Molecular Imaging of Pulmonary Disease *In Vivo*

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Characterization and noninvasive measurement of molecular pathways and biochemistry in living cells, animal models, and humans at the cellular and molecular level is now possible using remote imaging detectors. Positron and single photon emission tomography scanners, highly sensitive cameras for bioluminescence and fluorescence imaging, as well as high-magnetic-field magnetic resonance imaging scanners, can be used to study such diverse processes as signal transduction, receptor density and function, host response to pathogens, cell trafficking, and gene transfer. In many cases, images from more than one modality can be fused, allowing structure–function and multifunction relationships to be studied on a tissue-restricted or regional basis. “Molecular imaging” holds enormous potential for elucidating the molecular mechanisms of pulmonary disease and therapeutic response in intact animal models and humans.

Keywords: molecular imaging; positron emission tomography; optical imaging; magnetic resonance imaging; imaging reporter genes

Molecular imaging is broadly defined as the characterization and measurement of biological processes in living animals, model systems, and humans at the cellular and molecular level using remote imaging detectors. With refined genomic maps of human, mouse, and many pathogens completed, genetic information is expected to lead to new medical therapies, diagnostics, and ultimately to cures previously not imagined. In the post-genomic era, wherein functionality will be added to this vast array of genetic information, opportunity exists for imaging to play a significant role in basic and translational research as well as clinical care of patients. Molecular imaging offers an unprecedented opportunity to identify, follow, and quantify biologic processes at the cellular and subcellular level in intact organisms. In the long term, molecular imaging may provide a seamless translation for studies in cells to animals and, ultimately, to humans.

The ability to image fundamental biological processes, such as receptor occupancy, cell trafficking, and drug action, provides ample reason to employ molecular imaging strategies in studies of pulmonary disease. Molecular imaging capitalizes on recent advances in the techniques of molecular and cell biology, on new highly specific probes that serve as sources of imaging signal, and on significant improvements in imaging instrumentation specifically designed for small animal imaging. In this brief review, we highlight and illustrate a few of the general types of imaging studies that are being conducted (or could be) to investigate pulmonary disease. In-depth, general reviews of molecular imaging are available elsewhere (1–6).

GENERATING SIGNAL IN MOLECULAR IMAGING: APPRAISAL OF VARIOUS STRATEGIES

Overall, imaging reagents can comprise injectable and inhalable contrast agents, and radiopharmaceuticals, with or without activation strategies, or genetically encoded reporters. These reagents are all useful in biological studies, but injectable or inhalable agents have the potential to directly translate to the clinic, as has been the case for hyperpolarized helium-3 or xenon-129 (7). Except in the context of gene therapy, genetically encoded reporters are less likely to be used in humans, but possess a fundamental advantage in basic research in that, once validated, a genetically encoded reporter can theoretically be cloned into a variety of vectors and a broad array of regulatory pathways can be interrogated with the same validated reporter. In comparison to radiopharmaceuticals, genetically encoded reporters eliminate constraints inherent to traditional routes of synthesizing, labeling and validating a new and different radioligand for every new receptor or protein of interest. Genetically encoded imaging reporters also provide the potential for a stable source of signal, enabling longitudinal studies in living organisms with high temporal and, in some cases, high spatial resolution. This stands also in contrast to use of nanoparticles and magnetic resonance imaging (MRI) to study, for example, cell trafficking and stem cell proliferation over time *in vivo*. As the labeled cells divide and differentiate, cell-associated nanoparticles are diluted to the point of nondetectability within three to four cell cycles (8), limiting nanoparticle-based studies solely to the examination of early trafficking and targeting events.

Genetically encoded imaging reporters introduced into cells and transgenic animals can produce signal intrinsically (e.g., fluorescent proteins), through enzymatic activation of an inactive substrate (luciferases), by enzymatic modification of an active (e.g., radiolabeled) substrate with selective retention in reporter cells, or by direct binding or import of an active (e.g., radio-labeled, fluorophore) reporter substrate or probe (3). The most common reporters include firefly and click beetle luciferases (bioluminescence imaging), green and red fluorescence proteins (fluorescence imaging), herpes simplex virus-1 (HSV-1) thymidine kinase (positron emission tomography [PET]), sodium/iodide symporter (single photon emission computed tomography [SPECT]), and variants with enhanced spectral and kinetic properties optimized for use *in vivo*. When cloned into promoter/enhancer sequences or engineered into fusion proteins, imaging reporters enable fundamental processes such as transcriptional regulation, signal transduction cascades, protein–protein interactions, protein degradation, oncogenic transformation, cell trafficking, and targeted drug action to be temporally and spatially registered *in vivo*. Ideally, the magnitude and time course of reporter gene activity should parallel the strength and duration of expression of the endogenous target gene, which often requires more traditional experiments to validate the stringency of such reporters.

While image-enhancing or contrast agents provide molecular specificity to molecular imaging signals, these agents have inherent physicochemical differences producing their respective image contrast that in turn provide advantages or limitations for

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certain types of molecular imaging queries. In practice, choice of imaging modality and probe usually reduce to choosing between high spatial resolution and high sensitivity (Table 1 compares and contrasts several molecular imaging modalities). For example, PET and SPECT agents generally are synthesized at sufficiently high specific activity to enable use of tracer (sub-pharmacologic) concentrations of compound (picomolar) for detecting molecular signals and providing desired levels of image contrast. However, the physics of γ ray detection result in lower spatial resolution. Conversely, conventional MRI contrast agents may provide higher spatial resolution than PET or SPECT imaging. However, because of the indirect nature of enhancement produced by conventional MRI contrast agents (impacting water relaxivity), higher concentrations of material, on the order of 10 to 100 micromolar concentrations, generally are necessary to produce sufficient image contrast. These high levels of compound result in a stringent standard for MRI contrast agents in regard to risks of toxicity, cross-reactivity, and pharmacodynamic effects of the agents *in vivo*. High concentrations of contrast agents also may perturb the underlying molecular signal that is being monitored by MRI. This can be traversed by use of new hyperpolarized nuclei to generate signal, but these nuclei possess extremely short lifetimes at physiologic temperatures, which places severe constraints on the biochemical reactions and types of biology that can be studied by hyperpolarization contrast (7). Overall, when high sensitivity is desired, tracer technologies will provide

advantages for many molecular imaging applications. In contrast, when high spatial resolution is required, MRI may provide the necessary information. Similar tradeoffs extend to other contrast agents, optical imaging probes, and even X-ray contrast agents. However, fusion imaging strategies are rapidly becoming the norm. For example, the biochemical information contained in PET and SPECT images can be co-registered with the anatomic and spatial information contained in computed tomography (CT) images, which combines the strength of each. Because the goal of molecular imaging is to interrogate specific molecular signals, the underlying biomedical question, rather than the technology itself, will drive the choice of imaging agent and technique(s).

POSITRON EMISSION TOMOGRAPHY AND TRACER TECHNIQUES

PET is a quantitative, nuclear medicine imaging technique that can be used to study a variety of interesting and important problems in human and animal pathophysiology. Given its exquisite sensitivity and ability to locate events in three-dimensional space (9), it has great potential as a valuable tool for studying pulmonary pathology, biochemistry, inflammation, transgene expression, and cellular responses *in vivo* (10–13).

One of the most widely disseminated uses of PET imaging for a variety of diseases is measurement of glucose metabolism after the administration of the glucose analog F-18-fluorodeox-

TABLE 1. BRIEF COMPARISON OF VARIOUS MOLECULAR IMAGING MODALITIES USEFUL FOR PULMONARY-PATHOLOGY STUDIES *IN VIVO*

	Spatial Resolution	Temporal Resolution	Biochemical Sensitivity	Tomographic Potential	Biochemical S/N	Background/ Noise Sources in Pulmonary Imaging	Cost	Clinical Translatability
Fluorescence (microscopic)	μm	ms–min	++++*	Yes, but for < 1 mm depth	++++	Auto-fluorescence, Photobleaching, Movement artifacts	+++	Yes, but confined to superficial tissues
Fluorescence (macroscopic)	100 μm –mm	s–h	+++	Yes, to some extent	++	Auto-fluorescence, Photon scattering, Photobleaching	++	Future promise using NIRF probes
BLI	mm	s–h	++++	Possible	++++	Almost none, Photon scattering	++	Future potential
Nuclear (PET, SPECT, gamma scintigraphy)	mm–cm	min–h	++++	Yes	++	Metabolic uptake by liver, kidneys and thyroid; Excretion pathways via gut and urine, patient movement, tissue scattering, partial volume effect	++++	Yes
MRI	100 μm –cm	s–min	++	Yes	++	Patient/animal movement, Physiological motion, Air/tissue susceptibility artifacts, Blood flow	++++	Yes
X-ray CT	100 μm –cm	s–min	+	Yes	+	Patient/animal movement, Metallic objects	+++	Yes

Definition of abbreviations: BLI = bioluminescence imaging; CT = computed tomography; MRI = magnetic resonance imaging; NIRF = near-infrared fluorescent; PET = positron emission tomography; S/N = signal-to-noise ratio; SPECT = single photon emission computed tomography; US = ultrasound.

Biochemical sensitivity (the ability to detect minute quantities of target biomolecules *in vivo*) and biochemical signal-to-noise (strength of the target signal over background) are estimated in the context of a given instrument used with an appropriate contrast agent or imagable probe.

* Plus signs indicate the level of relevance of the attribute to the imaging technique, where + = low relevance and ++++ = high relevance.

yglucose ($[^{18}\text{F}]\text{FDG}$). $[^{18}\text{F}]\text{FDG}$ is transported into cells by the same family of GLUT transporter proteins as glucose (GLUT-1, GLUT-4). In the presence of hexokinase, cytosolic $[^{18}\text{F}]\text{FDG}$ is phosphorylated to $[^{18}\text{F}]\text{FDG-6-monophosphate}$ ($[^{18}\text{F}]\text{FDG-6-P}$). However, unlike glucose-6-phosphate, $[^{18}\text{F}]\text{FDG-6-P}$ cannot be metabolized further (14, 15). Therefore, in tissues that lack dephosphorylases (most tissues other than the liver), $[^{18}\text{F}]\text{FDG}$ is trapped intracellularly. A PET imaging signal can then be generated as $[^{18}\text{F}]\text{FDG}$ accumulates in the tissue over time. Quantification of actual glucose metabolic rates from data acquired via $[^{18}\text{F}]\text{FDG}$ -PET imaging sometimes requires use of a correction factor, which accounts for differences in the rate of transport and phosphorylation of $[^{18}\text{F}]\text{FDG}$ when compared with glucose itself (14). Corrections are important in tissues such as the myocardium that can switch between oxidative and nonoxidative metabolic pathways to meet energy needs, but this phenomenon is not known to occur in cells associated with inflammation such as neutrophils.

By and large, $[^{18}\text{F}]\text{FDG}$ is used for studying a variety of pulmonary-associated diseases, including extensive use in imaging respiratory inflammatory disease, lung cancer, and characterization of single pulmonary nodules. Uses of $[^{18}\text{F}]\text{FDG}$ in such studies are too numerous to include in this review, and thus, the reader is referred to several reviews of $[^{18}\text{F}]\text{FDG}$ imaging in lung cancer and assessment of single pulmonary nodules (16–18). As an example of how PET imaging might be employed to study inflammatory responses in respiratory disease, Schuster and colleagues reported the use of $[^{18}\text{F}]\text{FDG}$ to detect neutrophil activation in a mouse model of pneumonia (11). The use of FDG for this purpose is based on the dependence of neutrophils to use glucose to meet energy needs associated with cellular functions such as chemotaxis, phagocytosis, and microbial killing (19). Thus, neutrophil activation is associated with increased glucose uptake. In acute pneumonia, in both experimental animals and human patients, the rate of $[^{18}\text{F}]\text{FDG}$ uptake in the lungs increases 10 to 40 times above values obtained in normal controls (20). Evidence to date suggests that in states of acute inflammation (e.g., pneumonia), neutrophils are the primary cell type responsible for increases in $[^{18}\text{F}]\text{FDG}$ tissue uptake (20–23).

In the above mouse model of pneumonia (11), FDG-PET imaging was employed after airway instillation of *Pseudomonas aeruginosa*-laden agarose beads (24, 25). Two types of *Pseudo-*

monas strains were used: PA01 and M57-15. The M57-15 strain had been isolated previously from a patient with cystic fibrosis, while the PA01 strain is often used as a model organism because its complete genomic sequence has been reported. Lung uptake of $[^{18}\text{F}]\text{FDG}$ correlated significantly with the dose of bacteria in mice infected with the M57-15 strain of *Pseudomonas* ($R^2 = 0.62$), but not in mice infected with the PA01 strain. The overall lung uptake of $[^{18}\text{F}]\text{FDG}$ was higher in mice infected with the M57-15 strain than in those infected with the PA01 strain ($P < 0.05$). Total white blood cell concentrations in bronchoalveolar lavage fluid were also higher in the M57-15 infected mice. Interestingly, the PA01 strain has been characterized as minimally cytotoxic (26). These results suggest that $[^{18}\text{F}]\text{FDG}$ -PET imaging might be able to identify differences in innate immune response to different types of *Pseudomonas*, or more generally, bacterial infection.

Another recent, and particularly exciting form of molecular imaging seeks to follow the expression of specific genes via the non-invasive visualization of *in vivo* reporters (2–4, 6, 27). In one example, PET imaging of adenoviral-mediated transgene expression was demonstrated in rodent lungs using adenovirus particles (Ad) to deliver Herpes Simplex Virus type-1 thymidine kinase (HSV1-TK) as the PET reporter gene (Figure 1). Herein, HSV1-TK, unlike mammalian thymidine kinases, can efficiently phosphorylate administered nucleoside analogs (e.g., ganciclovir, penciclovir), as well as various radioactive derivatives such as (9-(4- $[^{18}\text{F}$]-fluoro-3-hydroxymethylbutyl)guanine ($[^{18}\text{F}]\text{FHBG}$)), which are then trapped and accumulate in cells expressing the viral kinase (28). This established proof-of-principle that an imageable transgene that also served as a therapeutic vector could be imaged by PET, thereby demonstrating that in the future PET could monitor the delivery, expression pattern, and duration of pulmonary gene therapy. This might apply to cystic fibrosis, for example. Several mutant HSV1-TK enzymes, such as sr39TK (29–31), have been developed to further improve the sensitivity of PET imaging to detect and quantify the cellular accumulation of these radioactive tracers.

Beyond PET imaging, other radionuclide-based methods currently being evaluated as platforms for imaging gene expression *in vivo* include planar γ scintigraphy and SPECT (32–34). SPECT imaging is generally a less sensitive and lower spatial resolution technique compared with clinical PET imag-

Figure 1. Positron emission tomography (PET) imaging of adenoviral infection of the lungs. Transaxial microPET images (upper row), light micrographs (middle row), and corresponding fluorescence micrographs (bottom row) obtained in one rat infected with AdCMV-null (left panel) and three rats respectively infected with 1×10^{10} , 5×10^{10} , and 1×10^{11} particles of AdCMV-mNLS-sr39tk-egfp (panels 2–4 from left to right). The PET images are transverse slices obtained at the mid-chest level. Light and fluorescent micrographs ($\times 10$ magnification) represent identical lung areas in the left lung from adjacent sections. Exposure time for each fluorescent micrograph was the same (960 ms). Adapted by permission from Reference 12.

ing. For PET, a higher percentage of annihilation events are able to be recorded. However, advances in collimator design of SPECT instruments (e.g., multi-pinhole collimators) have enhanced the resolution of SPECT to the millimeter level (35). These advances, coupled with many longer-lived SPECT isotopes, enable facile tracer delivery to imaging facilities (as opposed to the requirements for a cyclotron and devoted radiochemistry lab for several common PET isotopes) rendering SPECT imaging a viable choice for those engaged in pulmonary research. As an example of imaging endogenous gene expression in the lung, Duan and colleagues recently assessed whether there was a correlation between ^{99m}Tc -MIBI scintigraphy and expression levels of P-glycoprotein (assessed using immunohistochemistry of tumor isolates) in patients with primary lung cancer (37). ^{99m}Tc -MIBI is known to be outwardly transported by P-glycoprotein, the protein product of the multidrug resistance (*MDR1*) gene (36). The authors reported a significant positive correlation between ^{99m}Tc -MIBI tumor washout rate and P-glycoprotein expression, demonstrating the utility of SPECT imaging with ^{99m}Tc -MIBI to assess multidrug resistance in lung cancer (37).

OPTICAL IMAGING

Whole body imaging of light generated deep within the body from biochemical reactions and biological processes are now feasible in laboratory animals and potentially in humans, representing an interesting new approach to molecular imaging. Optical properties of tissues limit the ability to detect photons from weak light sources within the body. Fundamentally, constraints for optical imaging arise from the low levels of light emitted by internal sources and the high absorption and scatter of light traversing the body (38). To enable optical imaging *in vivo*, new sensitive instruments and imaging probes have recently been developed. Three general strategies have evolved for optical molecular imaging *in vivo*: use of endogenous fluorochromes; use of reporter genes that generate internal light from specific biochemical reactions (bioluminescence and fluorescent proteins); and use of injected optical contrast agents that incorporate visible light fluorophores, near-infrared fluorophores, or activatable fluorophores (3, 27, 38, 39). Optical imaging is relatively low in cost, highly versatile, and enables multichannel imaging through use of multiple probes with differing spectral characteristics.

Bioluminescence imaging (BLI) of luciferase reporters provides a relatively simple, robust, cost-effective, and extremely sensitive means to image fundamental biological processes *in vivo* due to exceptionally high signal-to-noise levels. Nevertheless, bioluminescence remains dependent on substrate pharmacokinetics, except in the case of bacterial *lux* operons, and in general, is usually represented as planar imaging datasets, therefore imposing some positional uncertainty of the attained signal. There are many luciferases with matching substrates available. However, most are blue/green and therefore are less suitable for deep tissue imaging. The luciferases that have been found to be most useful for molecular imaging are firefly (*Photinus pyralis*) luciferase, *Renilla* luciferase, green or red click beetle (*Pyrophorus plagiophthalmus*) luciferases, and *Gaussia* luciferase (40–42). However, both *Renilla* and *Gaussia* luciferases emit blue light, which is highly attenuated in living tissue, and possess high bursting activity, therefore requiring care and precision in timing the readout. New mutants of *Renilla* luciferase were recently reported, and while favorably shifted approximately 66 nm, are still rather green for optimal use *in vivo* (43). Moreover, the *Renilla* and *Gaussia* luciferase substrate, coelenterazine, has been shown to be transported by

the multidrug resistance transporter P-glycoprotein (44) as well as to interact efficiently with superoxide anion and peroxynitrate in light-producing reactions (45), thereby complicating certain applications of *Renilla* and *Gaussia* luciferases *in vivo*. Nonetheless, the favorable attributes of luciferin-based imaging provide a versatile platform for studying biology *in vivo*.

Bioluminescence is relatively simple to execute relative to PET or similar radionuclide-based imaging technologies because the substrates (D-luciferin for firefly luciferase and coelenterazine for *Renilla* luciferase) are commercially available and readily prepared for injection into whole animals. In addition, short image acquisition times (seconds to minutes), capacity for repetitive imaging after short time intervals, a platform for simultaneous acquisition of multiple animals at the same time, and a stable substrate, renders high-throughput imaging and screening feasible. However, the sensitivity of detecting these internal light sources is dependent upon many parameters, including the level of luciferase expression, the depth of labeled cells (host or pathogen) within the body (i.e., the distance that the photons must travel through tissue), and the sensitivity of the detection system. A key disadvantage of cooled solid-state cameras and bioluminescence imaging is the limited and wavelength-dependent transmission of light through animal tissues. As a rule of thumb, there is an approximate 10-fold loss of photon intensity for each centimeter of tissue depth (46). Nonetheless, as few as 100 transduced cells can be detected after injection into the peritoneal cavities of severe combined immunodeficient (SCID) mice (47). Also, images are surface-weighted, meaning that light sources closer to the surface of the animal appear brighter compared with deeper sources, due to tissue attenuation properties (48). Another minor disadvantage is the current limitation of planar display instead of the tomographic or three-dimensional displays typically seen with PET or MRI. Thus, while bioluminescent images typically lack depth information, recent technological advances with rotating mirrors and models of low-energy photon propagation through tissue enable tomographic bioluminescence imaging. As yet, clinical optical imaging of lung pathology remains theoretical, and thus, herein we focus on approaches that have enabled whole animal pulmonary imaging through the use of bioluminescence and fluorescent proteins and other agents. Indeed, luciferase reporters have been used in a variety of animal imaging applications (3). Bioluminescence enables monitoring throughout the course of disease, allowing localization and serial quantitation before killing the experimental animal (47, 49). Bioluminescence imaging can be used for real-time studies of cell trafficking (50), of various genetic regulatory elements in transgenic mice (51), and of *in vivo* gene transfer (40, 52, 53). For instance, Mizuno and coworkers have investigated the use of a dry powder inhalant, consisting of a chitosan–DNA mixture that had been passed through supercritical CO₂, as a potential gene delivery vector. The authors were able to observe in the lungs of mice significant levels of gene expression from a plasmid bearing a cytomegalovirus promoter driving luciferase that had been delivered using this strategy (54).

Also, bioluminescence methods have been used to study various signal transduction pathways *in vivo* (55, 56), some applicable to lung pathology. For example, in non-small cell lung cancer tumor xenografts (HCC827 and NCI-H1975), using a previously described split-luciferase reporter system (57) incorporated into a single-chain biosensor consisting of N- and C-terminal luciferase fragments flanking an FHA2 domain linked to a peptide sequence recognized by Akt (Aktpep), Zhang and coworkers have monitored *in vivo* activation of the serine/threonine kinase Akt (58). Differential sensitivity of the

two tumor lines to erlotinib, an anticancer drug targeting EGFR, was observed by bioluminescence imaging that correlated well with Western blotting analysis.

Bioluminescence also has been used to study patterns of bacterial and viral infections and their treatment using genetically engineered bioluminescent pathogens and whole body imaging (59–62). Using this approach, the spatiotemporal patterns of viral infection within the body can be elucidated. Tissue tropism and therapeutic response can be studied with these types of molecular imaging models (61). Using such a strategy, a report by Francis and colleagues described incorporation of the *LuxABCDE* operon into the *Streptococcus pneumoniae* genome and visualization of pneumococcal infections in the lungs of live mice using bioluminescence imaging. These studies have greatly enhanced the animal models being used to study gram-positive bacterial infection of the lungs (63).

More recently, use of transgenic animal models of human diseases promises to extend our understanding of the mechanisms of pathogenesis by placing target genes and processes within the appropriate physiologic milieu. Consequently, recent advances in reporter gene design allow integration of “imageable” reporters directly into transgenic mouse models of human physiology and diseases (3). For example, Pichler and colleagues generated a transgenic *Gal4-FLuc* universal reporter mouse strain that expresses the firefly luciferase gene (*Fluc*) under the regulatory control of the *Gal4* promoter ($Tg^{G4F(+/-)}$) (Figures 2A and 2B), and tested its usefulness by characterizing transactivation of the reporter in multiple tissues, including respiratory epithelia and the lungs (64). For this purpose, mice were administered an adenovirus (Ad-Gal4) engineered from a chimeric transcription factor comprising the DNA-binding domain from the yeast transcription factor Gal4 (Gal4BD) and the activation domain from the VP16 protein (VP16) of HSV-1 (65, 66). To control for nonspecific effects, $Tg^{G4F(+/-)}$ mice were also treated with an off-target virus (Ad-Cre) (64). Nasal delivery of Ad-Gal4 resulted in a steady signal increase when compared with the pre-treatment image on Day 0 (Figure 2C). Imaging signals were highest after 3 days, showing a 50-fold

increase over background (Figure 2D), but were only detectable in nasal passages in living animals. To determine whether signal was also induced in the lungs, several mice were killed on Day 3 and imaged through an open chest cavity. The reporter transgene was clearly activated in the lungs of Ad-Gal4-treated mice, while no signal was detected in the control Ad-Cre-treated animal (Figure 2E). Beyond studies of signaling cascades in cancer, the future of this technique will likely find use in assessments of viral progression and preclinical experimental therapeutics.

During fluorescence imaging, in contrast to bioluminescence imaging, an excitation light illuminates the subject and a solid-state camera detects the emitted fluorescence. Cells can be labeled with fluorescently labeled antibodies or express green fluorescence protein (GFP) or color-shifted/enhanced variants (yellow, cyan, red, eGFP, etc.). Like bioluminescence, the procedure generally involves transduction of the *gfp* gene into cells or tissues. Exciting the protein with appropriate blue light and detecting the ensuing fluorescence enables whole body imaging of GFP expression in living animals (67). Nonetheless, absorption and fluorescence of GFP in the visible region, coupled with increased light scattering, may hinder the use of GFP for high-resolution imaging of deep tissues *in vivo* (68). Recent advances in fluorescent proteins, however, include the engineering or discovery of variants with enhanced brightness, improved pH resistance, the ability to undergo photochemical color conversion, and red fluorescent emissions that have led to new applications in animal models (69).

Advantages of fluorescence imaging include the capacity for both living and fixed cells and tissues to be visualized, the lack of a substrate to generate the fluorescence phenomena and low cost of the animal imaging instruments (69). However, as with bioluminescence, the images are surface-weighted. Another disadvantage of fluorescence imaging is the higher background signal levels due to autofluorescence of tissues, especially in the blue and ultraviolet wavelengths compared with bioluminescence. Nonetheless, a variety of microbes have been transformed with GFP variants and have shown signifi-

Figure 2. Generation and analysis of a genomic imaging transgene. (A) Schematic representation of the reporter transgene vector that was injected into pronuclei of FVB oocytes. (B) PCR genotyping from a negative mouse (WT), a positive littermate ($Tg^{G4F(+/-)}$), and pGL3 as a positive control are shown. (C) $Tg^{G4F(+/-)}$ and WT mice inhaled 2×10^6 plaque-forming units/mouse of the adenovirus Ad-Gal4. Mice were imaged before treatment with virus (Day 0) and then followed over time, starting with 24 hours after inhalation treatment. Images from a representative $Tg^{G4F(+/-)}$ mouse are shown. (D) Signal progression of the mice was followed for 9 days until the signal declined back to almost pretreatment levels and is shown as total photon flux (photons/s) plotted over time. Data are presented as mean $\pm$ SEM ($n = 3$ each); error bars for WT are smaller than symbol size. (E) $Tg^{G4F(+/-)}$ mice administered the adenoviruses Ad-Gal4 or off-target Ad-Cre (as indicated) were imaged through an open chest cavity on Day 3. Signal was detectable from the nasal passages and lung of only the Ad-Gal4-treated mouse. Adapted by permission from Reference 64.

cant promise for the study of pathogenesis (70). Thus, the latest generation of GFP reporters allows the investigation of gene expression in individual bacterial cells within specific environments (70–74).

As noted earlier, direct optical imaging also can be performed after administration of exogenous optical contrast agents synthesized with fluorophores having emission characteristics in the visible and near-infrared (NIR) spectrum (700–900 nm). There is significant interest in applications in the near-infrared spectrum due to the high tissue penetration of light in this range as well as minimized autofluorescence. Many fluorophores are available for coupling to biologically targeted molecules such as antibodies and peptides (75, 76). In general, fluorescein, indocyanine green (ICG), and porphyrin derivatives are used as optical contrast agents for *in vivo* imaging, localizing to pathological tissues by a variety of targeting mechanisms (77, 78). For example, Bugaj and coworkers demonstrated the efficacy of a carbocyanine-somatostatin receptor-avid peptide conjugate for imaging receptor-mediated uptake in a rat pancreatic tumor model. They were able to demonstrate that the peptide conjugate was selectively retained in those tissues that overexpressed the somatostatin receptor type-2 (78).

Another approach with exogenous agents involves use of activated optical probes or optical beacons (79, 80). For nanoparticle strategies, graft copolymers (methoxy-polyethylene-glycol-derivatized poly-L-lysine) are appended with cleavable peptides conjugated between the polymer and near-infrared fluorescent (NIRF) fluorophores (such as tricarbo-cyanine and indocyanine green dyes). The spectral properties of these dyes produce self-quenching when the fluorophores are co-assembled on the polymer. However, upon proteolysis of the intervening target peptide sequence, fluorophores are released into the surrounding environment, enabling the generation of fluorescence signals. This approach has been used, for example, by Weissleder (39) and by Bremer and colleagues (79) to study tumor matrix metalloproteinase and cathepsin activities *in vivo*. These methods should be applicable in the future to the study of host and/or pathogen proteases activated during the course of pulmonary disease *in vivo*.

MAGNETIC RESONANCE IMAGING

MRI is another powerful and versatile imaging modality for noninvasive characterization of host structure and function. To date, most MRI experiments involve measuring the protons of water, which are ubiquitous and present in high concentrations in animal tissues and organs.

Imaging pathologic tissues in small animals presents some unique challenges for MRI, including respiratory and cardiac motion (81), leading to significant image blurring in the thorax and abdomen. Imaging of the lung, a common target for pathogens, suffers from two other factors: (1) relatively low tissue density (and therefore low water content), limiting signal-to-noise; and (2) variations in magnetic “susceptibility,” associated with the many air-tissue interfaces of the alveoli and bronchioles, creating local magnetic field heterogeneities (field gradients) that can lead to severe image degradation (13). In response to these challenges, researchers generally use very fast imaging sequences with short imaging times combined with respiratory synchronization (82). MRI has been used to assess edema and inflammation in the lungs after allergen or endotoxin challenges (83, 84).

Use of MR contrast agents to selectively label cells and change local T1 and T2 relaxation processes has enabled transgene expression (85) and cell migration and trafficking to be monitored *in vivo* by MRI (39). Most agents successfully used *in vivo* are

derived from iron oxides and shorten T2 relaxation processes (86). For example, *ex vivo* loading of T cells with a superparamagnetic iron oxide nanoparticle enabled MR imaging of T cells homing to the spleen (86). A related approach with labeled oligodendrocyte progenitor cells allowed migration of transplanted cells in the spinal cord to be followed noninvasively (87). Another strategy with magnetodendrimeric magnetic tags allowed *ex vivo* labeled human neural and mesenchymal stem cells to be followed *in vivo* for as long as 6 weeks after transplantation (88). Improvements with highly derivatized cross-linked iron oxide nanoparticles enabled trafficking of adoptively transferred CD8+ cytotoxic T lymphocytes to be monitored repetitively and semi-quantitatively within tumors in live mice (89). These approaches should be equally applicable to a variety of host–pathogen interactions and pulmonary responses to microbial infection in the future.

Reporter gene imaging with magnetic resonance (MR) also is possible (85), but this approach may not be suitable for pathogen studies since the receptor density on microorganisms may be too low relative to host tissues to generate a specific MR image. Nonetheless, all the above imaging approaches are relatively new, incompletely developed, and the advantages of one over another are still theoretical. Thus, further systematic studies of each are required to evaluate their relative sensitivity and accuracy.

MRI also can be used to measure morphologic properties, such as airway caliber after chronic infection with pathogens and functional assessments of ventilation and perfusion. MRI has the advantage of being able to generate images without the use of potentially damaging ionizing radiation. This may be especially important for small animal imaging studies conducted over multiple imaging sessions, as the radiation dose of X-ray CT in small animals can be considerable (90). On the other hand, there may be many instances in which the unique advantages of high spatial resolution of MRI can be combined with the biochemical information of PET, especially with new display software that allows anatomic images to be overlaid onto molecular images obtained with other techniques.

CONCLUSIONS

Overall, these innovative strategies offer a means to accurately analyze the dynamic nature of pulmonary physiology and disease, as well as a means to detect intracellular signaling and host–pathogen interactions, all within the complex environment of the intact animal. Integration of smart probes and reporters with animal models of respiratory disorders should enable investigators to address complex paradigms on microscopic and macroscopic scales and in four dimensions.

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