Visualization of pulmonary inflammation using noninvasive fluorescence molecular imaging

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Haller J, Hyde D, Deliolanis N, de Kleine R, Niedre M, Ntziachristos V. Visualization of pulmonary inflammation using noninvasive fluorescence molecular imaging. J Appl Physiol 104: 795–802, 2008; doi:10.1152/japplphysiol.00959.2007.—The ability to visualize molecular processes and cellular regulators of complex pulmonary diseases such as asthma, chronic obstructive pulmonary disease (COPD), or adult respiratory distress syndrome (ARDS), would aid in the diagnosis, differentiation, therapy assessment and in small animal-based drug-discovery processes. Herein we report the application of normalized transillumination and fluorescence molecular tomography (FMT) for the noninvasive quantitative imaging of the mouse lung in vivo. We demonstrate the ability to visualize and quantitate pulmonary response in a murine model of LPS-induced airway inflammation. Twenty-four hours prior to imaging, BALB/c female mice were injected via tail vein with 2 nmol of a cathepsin-sensitive activatable fluorescent probe (excitation: 750 nm; emission: 780 nm) and 2 nmol of accompanying intravascular agent (excitation: 674 nm; emission: 694 nm). Six hours later, the mice were anesthetized with isoflurane and administered intranasal LPS in sterile 0.9% saline in 25 μl aliquots (one per nostril). Fluorescence molecular imaging revealed the in vivo profile of cysteine protease activation and vascular distribution within the lung typifying the inflammatory response to LPS insult. Results were correlated with standard in vitro laboratory tests (Western blot, bronchoalveolar lavage or BAL analysis, immunohistochemistry) and revealed good correlation with the underlying activity. We demonstrated the capacity of fluorescence tomography to noninvasively and longitudinally characterize physiological, cellular, and subcellular processes associated with inflammatory disease burden in the lung. The data presented herein serve to further evince fluorescence molecular imaging as a technology highly appropriate for the biomedical laboratory.

in vivo small animal imaging; molecular tomography; cysteine proteases

THE RESPONSE TO PULMONARY inflammation as it occurs in healthy lungs is a highly coordinated and efficient mechanism of resolving infection. Poor regulation of the response can lead to a number of acute as well as chronic lung diseases, in particular ARDS, COPD, and asthma (16, 27). Our knowledge of the cascade of incapacitating events contributing to the whole of pulmonary inflammation has increased immensely during the last decade and it has become increasingly important to characterize cellular and subcellular mechanisms in situ in unperturbed environments. In vivo imaging of the inflammatory process as it occurs would permit mechanistic interroga-

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lungs, require several animals to build meaningful statistics and, importantly, do not allow following up of dynamic events and responses.

Fluorescence molecular tomography (FMT) is a technique developed to three-dimensionally visualize deeper into the animal, and it is herein considered as a noninvasive molecular lung imaging tool for small animal imaging. The technology uses multi-projection illumination of the animal thorax and combines multi-projection light measurements from the thorax with mathematical models of photon propagation in tissue in a reconstruction scheme that produces quantitative three-dimensional images of fluorochrome biodistribution in the lung. In this work we investigate noninvasive fluorescence macroscopic imaging as a tool for in vivo visualization of molecular biomarkers. By employing measurements in two separate wavelength bands we concurrently resolve activated cysteine pro tease in the lungs of a murine LPS model of acute inflammation and vascular changes in response to inflammation, the latter also serving as an internal control independently assessing molecular probe bio-distribution. In this capacity, fluorescence imaging renders itself herein as an adept laboratory approach for interrogation of lung disease at the physiological and molecular levels.

MATERIALS AND METHODS

Animal handling and image acquisition procedures. Animal studies were performed according to protocol approved by the Institutional Animal Care and Use Committee Review Board and Massachusetts General Hospital Center for Comparative Medicine. Female BALB/c mice (6–8 wk old, 20–25 g, n = 24) were obtained from Jackson Laboratories (Bar Harbor, ME) and had free access to food and water during experiments. At 18 h prior to imaging, mice were anesthetized with isoflurane and maintained in a supine position on a platform at an ∼45° angle. Upon sedation, mice (n = 16) received intranasal instillation of LPS (Escherichia coli, serotype O55:B5; Sigma, St. Louis, MO) in sterile 0.9% saline in one 25 μl aliquot per nostril, remaining anesthetized and upright for 5–10 additional minutes; four control animals were not challenged with intranasal instillation.

Imaging protocol. Twenty-four hours prior to imaging, while under isoflurane narcosis, the mice were shaved and injected intravenously with 2 nmol of an intravital cysteine protease probe (Prosense 750, VisEn Medical, Woburn, MA), a cathepsin activatable probe (excitation: 750 nm, emission: 780 nm) that remains optically silent until protease mediated activation produces fluorescence. Also 2 nmol intravascular agent [(AngioSense 670, VisEn Medical) excitation: 680 nm, emission: 720 nm], was injected at this time. Additionally, per intravascular agent (AngioSense 680, VisEn Medical) excitation: 680 nm, emission: 720 nm) was injected at this time. Additionally, per manufacturer’s report, the half-life of ProSense is 24 h in the blood and 96 h in the tissue, whereas that of AngioSense is 5 h in the blood. In vivo fluorescence imaging was performed with mice under ketamine-xylazine anesthesia. The imaging system used has been described in more detail (10, 24, 47). Briefly, fluorescence imaging was performed with the use of a modular home-built scanner capable of acquiring fluorescence and optical attenuation data in transillumination and epi-illumination modes in vivo. The unit used herein is an evolved version as described in the following. Excitation laser diodes at 672 nm and 750 nm, tailored to the imaging of Cy5.5 and Alexa Fluor 750, respectively, were used. The excitation system consisting of a set of two galvanometer controlled mirrors that scanned the laser beam in x-y direction to create a rectangular 6×9 source pattern illuminating the back of the animal. A slab geometry imaging chamber was used. For each of the 54 excitation point sources, two transillumination images were acquired, using two different band pass filters (Andover, Salem, NH) tuned at the excitation and emission peak of the fluorescent probes (AngioSense670-Cy5.5channel, excitation: 670 ± 5 nm, emission: 710 ± 10 nm and ProSense750-AF750 channel, excitation: 750 ± 5 nm, emission: 800 ± 20 nm). The images were formed with a 50 mm f/1.2 lens (Nikon, Japan) onto a 512×512 element, ultralow-noise, cooled charge-coupled device (CCD) camera (Vers-Array; Roper Scientific, Trenton, NJ). The exposure time was 0.1 s for the excitation channel and 1–3 s for the fluorescent channels, yielding a total acquisition time of 3 min/mouse. Additionally, reflectance (epi-illumination) images were acquired for both excitation and emission channels. The mice were partially immersed in the chamber filled with an Intralipid-ink solution (0.1% Intralipid and 250 ppm of india ink) with optical properties that yield similar attenuation of the mouse boundary. This matching fluid was used to simplify theoretical constraints associated with boundary modeling in a scattering medium. In particular, this matching medium sufficiently attenuates stray light reflecting off the mouse boundaries and the scanner walls and limits minimally attenuated light from the side boundaries of the animal from hitting the CCD camera. In addition, the fluid ensures that the dynamic range of the measurements is better interfaced to the dynamic-range of the CCD camera used for detection. To spectrally separate the signals, three-cavity 40-nm-wide band-pass interference filters were employed (Andover, Salem, NH) with center frequency 710 nm and 790 nm, respectively, for the Cy5.5 and AF750 fluorochromes.

Image processing. For each imaging study, the optical measurements acquired in vivo were processed to yield normalized transillumination and tomographic reconstructions (23). Normalized transillumination images were generated by dividing each fluorescence measurement with the corresponding optical attenuation image for each of the 54 illumination points employed and then adding all the ratio images. Prior to obtaining the ratio image, a threshold was applied set to 5% of the maximum to reject background noise. Correspondingly the threshold value was subtracted from each image (25). These normalized transillumination data were then tomographically processed using the normalized Born forward model (25). Inversion was based on a spatially regularized approach based on a Tikhonov regularization scheme and a conjugate gradient method. That is, inversion was executed using an approximate maximum likelihood solution based on the statistics of the Born ratio (14). A Tikhonov regularization term using the identity matrix stabilized the solution and provided robustness to noise. Regularization parameters were selected using L-Curve analysis (11). Each inversion used 50 iterations of the algorithm LSQR and required ~100 s on a 1.8 GHz Intel Core Duo with 1 GB of RAM.

Bronchoalveolar lavage. Directly following imaging, the animals were killed and BAL was performed. Simply, the lungs were excised along with attached trachea. Polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ) was inserted down through the tracheal opening prior to bifurcation into bronchi and tied off with 3–0 ethilon silk suture (Ethicon, Cincinnati, OH). The lungs were reinfated via two separate washes of 250 and 150 μl of phosphate buffered saline using a 1/2-ml syringe inserted into the tubing. Washes were collected for total white blood cell count, cytospin fixation, and staining for further (Diff-Quick stain kit, IMEB San Marcos, CA) analysis of cellular profile.

Ex vivo imaging and lung histology. On completion of the BAL procedures, the excised tissues were imaged ex vivo in epi-illumination mode to confirm the origin of the signal from the lungs. The images were acquired in a home-built reflectance imaging system. Two continuous wave laser diode sources (B&W Tek., Newark, DE) emitting at 670 and 750 nm were alternatively used to excite Angioscience680 (Cy5.5) and Proscence750 (Alexa750) probes, respectively. The laser source was coupled to a fiber bundle illuminator system to create a p-polarized slightly diverging illuminating field with an intensity of ~50 μW/cm². The objects/tissues were placed on a transparent plate against a black absorbing surface at the center of the illuminating field and the reflectance images were formed with...
over the more conventional epi-illumination modes to volu-
metrically sample through the entire lung, whereas normaliza-
tion is required to account for the heterogeneous attenuation of
light, as it propagates through different tissues, i.e., heart, other
muscles, lung, etc., to produce images of true fluorescence
bio-distribution.

The methodological steps that lead to lung imaging are
shown in Fig. 1, obtained from an LPS-challenged mouse,
visualized at the ProSense channel. Each imaging session
placed the mouse between the predefined source and detector
to be placed such that photon signals then collected had prop-
gated through and sampled the entire volume of the tho-
racic cavity. A photograph of the mouse obtained after
placement is shown in Fig. 1A. Trans-illumination measure-
ments were obtained at the excitation (Fig. 1B) and emission
wavelengths (Fig. 1C). The image collected at the excitation
wavelength represents an attenuation image and shows areas
of high absorption (dark) congruent with the presence of
liver and heart and areas of low absorption (light) through
lung and adipose tissue at the animal sides. Correspond-
ingly, the fluorescence transillumination image (Fig. 1C)
in this case does not reflect true fluorescence bio-distribution;
instead it looks very similar to the attenuation image of (Fig.
1B) and reflects fluorescence signals heterogeneously atten-
uated by tissue structures. In contrast, the normalized tran-
illumination image (Fig. 1D) better reflects overall fluores-
cence bio-distribution, showing an overall fluorescence ac-
tivity through regions of LPS passage, i.e., throughout the
tracheal route and the lung. Some liver activity is also
shown, since activatable probes are known to biodegrade
and become activated (fluoresce) by liver enzymes. Figure
1E shows a superposition of Fig. 1D onto Fig. 1A. Images
obtained in epi-illumination mode (not shown here, but see
also Fig. 2) did not reveal any activity from the lung and

zoom (20–90 mm) macro lens (Optem-Qioptiq Imaging Solutions,
Fairport, NY) in a 1.376×1.040 pixel CCD camera cooled to −70°C
(Cooke, Romulus, MI). The images were captured with the use of a
crossed polarizer to eliminate the reflections from the surface and with
three-cavity 10 nm band-pass filters (Andover Salem, NH) centered at
710 nm and 800 nm for the Cy5.5 and Alexa750 fluorescent dyes,
respectively. Additionally, the corresponding images of the tissue at the
excitation wavelength were acquired. The fluorescent images were
divided with an image recorded at the excitation wavelength to
compensate for the uneven illumination and spatial variation of tissue
absorption for enhanced image quality (25).

Histological preparation followed. Briefly, the excised tissue was
washed in PBS and fixed in 4% paraformaldehyde overnight. Subse-
quent PBS washes were performed the following day preceding
paraffin embedding, sectioning (5 μm), and hematoxylin and eosin
staining.

WESTERN BLOT. For immunoblotting, lungs were excised and homog-
enized in lysis buffer. Following total protein determination (Pierce
BCA protein assay, Pierce Biotechnology, Rockford, IL), 5 μg of total
protein lysate was subjected to electrophoresis on 12.5% SDS-PAGE
followed by transfer to methanol equilibrated PVDF membrane (Bio-Rad
Laboratories, Hercules, CA) according to the manufacturer’s instruc-
tions. Concentrations for blotting anti-cathepsin B as well as HRP-
labeled secondary antibody followed manufacturer’s recommenda-
tions (R & D Systems, Minneapolis, MN). The blots were developed
with the enhanced chemiluminescence system (Amersham Pharmacia
Biosciences, Piscataway, NJ). Immunoblotting of GAPDH (R & D
Systems) was used as a loading control.

RESULTS

In vivo fluorescence imaging. The methodology developed
for lung imaging combines measurements in the transillumi-
nation geometry with appropriate signal normalization pro-
cesses. This approach has been found essential herein to
accurately visualize the lung. Transillumination is required
over the more conventional epi-illumination modes to volu-

![Image](https://via.placeholder.com/150)

Fig. 1. Implementation of epi-fluorescence and tomo-
graphic imaging modalities: a systematic representation
of image collection for each animal included in the
study is displayed in the panels from left to right, shown
herein for the 750–780 nm spectral channel: epi-illumi-
nation (surface-reflectance) image taken at the excita-
tion wavelength, 750 nm (A), in vivo transillumination
images collected through the thoracic cavity at the excita-
tion wavelength, 750 nm (B) and the emission wave-
lengths, 780–820 nm (C), and the normalized transillu-
mination in vivo fluorescence image (D). A composite
image displaying the normalized image as an overlay
upon the epi-illumination image may be noted in E. The
fluorescent molecular tomographic (FMT) reconstruction
of the same animal in F provides a most accurate
and quantitative, 3-dimensional, volumetric indication
of the fluorochrome activity within the lungs, shown
here for a slice taken approximately from the middle of
the animal (6 mm depth).
they were surface weighted, i.e., they reflected fluorescence activity of the skin only (25).

Individual normalized transillumination data from each source were further combined into an FMT inversion scheme to provide tomographic reconstructions. By separating data from different laser point sources, each implementing different projections through tissue (47), a more accurate selection of lung signals is achieved. Figure 1F demonstrates a tomographic FMT slice, obtained from the lung volume, resolving the lung and upper tracheal fluorescence activity with improved resolution over the corresponding transillumination images.

Moreover, the protease-activatable probe may be noted here as evident in both the normalized transillumination (Fig. 1D) and FMT (Fig. 1F) data provided for the mouse depicted in Fig. 1 following LPS instillation. This systematic approach permitted visualization of the overall in vivo fluorescence generated as an indication of the corresponding cysteine protease activity within each animal included in the study.

**Experimental application.** While Fig. 1 represented the methodological steps required for reaching normalized transillumination and tomographic images, a representative example from a dual wavelength study from an experimental and a control mouse is shown in Fig. 2. A first observation of the benefit of the transillumination method of data collection over that of simple reflectance imaging is apparent here. That is, detection of the striking elevation of both cathepsin activity as well as blood volume evident in the LPS instilled mouse compared with the corresponding control was undetectable through observation of the accompanying epi-illumination fluorescence images shown in Fig. 2, A and F.

Thus, as depicted in Fig. 2, we were able to resolve heightened fluorescence (protease activity, imaged in the 780–820 nm channel derived from the cathepsin activatable probe, excitation: 750 nm, emission: 780 nm) within the lungs and upper respiratory tract of the mice following LPS instillation. Approximately a three-fold increase in detected fluorescence may be noted between the LPS exposed (14 μg; Fig. 2B) and the control animal (Fig. 2G). Specifically, the 24 h time point (following probe injection) at a dosage of 14–21 μg of LPS most consistently provided the two- to threefold detectable elevation in protease activity displayed here. That is, although a wide range of experimental time points and dosages were implemented, all proved less amenable in detection of probe activation (data not shown). Additionally, simultaneous imaging of the intravascular agent AngioSense (imaged in the 705–715 nm channel, excitation: 680 nm, emission: 720 nm) enabled observation of permeability and blood volume changes associated with the LPS challenge. The relevance of such was twofold, as it not only permitted verification of greater overall blood volume in the indicated inflamed lung regions [nearly double throughout the lung region following LPS exposure, comparing (Fig. 2C) and (Fig. 2h), respectively], but also functioned as an internal control for probe bio-distribution within the mice studied. This is because ProSense and AngioSense carry the same molecular structure, albeit AngioSense is loaded with only one or two fluorescent molecules so no quenching occurs. Therefore AngioSense accurately reveals the bio-distribution of ProSense. Lastly, three-dimensional reconstruction of the internal distribution of fluorochrome within the lung regions of these mice was possible using FMT (Fig. 2, D, E, and I, J). This served to corroborate the inflammatory response as indicated by the transillumination data; however, a remarkably more precise localization and quantification of the origin of the protease activity as well as bulk of the vascular volume within the lung region is evident and stands in stark contrast to that of the control animal (Fig. 2, I, J).

**Ex vivo.** The in vivo findings were confirmed by ex vivo fluorescence epi-illumination imaging to corroborate the origin of fluorescence signal within the lungs. The normalized fluo-
rescence images of the intact heart and lungs excised from the LPS-exposed animal (Fig. 3A) manifest a notably higher level of fluorescence activity than those of the control. The same pattern may be noted with regard to Fig. 3B, as the observed AngioSense signal indicated a greater volume of blood in the lungs of the LPS-exposed animal.

Western analysis and observed trends. Western Blot analysis further confirmed an inflammatory response by showing elevated proteases in challenged lungs, with the overall activity recorded scaling with the LPS dose. A characteristic Western blot depicted in Fig. 4A demonstrates the overall level of expression of the predominant mature single-chain form of the enzyme (29 kDa) resulting from cleavage of the (39 kDa) procathepsin B. Some indication of the mature heavy-chain (26 kDa) species may also be noted as the extent of processing may reflect variable decreases in pH within the inflamed lung (15, 17, 34). An LPS dose-dependent increase in band intensity is evident across lanes 1–3, corresponding with 7, 14, and 21 μg instillations, respectively, in contrast to that of an uninstalled control in lane 4. Densitometry was performed on radiographs generated from each of the separate Western analyses using Image J software. This permitted comparison of cathepsin B expression at different experimental dosages 7, 14, and 21 μg of LPS. A trend was indicated in the upregulation of cathepsin B expression at the protein level in vitro (Fig. 4B; data here plotted as density values from corresponding scans of Western blot films). Further analysis of data acquired in vivo, is depicted in Fig. 4, as fluorescence intensity values have been extracted using an in-house program designed in MatLab for derivation of average image values over user-selected regions corresponding to the known location of the lung, as identified by anatomical markers on the mouse photographs (visible location of rib cage) and further confirmed by MRI images obtained under identical mouse placement conditions (not shown here). Among the additional correlations identified by the data, we found that exposure to LPS induced a dose-dependent signal in both ProSense (cathepsin-activatable probe) and AngioSense (intravascular agent) channels as indicated in Fig. 4, C and D, respectively. Also regarding the performance of the accompanying AngioSense intravascular agent, the reported values reflect more of a constant to slightly elevated signal as increasing doses of LPS were given (Fig. 4D). As the overall permeability and perfusion of the inflamed lungs remained higher than those of control animals, this typifies what has been reported of LPS type acute lung inflammation in which continual and even elevated perfusion to dependent, poorly aerated lung regions occurs in contrast to the normal arterial constriction and shunting of blood from regions of local hypoxia functional during other forms of lung injury (7).

Correlative histology. The published parameters of an acute inflammatory response, characteristic of introduction of LPS into the airways, typically feature regions of alveolar wall thickening and collapse with accompanying edema and inflammatory cell penetration (see online supplementary Fig. 5, a and b; Refs. 8, 18, 19, 44). Mice exposed to intranasal LPS at each of the experimental dosages demonstrated significant increase in total BAL white cell counts with a characteristic polymorphonuclear leukocyte infiltrate reported as indicative of an acute inflammatory response (1, 9). On average, BAL inspection showed total white cell counts (following red blood cell lysis), nearly 10-fold higher after 14 and 21 μg LPS exposure than that of control animals (data not shown). The comparative views of BAL collections from control (Fig. 5, e and f) and LPS-treated mice (Fig. 5, g and h) are provided, each as representative of the stained cytospin preparations produced 24 h after LPS instillation in four separate experiments including a total of 16 mice. The overall change in cellular profile from predominantly mononuclear and resident macrophage inhabitants in lungs of control animals to that of increased cellularity upon LPS exposure reflects the invasion of a large number of polymorphonuclear inflammatory cells.

DISCUSSION

Imaging and quantification of cellular and subcellular processes in appropriate models of airway inflammation will permit in vivo insights to be gained at the system level as to the

![Fig. 3. Ex vivo experimental confirmation: ex vivo epi-illumination images of excised tissues collected immediately after imaging an LPS instilled (14 μg) mouse and corresponding control animal. Tissues include anatomically oriented intact right and left lungs (solid white arrowheads) with heart (white outlined arrowhead). A: brightened activity of cysteine protease-activatable probe (ProSense; Visen Medical, Woburn, MA), noted in the lungs of the LPS-exposed mouse, both excitation wavelength (750 nm) and normalized image data provided. B: activity of intravascular agent (Angiosense, Visen Medical, Woburn, MA) as an indication of increased vascular volume in the inflamed lungs of the LPS-exposed mouse relative to the control, excitation wavelength (680 nm) and normalized image displayed.](image-url)
role of proteases specifically in respiratory pathophysiolo-
gy. Furthermore, the papain-like cysteine proteases (cathepsins B, 
H, K, L, and S) have already been well correlated with a 
growing number of inflammatory pathologies, namely: osteo-
porosis, rheumatoid arthritis, osteoarthritis, bronchial asthma,
neurodegenerative disease, and cancer (2, 3, 28, 35, 37, 50).
Additionally, compounds targeting cysteine proteases are 
currently under preclinical and clinical evaluation (5, 41–
43, 45, 46).

Thus, with the introduction of near-infrared fluorochromes 
having molecular sensitivity to several proteases and other 
molecular targets, it becomes possible to visualize key pro-
cesses associated with inflammation and overall lung chal-
lenge. To do so, such limitations of epi-fluorescence imaging 
as surface weighted and reduced contrast as a function of the 
2- to 3-mm sampling depth must be overcome. In direct 
response, transillumination-based imaging collects light that 
has fully propagated through the tissue or animal, bearing 
information from the entire volume sampled during its propa-
gation. FMT applied here in the slab geometry proved useful 
to inspect lung fluorescence activity compared with epi-illumina-
tion methods that could not distinguish lung activity between 
experimental and control animals. By further using normaliza-
tion approaches and physical models of photon propagation in 
tissue, FMT then accounts for the nonlinear dependence of 
fluorescence intensity to depth (48) and optical heterogeneity 
(39, 47). The use of fluorescence “normalization” with signals of 
background optical attenuation in particular is important in 
either transillumination or FMT imaging modes to obtain 
fluorescence bio-distribution images that are independent of 
the varying light attenuation in tissues, for example the differ-
etial attenuation between the highly absorbing heart or the 
highly scattering lung. This correction enables not only images 
of higher fidelity but improves the accuracy of fluorochrome 
quantification as well (25, 39).

Herein, linear trends were noted with regard to fluorescence 
signal collected as a function of both LPS dosage and the 
corresponding underlying cathepsin B upregulation. Impor-
tantly, by means of the dual wavelength approach, an intravas-
cular agent was resolved to serve as an independent monitor of 
activatable probe bio-distribution and for confirming the pres-
ence of increased blood volume of the injured lungs. The use 
of dual-wavelength scheme can concurrently serve as an inter-
nal control for quantifying protease activity by independently 
assessing probe delivery and for visualizing hemodynamics.

While the imaging modality implemented for this study 
proved capable of accurately portraying underlying fluores-
cence activity, varying contributions on behalf of the sum total 
of disease-associated proteases capable of activating the probe 
may be present in the data. That is, fluorescence may occur as 
the probe is activated by elevated levels of cathepsins B, L, or 
S, as well as plasmin. The latter most of these, plasmin, may 
play a role in probe activation since the animal model used 
herein achieves increased blood flow to dependent nonaerated 
lung regions through a highly compromised alveocapillary 
barrier permitting leakage of plasma and allowing formation of 
isoluble fibrin clots in the airways (7). Plasmin, activated to 
hydrolyze the accumulating fibrin, may become an additional 
source of probe activation. Cathepsin L and S expression are 
also likely to contribute to the probe activation noted, as 
cathepsin L upregulation has specifically been observed 
through Western analysis (data not shown). Therefore, while

Fig. 4. Autoradiograph of Immunoblot (12.5% SDS-PAGE) of cathepsin B expression and observed correlations: homogenized lung lysates of LPS instilled mice 
exhibited a dose-dependent increase in cathepsin B expression relative to that of a control animal (A). Protein bands immunoreactive to mouse anti-cathepsin B were 
detected at molecular weights corresponding with those indicated by the accompanying molecular weight standards provided at left of immunoblot. The sizes of these 
protein bands are consistent with the pro-form (39 kDa), single (29 kDa) and heavy (26 kDa) chain forms as previously reported (15). Specifically, lung lysates, prepared 
following 7, 14, or 21 μg LPS instillation, (top, panel A, lanes 1–3, respectively), predominantly express single-chain cathepsin B indicated in proximity of 32-kDa 
marker; same noted for probe-only control (lane 4). GAPDH load control (bottom, panel A, lanes 1–4), 5 μg total protein loaded per well (12.5% SDS-PAGE), 
HRP-labeled secondary antibody used. Densitometry was performed on the autoradiographs generated from each of four experiments for n = 16 mice. All samples for 
Western blotting were tested in triplicate. An LPS dose-dependent upregulation of cathepsin B expression was noted in vitro (plotted as density values from 
corresponding scans of Western blot films; B). Also dose dependency of fluorescence intensity, ProSense (cathepsin-activatable probe) (C) and AngioSense (intravascular 
agent) (D) was detected following LPS exposure of 7, 14, or 21 μg, respectively, with mean right and left lung values from a total of 16 mice plotted (all values derived 
for user-selected regions coregistered with MRI images obtained under identical placement conditions, with an in-house program designed in MatLab).

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we have successfully demonstrated noninvasive in vivo imaging of lung inflammation, the complexity and progression of the disease process may simply exceed correlation to that of a single parameter (cathepsin B).

Improved specificity may be achieved through the use of fluorescent probes designed to be specific to particular proteins or through the utilization of fluorescent proteins that can accurately report on transcription, regulation, and protein function, or cellular motility. The emergence of novel bright fluorescence protein mutants emitting in the near-infrared region offers a platform by which a versatile range of cellular processes can be visualized with high detection sensitivity compared with using fluorescent proteins emitting in the visible (4, 38, 49). Overall, this approach further enables imaging of generic physiological and molecular biomarkers using extrinsically administered probes in the 700–850 nm spectral range, whereas specific molecular processes can be visualized using currently available red-shifted fluorescence proteins such as mCherry and mRaspberry (4) in the 600–700 nm spectral window.

In summary, the present results indicate that FMT is a potent modality for noninvasively studying lung processes in animals. While the technology is not likely to migrate to the clinical setting, due to the significant change in dimensions from the small animal case, it nevertheless allows for a highly accessible tool appropriate for the biomedical laboratory. The insights gained by visualizing physiological, cellular, and subcellular processes in vivo in a noninvasive fashion lead to a more accurate depiction of the dynamic molecular mechanisms involved in disease progression without the potential for distortion and destruction of native properties or the true evolution and quantification of biomarkers that may occur with other ex vivo or in vitro approaches. In addition, multiple readings as a function of time can be obtained from the same animal, further minimizing the need for large animal cohorts to piece together meaningful statistics, as is more common in in vitro-based animal data analyses. We demonstrated the ability to exceed the boundaries of simple reflectance imaging through accurate detection and quantification of an appropriate inflammatory marker within a relevant animal model of acute lung injury. This may well serve in future applications of assessing response to drugs or other forms of treatment by drawing on this noninvasive technique for characterization of functional and molecular activity.

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