Mizuno T, Mohri K, Nasu S, Danjo K, Okamoto H. Dual imaging of pulmonary delivery and gene expression of dry powder inhalant by fluorescence and bioluminescence. *Journal of Controlled Release.* 2009; 134(2):149–154. [PubMed: 19100298]
83. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of aav serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther.* 2008; 16(6):1073–1080. [PubMed: 18414476]
84. Klerk CP, Overmeer Rm, Niers Tm, et al. Validity of bioluminescence measurements for noninvasive in vivo imaging of tumor load in small animals. *Biotechniques.* 2007; 43(1 Suppl):7–13. 30. [PubMed: 17936938]
85. Paroo Z, Bollinger RA, Braasch DA, et al. Validating bioluminescence imaging as a high-throughput, quantitative modality for assessing tumor burden. *Mol Imaging.* 2004; 3(2):117–124. [PubMed: 15296676]
86. Wetterwald A, Van Der Pluijm G, Que I, et al. Optical imaging of cancer metastasis to bone marrow: A mouse model of minimal residual disease. *Am J Pathol.* 2002; 160(3):1143–1153. [PubMed: 11891210]
87. Shi HY, Liang R, Templeton NS, Zhang M. Inhibition of breast tumor progression by systemic delivery of the maspin gene in a syngeneic tumor model. *Mol Ther.* 2002; 5(6):755–761. [PubMed: 12027560]

88. Zhang M, Sheng S, Maass N, Sager R. Mmaspin: The mouse homolog of a human tumor suppressor gene inhibits mammary tumor invasion and motility. *Mol Med*. 1997; 3(1):49–59. [PubMed: 9132279]
89. Xu Y, Piston DW, Johnson CH. A bioluminescence resonance energy transfer (bret) system: Application to interacting circadian clock proteins. *Proc Natl Acad Sci U S A*. 1999; 96(1):151–156. [PubMed: 9874787]
90. Koterba KL, Rowan BG. Measuring ligand-dependent and ligand-independent interactions between nuclear receptors and associated proteins using bioluminescence resonance energy transfer (bret). *Nucl Recept Signal*. 2006; 4:e021. [PubMed: 17016546]
91. De A, Ray P, Loening AM, Gambhir SS. BRET3: A red-shifted bioluminescence resonance energy transfer (BRET)-based integrated platform for imaging protein-protein interactions from single live cells and living animals. *FASEB J*. 2009 * The most recently published class of BRET reporters with red-shifted emission for enhanced tissue penetration.
92. De A, Loening AM, Gambhir SS. An improved bioluminescence resonance energy transfer strategy for imaging intracellular events in single cells and living subjects. *Cancer Res*. 2007; 67(15):7175–7183. [PubMed: 17671185]
93. Rogers KL, Martin JR, Renaud O, et al. Electron-multiplying charge-coupled detector-based bioluminescence recording of single-cell Ca²⁺ J Biomed Opt. 2008; 13(3):031211. [PubMed: 18601535]
94. Iglesias P, Costoya JA. A novel bret-based genetically encoded biosensor for functional imaging of hypoxia. *Biosensors and Bioelectronics*. 2009; 24(10):3126–3130. [PubMed: 19423327]
95. Seemann MD. Whole-body PET/MRI: The future in oncological imaging. *Technol Cancer Res Treat*. 2005; 4(5):577–582. [PubMed: 16173829]
96. Gilad AA, Winnard PT Jr, Van Zijl PCM, Bulte JWM. Developing MR reporter genes: Promises and pitfalls. *NMR in Biomedicine*. 2007; 20(3):275–290. [PubMed: 17451181]
97. Cohen B, Ziv K, Plaks V, Harmelin A, Neeman M. Ferritin nanoparticles as magnetic resonance reporter gene. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*. 2009; 1(2):181–188. [PubMed: 20049789]
98. St. Pierre TG, Chan P, Bauchspiess KR, et al. Synthesis, structure and magnetic properties of ferritin cores with varying composition and degrees of structural order: Models for iron oxide deposits in iron-overload diseases. *Coordination Chemistry Reviews*. 1996; 151:125–143.
99. Genove G, Demarco U, Xu H, Goins WF, Ahrens ET. A new transgene reporter for *in vivo* magnetic resonance imaging. *Nat Med*. 2005; 11(4):450–454. [PubMed: 15778721]
100. Cohen B, Ziv K, Plaks V, et al. MRI detection of transcriptional regulation of gene expression in transgenic mice. *Nat Med*. 2007; 13(4):498–503. [PubMed: 17351627]
101. Bartzakis G, Aravagiri M, Oldendorf WH, Mintz J, Marder SR. Field dependent transverse relaxation rate increase may be a specific measure of tissue iron stores. *Magnetic Resonance in Medicine*. 1993; 29(4):459–464. [PubMed: 8464361]
102. Deans AE, Wadghiri YZ, Bernas LM, Yu X, Rutt BK, Turnbull DH. Cellular MRI contrast via coexpression of transferrin receptor and ferritin. *Magnetic Resonance in Medicine*. 2006; 56(1): 51–59. [PubMed: 16724301]
103. Arosio P, Levi S. Ferritin, iron homeostasis, and oxidative damage. *Free Radic Biol Med*. 2002; 33(4):457–463. [PubMed: 12160928]
104. Zurkiya O, Chan Aw, Hu X. MagA is sufficient for producing magnetic nanoparticles in mammalian cells, making it an MRI reporter. *Magn Reson Med*. 2008; 59(6):1225–1231. [PubMed: 18506784]
105. Watson CM, Trainor PA, Radziewicz T, et al. Application of LacZ transgenic mice to cell lineage studies. *Methods Mol Biol*. 2008; 461:149–164. [PubMed: 19030795]
106. Bogdanov A, Weissleder R. *In vivo* imaging of gene delivery and expression. *Trends in Biotechnology*. 2002; 20(8):S11–S18.
107. Moats RA, Fraser SE, Meade TJ. A “smart” magnetic resonance imaging agent that reports on specific enzymatic activity. *Angewandte Chemie International Edition in English*. 1997; 36(7): 726–728.

108. Urbanczyk-Pearson LM, Femia FJ, Smith J, et al. Mechanistic investigation of β -galactosidase-activated MR contrast agents. *Inorganic Chemistry*. 2008; 47(1):56–68. [PubMed: 18072754]
109. Urbanczyk-Pearson LM, Meade TJ. Preparation of magnetic resonance contrast agents activated by beta-galactosidase. *Nat Protoc*. 2008; 3(3):341–350. [PubMed: 18323804]
110. Alauddin MM, Louie AY, Shahinian A, Meade TJ, Conti PS. Radiolabeling of a mri contrast agent with in-111, and its biological evaluation in vitro and in vivo. *J. Nucl. Med*. 2002; 43(5): 503.
111. Alauddin MM, Louie AY, Shahinian A, Meade TJ, Conti PS. Receptor mediated uptake of a radiolabeled contrast agent sensitive to b-galactosidase activity. *Nuclear Medicine and Biology*. 2003; 30(3):261–265. [PubMed: 12745017]
112. Chang Y-T, Cheng C-M, Su Y-Z, et al. Synthesis and characterization of a new bioactivated paramagnetic gadolinium(III) complex [Gd(DOTA-FPG)(H₂O)] for tracing gene expression. *Bioconjugate Chemistry*. 2007; 18(6):1716–1727. [PubMed: 17935289]
113. Tung C-H, Zeng Q, Shah K, Kim D-E, Schellingerhout D, Weissleder R. In vivo imaging of {beta}-galactosidase activity using far red fluorescent switch. *Cancer Res*. 2004; 64(5):1579–1583. [PubMed: 14996712]
114. Jossierand V, Texier-Nogues I, Huber P, Favrot Mc, Coll JL. Non-invasive in vivo optical imaging of the LacZ and Luc gene expression in mice. *Gene Ther*. 2007; 14(22):1587–1593. [PubMed: 17882264]
115. Zhang G-J, Chen T-B, Connolly B, et al. In vivo optical imaging of lacZ expression using LacZ transgenic mice. *ASSAY and Drug Development Technologies*. 2009; 7(4):391–399. [PubMed: 19689207]
116. Wehrman TS, Von Degenfeld G, Krutzik PO, Nolan GP, Blau HM. Luminescent imaging of [beta]-galactosidase activity in living subjects using sequential reporter-enzyme luminescence. *Nat Meth*. 2006; 3(4):295–301.
117. Yao H, So MK, Rao J. A bioluminogenic substrate for in vivo imaging of beta-lactamase activity. *Angew Chem Int Ed Engl*. 2007; 46(37):7031–7034. [PubMed: 17676567]
118. Zlokarnik G, Negulescu PA, Knapp TE, et al. Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter. *Science*. 1998; 279(5347):84. [PubMed: 9417030]
119. Xing B, Khanamiryan A, Rao J. Cell-permeable near-infrared fluorogenic substrates for imaging beta-lactamase activity. *J Am Chem Soc*. 2005; 127(12):4158–4159. [PubMed: 15783183]
120. Lee S, Park K, Kim K, Choi K, Kwon IC. Activatable imaging probes with amplified fluorescent signals. *Chem Commun (Camb)*. 2008; (36):4250–4260. [PubMed: 18802536]
121. Maxwell D, Chang Q, Zhang X, Barnett Em, Piwnica-Worms D. An improved cell-penetrating, caspase-activatable, near-infrared fluorescent peptide for apoptosis imaging. *Bioconjugate Chemistry*. 2009; 20(4):702–709. [PubMed: 19331388]
122. Segal EI, Low PS. Tumor detection using folate receptor-targeted imaging agents. *Cancer Metastasis Rev*. 2008; 27(4):655–664. [PubMed: 18523731]
123. Ke S, Wen X, Gurfinkel M, et al. Near-infrared optical imaging of epidermal growth factor receptor in breast cancer xenografts. *Cancer Res*. 2003; 63(22):7870–7875. [PubMed: 14633715]
124. Mahmood U, Weissleder R. Near-infrared optical imaging of proteases in cancer. *Mol Cancer Ther*. 2003; 2(5):489–496. [PubMed: 12748311]
125. Stearns ME, Wang M. Type IV collagenase (Mr 72,000) expression in human prostate: Benign and malignant tissue. *Cancer Res*. 1993; 53(4):878–883. [PubMed: 7679051]
126. Hu L, Roth JM, Brooks P, Luty J, Karparkin S. Thrombin up-regulates cathepsin D which enhances angiogenesis, growth, and metastasis. *Cancer Res*. 2008; 68(12):4666–4673. [PubMed: 18559512]
127. Hotary K, Allen E, Punturieri A, Yana I, Weiss SJ. Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. *J. Cell Biol*. 2000; 149(6):1309–1323. [PubMed: 10851027]
128. Whittaker M, Floyd CD, Brown P, Gearing AJH. Design and therapeutic application of matrix metalloproteinase inhibitors. *Chemical Reviews*. 1999; 99(9):2735–2776. [PubMed: 11749499]

129. Kato D, Boatright KM, Berger AB, et al. Activity-based probes that target diverse cysteine protease families. *Nat Chem Biol.* 2005; 1(1):33–38. [PubMed: 16407991]
130. Blum G, Von Degenfeld G, Merchant MJ, Blau HM, Bogoy M. Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes. *Nat Chem Biol.* 2007; 3(10):668–677. [PubMed: 17828252]
131. Greish K. Enhanced permeability and retention of macromolecular drugs in solid tumors: A royal gate for targeted anticancer nanomedicines. *Journal of Drug Targeting.* 2007; 15(7–8):457–464. [PubMed: 17671892]
132. Weissleder R, Tung CH, Mahmood U, Bogdanov A Jr. In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat Biotechnol.* 1999; 17(4):375–378. [PubMed: 10207887]
133. Tung CH, Mahmood U, Bredow S, Weissleder R. In vivo imaging of proteolytic enzyme activity using a novel molecular reporter. *Cancer Res.* 2000; 60(17):4953–4958. [PubMed: 10987312]
134. Bremer C, Ntziachristos V, Weitekamp B, Theilmeyer G, Heindel W, Weissleder R. Optical imaging of spontaneous breast tumors using protease sensing ‘smart’ optical probes. *Invest Radiol.* 2005; 40(6):321–327. [PubMed: 15905717]
135. Bremer C, Tung C-H, Weissleder R. In vivo molecular target assessment of matrix metalloproteinase inhibition. *Nat Med.* 2001; 7(6):743–748. [PubMed: 11385514] * The use of a MMP-2 cleavable NIRF probe to evaluate the changes in protease activity in tumors after treatment of animals with an MMP inhibitor.
136. Jastrzebska B, Lebel R, McIntyre OJ, et al. Monitoring of MMPs activity in vivo, non-invasively, using solubility switchable MRI contrast agent. *Adv Exp Med Biol.* 2009; 611:453–454. [PubMed: 19400263]
137. Lebel R, Jastrzebska B, Theriault H, et al. Novel solubility-switchable MRI agent allows the noninvasive detection of matrix metalloproteinase-2 activity in vivo in a mouse model. *Magn Reson Med.* 2008; 60(5):1056–1065. [PubMed: 18956456]

Executive summary

- Advances in chemistry, physics, engineering and biology have led to advances in optical imaging technologies to visualize molecular events using near-infrared probes and fluorescence molecular tomography.
- High field strength magnetic resonance imaging has increased resolution to detect molecular events in small animals.
- The spectral range of fluorescent proteins has become a standard laboratory technique to label whole cells, or proteins.
- FRET reporters have been used to visualize transient signals, enzymatic activity and protein-protein interactions.
- Genetically encoded bioluminescent reporter genes are advantageous in that no light excitation is required for emission, but is generated from a chemical reaction.
- BRET indicators have the same advantage and bioluminescence and have been modified to emit in the NIR.
- Metalloproteins ferritin and MagA have been used as MRI reporter genes as the accumulation of these superparamagnetic protein clusters results in contrast enhancement.
- MRI and optical β -galactosidase contrast agents have emerged as an exogenous source of contrast. Enzymatic cleavage by the reporter protein results in a change in contrast by the administered agent.
- Exogenous bioluminogenic contrast agents for the detection of β -lactamase utilize a dual reporter system, where the contrast agent is first cleaved by β -lactamase to generate the luciferase substrate to subsequently produce light.
- Exogenous contrast agents for the detection of reporter genes led to contrast agents to detect changes in endogenous gene expression associated with disease.
- MMP2-cleavable near-infrared probes have been used to detect changes in protease activity in cancer and to evaluate the efficacy of MMP inhibitors.
- With the evolution of molecular imaging agents and high resolution imaging instruments become amenable to human use will have a high impact on diagnosis of disease and the subsequent route of treatment and prevention.

Figure 1.
Current molecular imaging instrument and technology resolution and sensitivity limitations.

Figure 2. Schematic diagram of mechanism of RET-based probes. In the absence of binding ligand no emission from the acceptor is observed. With the addition of the binding ligand the donor and acceptor molecules are in close proximity for resonance energy transfer and emission from the acceptor. (A) CFP is the FRET donor molecule, and YFP the FRET acceptor. (B) RLuc is the BRET donor that produces light when the coelenterazine substrate is present. RFP is the BRET acceptor molecule.

Figure 3.

(A) Proposed mechanism of activation of β -EgadMe. (B) Proposed mechanism of activation of α -EgadMe.