Towards *In Vivo* microimaging of Lung Inflammation and Repair: Current issues and future directions

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**Rationale:** a new lung imaging technique based on endoscopic confocal fluorescence microscopy (ECFM) is presented, which is a new method able to provide cellular and structural assessment of living tissue using a miniaturized confocal probe in direct contact with the visceral pleura or introduced through the airways down to the airspaces.

**Objectives:** To observe distal airspace structure and cellular and molecular conditions in normal and injured lungs (hyperoxic and bleomycin challenged), using fluorescent-specific marker contrast and ECFM. To avoid surgical open lung biopsies and tissue fixation procedures, and directly visualize real-time condition of selected regions of interest.

**Methods:** Alveolar space ECFM (using a Five-1® system) with spectral analyses are performed at 488-nm excitation using FITC-labeled markers or naturally fluorescent dyes. The normal lung is compared with the sick lung, where *in vivo* imaging experiments correlated well with results obtained with corresponding *ex vivo* conventional histological assays. Comparisons with current radiological lung imaging procedures, and with alternative ECFM techniques presently available are given.

**Data:** Main elements pertaining to the acute lung injury/acute respiratory distress syndrome (ALI/ARDS) pathophysiology and established early key events, such as inflammation and repair, can be specifically studied: alveolar epithelial membrane phenotype, lung cell apoptosis, neutrophil recruitment, and edema. ECFM allows visualization of: i) fine-tuned ultrastructural lectin (RCA-1, PSA) epithelial cell membrane expression, ii) YO-PRO®-1-related DNA linking of lung cell apoptosis, iii) PKH2 green fluorescent cell linker-labeled neutrophil tracking in lung microcirculatory network and airspaces, iv) FITC-dextran plasma contrast and extravasation with edema formation. ECFM provides reliable and comparable results to corresponding *ex vivo* fluorescent methods.

**Conclusions:** ECFM, using a minimally invasive optical instrument and specific fluorescent markers, is able to provide real-time revolutionary imaging of live unfixed normal and injured lung tissue with promising developments for improving bedside diagnostic and decision-making therapeutic strategy in patients with acute lung injury (ALI). Works are in progress to establish a user-friendly multimodal interface of macro- and microimaging of the lung, including ECFM, and using a “google earth” approach.

**Keywords:** Lung microimaging *in vivo*, ALI/ARDS, epithelium, neutrophils, edema

Implementing better methods for understanding tissue repair and regeneration is necessary to further scientific knowledge about human disease and is especially required to develop new therapeutic approaches for use by bedside physicians. Some human tissue properties, such as the skin, have been extensively studied, but lung repair is still a developing and promising field. This non-exhaustive review discusses current knowledge of lung inflammation and repair, methods of assessing this condition in the clinical setting, as well as commonly used lung imaging techniques and their use in the evaluation of lung repair and imaging techniques currently in development. In particular, new technologies allowing *in vivo* imaging of lung inflammation/repair are now available, opening many new research possibilities and eventually new clinical tools for the assessment and treatment of acute lung diseases at bedside. In this respect, the 2007 American Thoracic Society (ATS) proceedings on small animal imaging of the lung includes among its recommendations for future directions, to “utilize imaging modalities to investigate intracellular lung pathophysiology in vivo and in real time”[1]. This new technology area shows several advantages over older imaging procedures, but obstacles must still be overcome before it integrates regular medical practice.

Current understanding of lung inflammation/repair stems from the knowledge acquired in the study of several lung diseases whose physiopathology involves inadequate, dysfunctional or inappropriate tissue repair. Examples include acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), chronic obstructive lung disease (COPD), asthma, idiopathic pulmonary fibrosis and other interstitial lung diseases. Specifically, knowledge acquired from the study of ALI using animal models (e.g. the bleomycin rat model) has given us greater insight into lung repair. Therefore, much of the information on the mechanisms of lung repair provided in this article is derived from ALI animal model studies [2-4].
Mechanisms of lung repair

The human respiratory system is composed of airways leading to a fine network of blood vessels and alveoli. This fragile system must act as an efficient barrier to fight exterior microorganisms and chemical aggressors, as well as to retain its intended function in maintaining gas exchange and homeostasis. When needed, normal lung tissue must respond to exterior aggression and initiate repair while preserving the delicate interface between the epithelial and endothelial lumen. In addition, all cellular components must function in the constantly mobile environment of the breathing lung, in contrast to the mostly static environment of most tissues.

Lung repair is made of a series of complex processes that begins with a response to aggression and involves tissue inflammation with recruitment of white blood cells, migration of epithelial cells and fibroblasts and extracellular matrix deposition. Interactions between these committed cells are mediated through cytokines and growth factors which are still being studied and elucidated. Known mechanisms of lung repair in the setting of different disease models have been extensively reviewed, and it is beyond the scope of this article to describe in detail the different healing pathways. Nevertheless, the general mechanisms will be discussed as a starting point for future directions in in vivo lung imaging.

In summary, lung repair is composed of complex, dynamic and partly understood processes which occur at the cellular and molecular levels. Only by studying it at this microscopic and molecular level can we truly aim to comprehend and influence it.

The initial triggering event may take diverse forms such as microorganisms (chemical and physical), aggressors, allergens or circulating immune complexes. Whichever the triggering event, it results in a disruption of the integrity of the alveolar-capillary membrane complex, with alveolar type I, type II and endothelial cell damage, necrosis and apoptosis as well as basement membrane exposure. Regulation of apoptosis is thought to play a major role in tissue repair [5, 6]. All lung cell types can undergo apoptosis in particular settings. Initial injury, especially if of an inflammatory nature, may induce apoptosis rather than necrosis of alveolar epithelial cells, such as in ALI. Clearance of excess neutrophils, fibroblasts and alveolar epithelial type II cells is thought to be regulated by apoptosis. The apoptotic pathways (such as Fas/FasL ligand, bax/bcl-2 and myc proto-oncogen systems) are extremely complex and the role and mechanisms of each throughout the process of lung repair are largely unknown. Structural cell apoptosis (i.e. epithelial and endothelial) occurs very rapidly, making it very difficult to study with traditional methods, whereas neutrophil apoptosis is delayed in ARDS as well in sepsis [7-8]. In this respect, real-time in vivo microscopy of apoptotic cells may provide more insight in this process.

The inflammatory phase is initiated by the damaged, necrotic and apoptotic cells and exposed extracellular matrix components. It is usually characterized by a protein rich exudate and infiltration of white blood cells such as neutrophils and macrophages. The type of white blood cell infiltrate will be influenced by the type of triggering event. All implicated cell types (type I and II alveolar epithelium, endothelium and white blood cells) will produce the cytokines and other molecules responsible for the initiation of the repair process and the influx of other cells. Tumor necrosis factor (TNF-α) and interleukins (e.g. IL-1β, IL-6, IL-8) play major roles in promoting inflammation while IL-10 is thought to play an anti-inflammatory role. The balance of pro- and anti-inflammatory mediators will then determine if the response is appropriate, self-limited or inappropriate and inadequately sustained.

The proliferative phase is characterized by epithelial type II cell proliferation and influx of fibroblasts. Alveolar type II cells proliferate, migrate to the damaged area, recreate and remodel the underlying basement membrane (partly with the help of fibroblasts) and finally differentiate into type I cells. Extracellular matrix deposition and remodelling is a careful balance mediated by alveolar type II cells, fibroblasts and macrophages through molecules such as transforming growth factor (TGF-β), macrophage derived platelet derived growth factor (PDGF), interferon-γ, TNF-α and IL-1β. This is a crucial phase which leads to restoration of tissue integrity and function or, in examples such as ARDS and interstitial lung diseases, to pulmonary fibrosis.

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Evaluation of lung repair in the clinical setting

There are currently no methods for direct assessment of lung repair in the clinical settings. Clinical tools are directed at correct diagnosis in order to proceed to adequate treatment. Response is then assessed by history, physical exam, imaging and function tests. There are no available methods to evaluate the state of lung repair in a patient, either to use as guidance for the best timing in initiating treatment or to monitor treatment response. All current evaluation methods do not permit a clear understanding of the repair process, only showing the initial consequences of loss of function, then monitoring the return to normal function or further deterioration.

Bedside clinical evaluation tells the physician how much the lung function is deteriorating after the initial insult or inadequate repair. In a highly simplified example, as a patient shows increasing dyspnea, low blood oxygen saturation
and arterial blood gas indicating hypoxemia, his treating physician will suspect impaired oxygen exchange, and direct further investigation at identifying the cause and treatment at restoring the lung’s impaired function. Patient’s symptoms, vitals, and blood gases will inform the physician if lung function is improving or has returned to normal. It will be assumed that the lung has repaired, without any direct assessment of lung repair itself. Commonly used investigative tools have the same weakness, showing consequences of injury and repair in greater detail, but not the underlying state and mechanisms of repair itself.

Chest imaging is an essential part of the lung diagnostic workup [9-11]. Standard chest x-ray will show infiltrates, patterns of fibrosis or changes in lung volumes. Alveolar or interstitial infiltrates suggest local inflammation or main the potential morbidity related to the use of an invasive technique on already critically ill patients, and the to use open lung biopsy in this context [14]. However, disadvantages limiting the clinical use of surgical lung biopsy are

Non-imaging methods of lung investigation include bronchoalveolar lavage and open lung biopsy. Broncholaveolar lavage is diagnostically useful by providing white blood cell counts and most importantly adequate material for microbiological cultures. In the research setting, it is often used to isolate inflammatory molecules such as TNF-α or IL-

MRI. Magnetic resonance imaging (MRI) is also a progressively more widespread technique. In its beginnings, MRI had low intensity, due to low density of protons and tissue inhomogeneity. These problems are very present in the air-filled lung, as the air itself is not easily detectable by MRI. Furthermore, the fluid-air interfaces cause a phenomenon called susceptibility artefacts [16]. When two tissues at an interface have different magnetic susceptibility (intern animation due to magnetic field) it creates a magnetic distortion. In some cases, the low proton density in lung can be an advantage because certain inflammatory and neoplastic processes increase the number of protons [15]. Hyperpolarized gases, the most used being 129Xe, have further increased the MRI resolution. The polarization of these gases has been enhanced by a factor of up to 100,000 to extend their detection [1]. Another technique used to increase the resolution is Fast-MRI. The lung is constantly in motion, therefore causing movement artefacts. Fast-MRI uses new receptors and an HF antenna to attenuate these artefacts. MRI is also used to ascertain more complex lung characteristics, by measurements of local lung physiology. For example, the pulmonary blood flow can be measured, but this presents its own difficulties due to low proton density [17]. MRI’s advantages over CT include the improved acquisition speed and absence of radiation.
Micro-PET. New positron emission tomography (PET) technology, especially the combined CT/PET, has permitted new results in small animal imagery. PET allows the visualisation of in vivo mechanisms and has numerous applications (e.g. brain injury, bone degeneration) [1]. The main problem with PET is its low sensitivity to small signals. Furthermore, the dose of radiation can have an influence on the results. To obtain the radioisotope, a cyclotron is needed, and the cost is high. One of its derived techniques is single photon emission CT (SPECT) [18]. In spite of having decreased resolution and a longer acquisition time compared to CT, SPECT allows the simultaneous visualisation of some mechanisms such as blood and air circulation. A significant advantage of SPECT is 3D reconstruction which gives tridimensional information on the pulmonary structure or on the precise configuration of abnormalities such as lung tumours. PET is used to get some lung parameters, such as the regional effective alveolar ventilation-perfusion (VA/Q), and even the degree of bronchoconstriction [19].

Echography. Echography can also be used to image the lung, but it is mostly restricted to the visualisation of the pleural space because of the poor echogenicity of air in the lung. A new technique, called ultra sound lung comets (ULCs), allows the measurement of the extra vascular water in the lung. This imaging modality is well adapted with ARDS because water is very present, due to the thickening of the alveolar wall. With a pulmonary injury model reproducing the first stage ARDS, Gargani has proven that ULCs are capable of quickly detecting the extra vascular water in pigs [20].

Molecular markers. The lung has a low background signal, allowing the use of molecular markers. There are two types of molecular imaging: one uses the endogenous targets (direct imaging), and the other uses a reporter gene (indirect imaging). The principle of direct imaging hinges upon the presence of receptors such as enzymes or kinases in the target tissue. Indirect imaging is based on gene expression or protein-protein interactions [1]. This technique can be compared with PET (with FDG) and is very useful in several lung diseases. The molecular markers are particularly useful in staging non-small cell lung cancer and searching for metastasis [21].

The above techniques are very useful to obtain structural and physiological information of the lung, but they don’t allow the evaluation of the state of pulmonary repair. Microscopic in vivo imaging shows great promise in complementing these techniques.

**Imaging modality: microscopic in vivo imaging**

Developments in microscopic in vivo imaging are booming. Its potential to evaluate pulmonary repair is high. The molecular or cellular information that physicians can obtain from this technique is very important in ARDS. It is with the presence (or the absence) and number of certain cells that the physicians can evaluate with precision if the lung is repairing or not. This technique can be divided in two subcategories: transthoracic and endobronchial imaging. The approach in both cases is totally different but the information is quite similar. Two companies have developed systems that can be used to lung in vivo microscopy: Mauna Kea (France) and Optiscan (Australia). Both systems are fibered confocal fluorescence microscopes (FCFMs).

Mauna Kea has chosen the endobronchial approach. They have introduced the “alveoloscopy” term, because their instrument can go down the lung airways to the alveoli. The system is equipped with very small probes (350 to 1800µm, depending of the probe), allowing physicians to observe very small airspaces. This system can record video sequences due to its fast acquisition rate (12 frames/sec) [22]. It mainly depends on endogenous fluorescence produced by the elastin component of the basement membrane zone [23]. It has a good spatial resolution (lateral resolution of 3.5µm) and a field of view of 600µm, allowing the observation of very small structures.

The Optiscan instrument has two configurations: a flexible endoscopic system for clinical use marketed by Pentax and a rigid pen-like probe (6mm diameter) for research applications. In both cases, a transthoracic approach is required. The size and rigidity of the probe precludes an endobronchial approach, so the probe must be applied directly on the pleura. Lateral resolution is 0.7µm and axial resolution is 7µm. Due to the acquisition speed (0.7 to 1.4 frames/sec), the recording of video sequences is not allowed, but with several successive images and appropriate software, video sequence reconstruction is possible. An advantage of this system is its ability to image through tissue, as it is a true confocal microscope with adjustable imaging depth. Indeed, the focal plane can be moved from the surface to 250µm in depth, allowing the tridimensional structure of the lung to be better observed. However, the system cannot detect endogenous fluorescence, so that the injection of contrast agents is necessary.

Other advances in intravital microscopy (IVM) require a window-based preparation for the visualisation of subpleural lung microvessels, for example the implantation of flanged steel windows in the thoracic wall. Stabilisation is assured via suction through a borehole. With window approaches, blood flow and blood cells can be visualised more easily [24].

Real-time fluorescence imaging (RFI) allows the visualisation of lung cellular or molecular responses, e.g. the determination of cytosolic Ca²⁺ levels. RFI has been coupled with immunofluorescence, for observation of leukocyte adhesion in lung [24].
Microscopic *in vivo* technique is a judicious choice for imaging the lung in the setting of lung injury, such as in ARDS. This technique brings important information not only concerning the structure of the lung, but especially its state of repair.

**Advantages, limits and future directions for in vivo microimaging**

In the study of lung inflammation/repair, microscopic *in vivo* imaging shows major advantages over traditional imaging techniques as well as techniques still in development. It is the only imaging modality able to visualise microscopic lung architecture without the need for collecting actual tissue, as in open lung biopsy. With the use of *in vivo* markers, it has the potential of providing at least as much information as a biopsy specimen, with the added benefit of visualising actual live tissue. *An in vivo* image is not altered in any way, showing cells in their natural homeostasis. Surrounding molecules such as cytokines, growth factors and hormones are also intact. *In vivo* imaging eliminates confounding factors related to laboratory *in vitro* techniques. With the additional benefit of real-time imaging, potentially any *in vivo* cell movement is visible to the examiner. Migrating leucocytes, creeping/repairing type II alveolar epithelial cells, apoptosis and edema could be studied directly (e.g. figure 1 A-E). With the use of modern fluorescent biomarkers, images of targeted circulating molecules and their site of action may someday be obtained for real-time functional studies. The potential for acquiring new knowledge of lung repair and eventually using it for the benefit of patients is immense, making *in vivo* microscopy a promising new research tool.

**Figure 1 A:** *Ricinus Communis Agglutinin I* lectin labeling of lung epithelial cells. *In vivo* (a) normal lung (b) bleomycin lung: enhanced parenchymal thickness together with macrophage and metaplastic epithelial cell staining. *Ex vivo*, with DAPI; (c) normal lung (d) bleomycin lung. (b) and (d): conjunction of dense/continuous linear; irregular/discontinuous; and no labelling.

**Figure 1 B:** RTI-40 lung epithelial cell membrane labeling. *In vivo* (a) normal lung: linear/apical staining of type I epithelial cells; (b) bleomycin lung: disorganized multilayer stratified labeling. *Ex vivo* with DAPI (c) normal lung: type I cell membrane staining exhibiting similar pattern to (a); (d) bleomycin lung. (b) and (d): conjunction of dense/continuous linear; irregular/discontinuous; and no labeling.
Figure 1 C: Lung neutrophil’s labeling with PKH2. In vivo (a) normal lung; a few circulating cells can be viewed; (b) bleomycin lung; more labeled white circulating cells including some translocated into airspaces; (c) Ex vivo green neutrophils crossing normal lung; (d) same as (c) in bleomycin lung with more labeled cells.

Figure 1 D: Visualization of lung cell apoptosis (using two different DNA end-terminal markers). YO-PRO®-1 (I/T instilled) labeling with FITC-dextran (I/V) counterstaining in normal (a); bleomycin (b); and hyperoxic lungs (c). Ex vivo (d), (e), (f) same as (a), (b), (d) using TUNEL.

Figure 1 E: Normal rat lung vs. lung edema (after 72hrs hyperoxia) after FITC-dextran I/V injection. Using Five1® FCFMs: (a) normal lung with interstitial microcirculatory network and moving blood cells negatively visible, and (b): 72hrs-hyperoxia-exposed lung with heterogeneous diffusion of dextran outside microvessel network leading to interstitial and alveolar space labeling with empty capillaries and leaking dextran. Using ex vivo H&E staining and conventional OM: (c) normal lung and (d) lung congestion, and interstitial/ alveolar edema.

Before in vivo imaging is commonly used by researchers, and later at the bedside, the current limits of the technique must be overcome. Images of lung obtained by in vivo microscopes, with or without fluorescent markers, have so far never been seen. Experience at interpreting the images must be solidly acquired before interpretation guidelines and diagnostic criteria as well established as those of conventional microscopy may be published. The use of fluorescent in
in vivo markers is still recent and much research is needed before an extensive and well studied panel is available. Compared with traditional histology, in vivo marking is faced with the major challenge of having to contend with the lung’s natural epithelial, endothelial and cellular membrane barriers. In addition the markers must also be proven safe for human use. Other limitations of in vivo imaging are mechanical. The constant movement of live breathing lung renders stabilisation of probes on a particular segment of tissue difficult. Also, the speed of image capture is often too slow for rapid breathing movements, creating a kinetic blur and rendering image acquisition of the lung problematic. Future advances in the probe technologies should soon correct these limitations. Greater mechanical limits are those imposed by lung diseases with a patchy damage pattern such as ALI/ARDS. An ideal site of damaged lung must be correctly identified before applying the probe. This is especially relevant in the case of the transthoracic approach, in which a small opening in the thorax must be performed. Once the ideal site is identified and the first images are obtained, the same site must be found again in order to conduct follow-up imaging, representing yet another significant challenge at the microscopic level. Combination of traditional imaging technique like CT scanning with in vivo microscopy may be useful in this setting. These are only some examples of the obstacles that must be overcome. New challenges will likely surface as the technology and research continue to develop.

In the path to developing new treatments in lung diseases, better understanding of lung repair is a logical step. Current imaging technologies are incapable of adequately assessing lung repair by the clinician. This clearly demonstrates a great gap in bedside assessment that may be filled with in vivo microimaging. Continuing research will determine its role in diagnosis. Increasing knowledge of the complex mechanisms involved in lung inflammation/repair, will lead to the identification of more precise turning points in the repair stages, as well as description of precise molecular inflammatory patterns and their variations depending on stage and disease. In cases of acute respiratory failure or chronic lung diseases in which diagnosis is unclear, in vivo microimaging might prove to be a new indispensable tool, especially in patients whose condition precludes more invasive procedures. In patients with clear diagnosis, in vivo microimaging might be used as a prognostic indicator and as a guide in the timing of treatment initiation. For example, the use of corticosteroids is ARDS has been the subject of debate, with large studies showing no clear benefit [25]. Corticosteroids might still be useful in certain subsets of patients and in vivo imaging may aid in identifying them. A similar example of a chronic disease would be in the case of idiopathic pulmonary fibrosis. Identification of specific inflammatory patterns and lung repair stages could also guide the use of future treatment options such as biologicals and other immunomodulators. Better evaluation of tissue repair may help the physician decide which treatment to use, when to initiate and when to cease or modify it.

The study of lung repair shows great potential benefit in aiding patients stricken with acute and chronic lung diseases. It is in the interest of these patients that research involving in vivo microimaging of the lung be pursued.

References


