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Multidimensional lung imaging: integrated preclinical platforms enabling the identification of translational biomarkers for pulmonary research

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Abstract

Background Medical imaging is changing diagnostics, elucidating molecular disease mechanisms, supporting patient stratification, and advancing drug development toward personalized medicine across multiple therapeutic areas. Regrettably, in respiratory research, it is rarely used as a primary endpoint in clinical trials, despite the pressing need for non-invasive biomarkers, particularly in pulmonary disease, where anatomical complexity and patient risk often preclude lung biopsies.

Main body This review describes how biomarkers derived from preclinical imaging, when combined with end-stage readouts such as OMIC data within multi-integrative platforms, can provide a comprehensive and multiscale understanding of lung pathology. Focusing on rodent models, we survey a range of imaging modalities, including anatomical (micro-CT, MRI), optical (BLI, FLI), and functional (PET, SPECT), emphasizing their role in longitudinal in vivo studies. These approaches are complemented by end-stage bioanalytical tools, such as histology, tissue clearing, and spatial omics, implemented within scalable workflows. The feasibility and translational aspects of these technologies, including considerations related to dose, operational requirements, and emerging needs for protocol standardization, are also examined, as these factors critically influence data robustness and reproducibility. A key component of these multi-level platforms is the systematic matching and integration of in vivo imaging with end-stage data, enabling quantitative pathology validation, the acquisition of etiopathological insights, as well as biomarker discovery. These multilayered platforms also take advantage of advanced computational tools, including machine learning and explainable AI, which improve interpretability, reproducibility, and translational relevance of the data in the context of personalized medicine. These strategies further strengthen early disease assessment, improving diagnostic precision and informing therapeutic development.

Conclusion Overall, imaging-driven, integrated preclinical investigation strategies represent a powerful and ethically responsible approach to refining disease modeling and accelerating drug development in pulmonary medicine.

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Introduction

Pulmonary diseases, including lung cancer, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF), are a major global health burden, contributing significantly to global morbidity and mortality. The development of effective therapies depends on robust and reproducible experimental animal models, accurate disease assessment, and successful translation to human clinical trials. Despite significant investments, often exceeding a decade and over one billion dollars per compound [1], many drug candidates still fail in late-stage clinical trials, as seen with the autotaxin inhibitor ziritaxestat and the anti-CTGF antibody pamrevlumab for IPF [2–5], and CXCR2 antagonists for COPD [6]. These failures underscore a persistent gap in translational research, namely, the inability of current preclinical models to reliably predict clinical efficacy [5, 7].

The assessment of disease progression and therapeutic response at the preclinical level remains a critical bottleneck. Conventional end-stage methods, such as histopathology and biochemical assays, are inherently invasive, typically involve partial tissue sampling, and are limited to single timepoints. More advanced techniques, such as 3D histology (e.g., light-sheet fluorescence microscopy), despite their ability to provide spatially resolved, quantitative insights into tissue architecture, remain destructive and labour-intensive, with limited suitability for high-throughput or longitudinal studies. Additionally, while lung function is routinely assessed in humans using non-invasive spirometry, preclinical evaluation of pulmonary functionality relies on invasive techniques such as forced oscillation using commercially available systems (e.g., FlexiVent), which require terminal procedures and are unsuitable for repeated measurements on the same animal. These limitations hinder longitudinal studies and increase animal usage, raising both scientific and ethical concerns [8, 9].

A growing body of research points to the integration of complementary technologies capable of capturing both anatomical and functional features of lung disease over time as an effective means to face these limitations. In particular, in vivo imaging has emerged as a transformative approach enabling non-invasive, longitudinal monitoring of disease progression within the same animal, thus aligning with the 3Rs principle (Replacement, Reduction, Refinement) [10]. High-resolution anatomical investigation approaches such as micro-computed tomography (micro-CT) and magnetic resonance imaging (MRI) are complemented by functional and molecular imaging techniques, including positron emission tomography (PET), single photon emission computed tomography (SPECT), and optical imaging (e.g., fluorescence- and bioluminescence-based techniques). When used in combination, these multimodal approaches enable a more comprehensive characterization of disease dynamics, capturing both structural and functional alterations over time.

In addition to imaging, omics-based technologies, including genomics, transcriptomics, proteomics, and metabolomics, are being increasingly integrated into preclinical investigation workflows. The resulting multimodal platforms enable a systems-level understanding of disease biology and therapeutically amenable etiopathogenetic mechanisms, while also aiding the identification of novel candidate biomarkers suitable for clinical translation [11–13]. Omics data, in particular, when combined with imaging and ex vivo analyses, can significantly enhance the interpretability and contextualization of preclinical models at the molecular level.

An overview of these complementary and, to a large extent, synergistic technologies, along with their integration in the framework of preclinical pulmonary research, is presented in Fig. 1. The diagram illustrates the interplay between longitudinal in-vivo imaging and classical as well as advanced ex-vivo end-stage techniques, which are instrumental to gain higher-resolution insights. Together, these combined approaches highlight the advantage of a multiscale, multimodal platform for comprehensive disease modeling and evaluation.

This review aims to provide a unified, translation-oriented perspective on the integration of in vivo and ex vivo technologies in preclinical pulmonary research. Particularly emphasized is the complementarity of imaging, histology, and omics, combined within a multiscale framework capable of capturing anatomical, functional, and molecular features of lung disease. Special attention is given to imaging technologies and integrated workflows that can deepen biological mechanistic insight, improve reproducibility, comply with current ethical standards, and strengthen overall translational relevance, including the discovery of novel biomarkers, in the context of preclinical pulmonary research.

End-stage techniques

A concise overview of the main end-stage techniques, including their measurable biomarkers, advantages, limitations, and spatial resolution, is provided in Table 1.

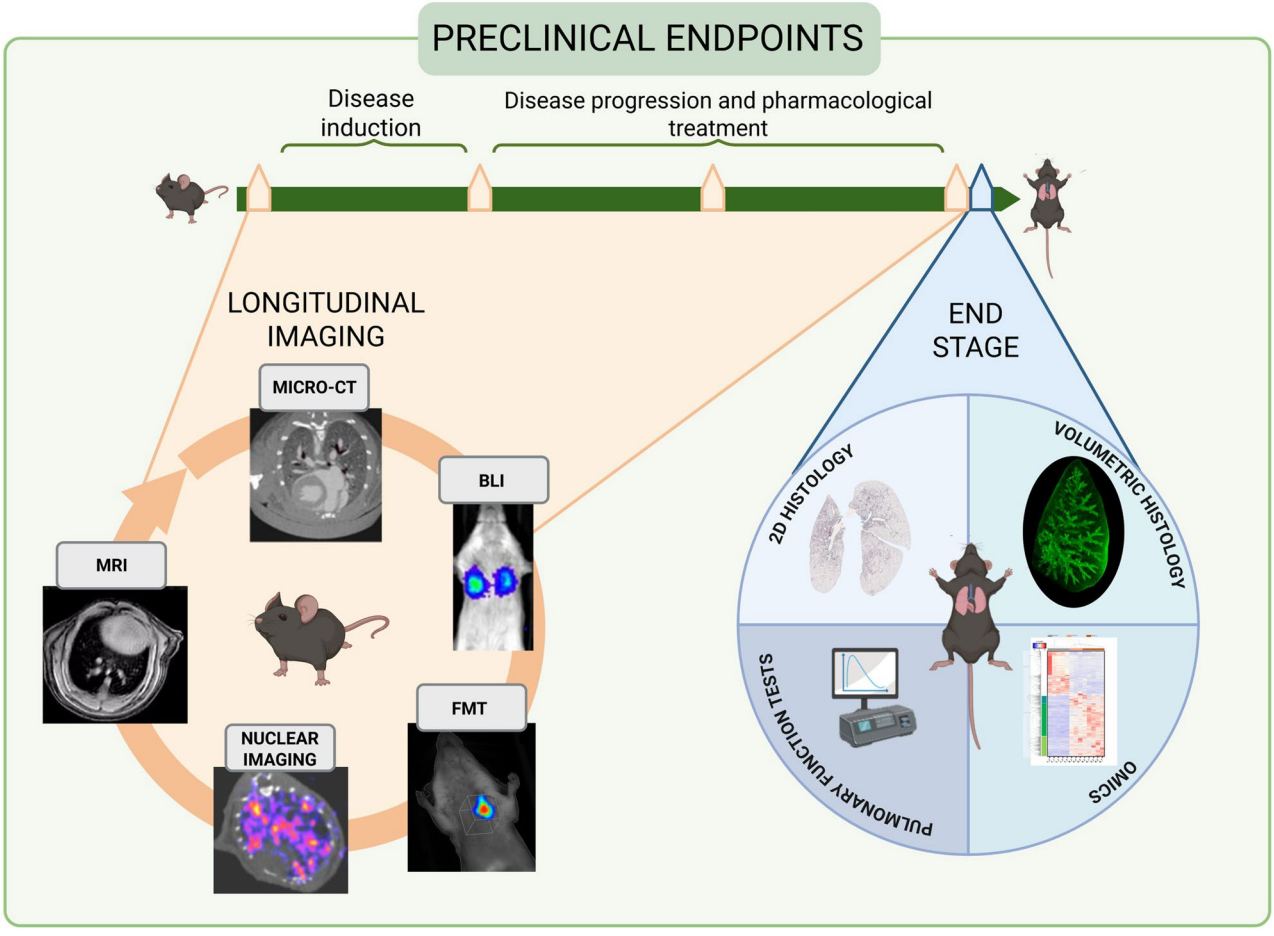

Fig. 1 Overview of preclinical endpoints in experimental models of lung disease. The figure summarizes the typical workflow of preclinical studies, highlighting how different methodologies are applied at distinct phases of disease modeling and therapeutic evaluation. On the top, the timeline shows the two major phases in which the experimental protocols are divided: disease induction and disease progression/pharmacological treatment, culminating in end-stage evaluation. A suite of non-invasive imaging techniques supports longitudinal in vivo assessments across the disease course. These include high-resolution anatomical investigation approaches, such as micro-computed tomography (micro-CT), magnetic resonance imaging (MRI)[163], as well as functional and molecular imaging modalities, such as nuclear imaging, fluorescence molecular tomography (FMT) and bioluminescence imaging (BLI). These approaches enable real-time monitoring of pathological changes and therapeutic responses. At the end of the study, end-stage analyses are conducted to validate and complement imaging data with high-resolution anatomical and molecular-level information

Table 1 End-stage techniques in preclinical pulmonary research

Technique	Biomarkers	Advantages	Disadvantages	Resolution
Lung function measurements	Lung mechanics and volumes	Widely validated; highly translational	Invasive and terminal; requires ventilatory maneuver; no regional resolution; technical variability [14,16–19]	Whole lung
Histology (2D)	Cellular morphology and organization	High-resolution; widely validated in pathology; compatible with molecular labeling [24,25]	Terminal; sampling bias; intra- and inter-observer variability [25,26]	Cellular and subcellular (0.1–0.5 µm); single section
Volumetric histology	Cellular morphology and organization within a 3D spatial context	High-resolution; preserves 3D spatial context; whole-organ coverage; captures tissue heterogeneity; compatible with multiplex labeling [35,40,41]	Terminal; technically complex; generates large data volume; requires advanced computational tools [35,38–40,42]	Cellular (10–20 µm); 3D volume coverage
Omics (bulk and single cell)	Molecular (gene expression patterns, relevant proteins, and metabolites)	Unbiased and high-dimensional; facilitates identification of biomarkers and etiopathogenetic mechanisms [43–47,49]	Sample heterogeneity; data complexity; bulk omics lose spatial/cell-type resolution; single-cell omics are costly and data-intensive [43–47,49]	Molecular, bulk (tissue-level) or single cell

Pulmonary function measurements

Pulmonary function measurements, including forced oscillation technique (FOT), negative pressure-driven forced expiratory (NPFE) measurements, and pressure-volume (PV) curves, provide a quantitative assessment of respiratory mechanics in both preclinical and clinical settings [14–16]. FOT, in particular, applies pressure oscillations to the lungs via a mouthpiece or tracheostomy and derives mechanical impedance spectra through Fourier analysis. These spectra are deconvoluted to yield parameters such as Newtonian flow resistance, tissue damping, tissue elastance, and gas inertance, which collectively reflect airway obstruction, parenchymal stiffness, and lung expandability [17, 18]. In small laboratory animals, the constant-phase model is used to fit multi-frequency impedance data, while in humans, the technique can be applied non-invasively during spontaneous breathing, as oscillation frequencies exceed the natural breathing rate. Under controlled mechanical ventilation, quasi-static pressure-volume loops and single-frequency oscillations allow for the extraction of dynamic data such as respiratory system resistance and elastance, with quasi-static compliance of the respiratory system calculated at zero flow. Additional indexes such as forced expiratory volume at 0.1 s ($FEV_{0.1}$) and forced vital capacity (FVC) provide insights into expiratory airflow limitation and lung capacity, respectively [18]. Collectively, these metrics capture both central airway function and peripheral parenchymal mechanics, which are known to be sensitive indicators of structural and functional alterations associated with various lung diseases. Despite their undisputable diagnostic value, these techniques are invasive and terminal, precluding their application in longitudinal studies. Furthermore, while FOT provides detailed insights on respiratory mechanics, it lacks spatial resolution, which represents a critical issue in tissue-heterogeneous diseases such as pulmonary fibrosis [19]. Moreover, results can vary due to technical factors such as depth of anesthesia, ventilator settings, and operator expertise, in line with the "phenotyping uncertainty principle", which highlights the trade-off between measurement precision and physiological relevance [20]. Interpretation of the parameters derived from this kind of measurement also requires caution, as similar mechanical changes may arise from distinct pathological processes such as inflammation, edema, or fibrosis.

To address these limitations, non-invasive alternatives such as whole-body, head-out, and double-chamber plethysmography have been employed for longitudinal monitoring of breathing patterns and derivation of related indices such as tidal volume, respiratory rate, and enhanced pause (Penh). However, the physiological relevance of certain parameters, most notably Penh, remains controversial [21–23].

In summary, while pulmonary function tests remain a cornerstone for the functional characterization of pulmonary physiology in preclinical models, their information potential is maximized when integrated with complementary imaging or histopathological measurements, which can provide essential spatial and morphological context to otherwise whole-tissue functional readouts.

Histology

Histology remains a pivotal tool for detecting microstructural alterations in preclinical lung research and providing direct visualization of tissue architecture as well as cellular organization. Standard techniques such as hematoxylin and eosin (H&E), Masson's trichrome, alcian blu-PAS, and immunohistochemical analysis of specific cellular markers are widely used across a variety of disease models. In fibrotic models, semi-quantitative scoring systems, most notably the Ashcroft score, are commonly used to estimate fibrotic burden. However, recent guidelines have pointed out that histological scoring alone may be insufficient. Indeed, although some researchers believe that hydroxyproline (HYP) can be a reliable fibrosis marker, other researchers highlighted the risk of possible misinterpretations [24]. Furthermore, because of the destructive nature of the procedure utilized for HYP quantification, histological analysis and hydroxyproline determination cannot be performed on the same sample.

In models of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), histology allows identification of key features such as alveolar epithelial injury, intra-alveolar proteinaceous fluid and debris accumulation, hemorrhage, and neutrophilic infiltration, all of which reflect disruption of the alveolar-capillary barrier. The 2022 ATS Workshop recommended assessing at least three of four domains (histological injury, barrier dysfunction, inflammation, and physiological impairment) to rigorously define experimental ALI, with validated histological alterations ranked among the most reliable indicators [25]. Histology is also central to models of asthma and allergic inflammation, where it can reveal specific features such as goblet cell metaplasia, smooth muscle hypertrophy, and subepithelial fibrosis, as well as in models of infectious diseases, where it can capture immune cell dynamics and localized cellular damage, including necrosis and consolidation [26].

Despite its versatility and high spatial resolution, histological analysis faces significant limitations. The limited size of tissue sections introduces sampling bias, while semi-quantitative scoring remains subjective and prone to interobserver variability. Despite recent efforts toward digital image analysis and AI-driven quantification [27, 28], both approaches still require careful validation and

standardization. Moreover, as a terminal endpoint, histology provides only a static snapshot of disease, precluding longitudinal assessment as well as detailed insight into dynamic processes such as disease progression and/or response to therapeutic interventions. As highlighted by ATS guidelines, no universally accepted scoring system with a validated intra- and inter-observer reproducibility is currently available. As a result, blinded scoring of multiple non-overlapping fields using clearly defined criteria remains the preferred approach [25]. Variability in tissue processing and staining protocols also remains a technical hurdle, further underscoring the need for stringent methodological controls.

In summary, histology provides detailed cellular insights across a range of pulmonary disease models and remains a critical endpoint for tissue architecture evaluation. Its major strengths are related to its versatility, high resolution, and compatibility with molecular labeling. However, limitations related to its inherent invasiveness, sampling bias, and operator subjectivity highlight the importance of combining histology with complementary quantitative analytical approaches and tightly adhering to standardized protocols to ensure reproducibility and robustness.

Volumetric histology

Volumetric histology refers to advanced imaging strategies that combine high-resolution cellular detail with a three-dimensional spatial context, thereby allowing for overcoming the intrinsic sampling limitations of conventional 2D histology. Although the term *volumetric histology* is not yet universally adopted in the literature, it effectively captures the conceptual integration of tissue clearing, large-scale imaging, and 3D reconstruction in the histopathological analysis of whole organs.

Light-Sheet Fluorescence Microscopy (LSFM)

Among these advanced imaging strategies, light-sheet fluorescence microscopy (LSFM) combined with optical tissue clearing has emerged as a cornerstone for whole-lung 3D imaging, enabling detailed visualization of alveolar architecture, fibrotic foci, immune cell infiltrates and vascular networks across intact lung lobes [29–31]. In murine models of lung fibrosis, LSFM has revealed spatially confined foci of collagen deposition and extracellular matrix remodeling, especially around airways and blood vessels, which are often missed in conventional 2D Sect [32]. In infectious and allergic inflammation models, volumetric approaches have revealed complex immune cell distributions shaped by lung anatomy. For example, multiplexed LSFM enabled compartment-specific localization of tumour-associated macrophages and therapy-driven immune reorganisation in lung carcinoma models [33], while a multiscale pipeline combining LSFM and confocal microscopy allowed visualization of $CD45^+CD3^+$ lymphocytes preferentially accumulating near the airways and pulmonary arteries in asthmatic lungs [34].

Cryo-Fluorescence Tomography (CFT)

CFT has emerged as an effective complement to LSFM and a powerful 3D histological technique bridging the gap between in-vivo imaging and conventional histology. In CFT, the entire animal or intact organ is embedded *en bloc* in cryo-media, and the frozen bloc is serially sectioned from top-to-bottom at 50–100 μm intervals using a robotic cryo-macrotome. After each section, white-light and multispectral fluorescence images are acquired directly from the block face using an overhead camera. The resulting data are then reconstructed into high-resolution volumetric datasets that retain structural and molecular information throughout the entire specimen [35]. CFT provides intermediate-to-high resolution imaging of whole animals or organs, with the added advantage of preserving full compatibility with fluorescence-based probes, thus making it suitable for mapping molecular and cellular events within the spatial context of intact tissue [36]. Although its application to pulmonary disease models remains limited, recent studies have demonstrated its usefulness for capturing distributed inflammatory signals and tracer accumulation in lung tissue, suggesting promising applications in respiratory research [37–40].

In vascular studies, multiscale imaging combining LSFM, optical projection tomography, and ex vivo micro-CT enables detailed reconstructions of the pulmonary vasculature, while stand-alone LSFM has been used to quantify neointimal hyperplasia and adventitial neovascularization in models of vascular remodeling [41]..

When applied in parallel, volume electron microscopy techniques such as serial sectioning transmission electron microscopy (serial-section TEM) offer nanoscale 3D resolution of lung structure. Although labor-intensive, these methods provide excellent lateral resolution and compatibility with immunolabeling, thus allowing a detailed analysis of alveolar–capillary units, epithelial differentiation, and fibroblast–epithelial interactions [42].

Integrative perspective and methodological trade-offs

In summary, volumetric histology, particularly when enhanced by automated segmentation, multiplexed labeling, and computational analysis, enables high-content, organ-scale phenotyping while preserving the native 3D lung architecture. Its key advantages include the ability to map disease features across whole organs, capture spatial heterogeneity, and integrate structural, cellular, and molecular data within a single imaging pipeline. While no single mode of operation can fully address all analytical needs, each one features distinct strengths and specific

advantages. LSFM allows exceptional preservation of tissue morphology and spatial context, but is essentially a one-way approach, since clearing protocols interfere with downstream histological or omics analyses. By contrast, cryo-fluorescence tomography (CFT) enables whole-organ 3D reconstruction, preserving fluorescence signals as well as anatomical integrity, and thus represents a valuable compromise between spatial resolution and compatibility with molecular analyses. These trade-offs highlight the complementary nature of volumetric and section-based strategies and suggest that future workflows will increasingly integrate multiple modalities.

As illustrated in Fig. 2, the diversity of available ex vivo analytical strategies that can be applied following lung extraction enables a comprehensive structural and molecular characterization at multiple scales. Different tissue processing procedures, including optical clearing, FFPE fixation, and O.C.T. embedding, can be best applied to, and merged with, distinct downstream applications, including LSFM, CFT, conventional histology, and transcriptomic profiling (e.g., RNA-seq). This modular framework exemplifies how specific experimental goals guide the selection of the most appropriate analytical route that best supports multiscale lung disease modeling.

Nonetheless, significant challenges remain, including technical complexity, handling large data volume and resource demands, as well as the need for standardized protocols and computational tools for robust quantification and cross-study comparability.

Omics technologies

Omics technologies, encompassing transcriptomics, proteomics, and metabolomics, offer high-throughput and unbiased insights into molecular pathways involved in disease progression and response to treatment. By allowing the identification of gene expression signatures,

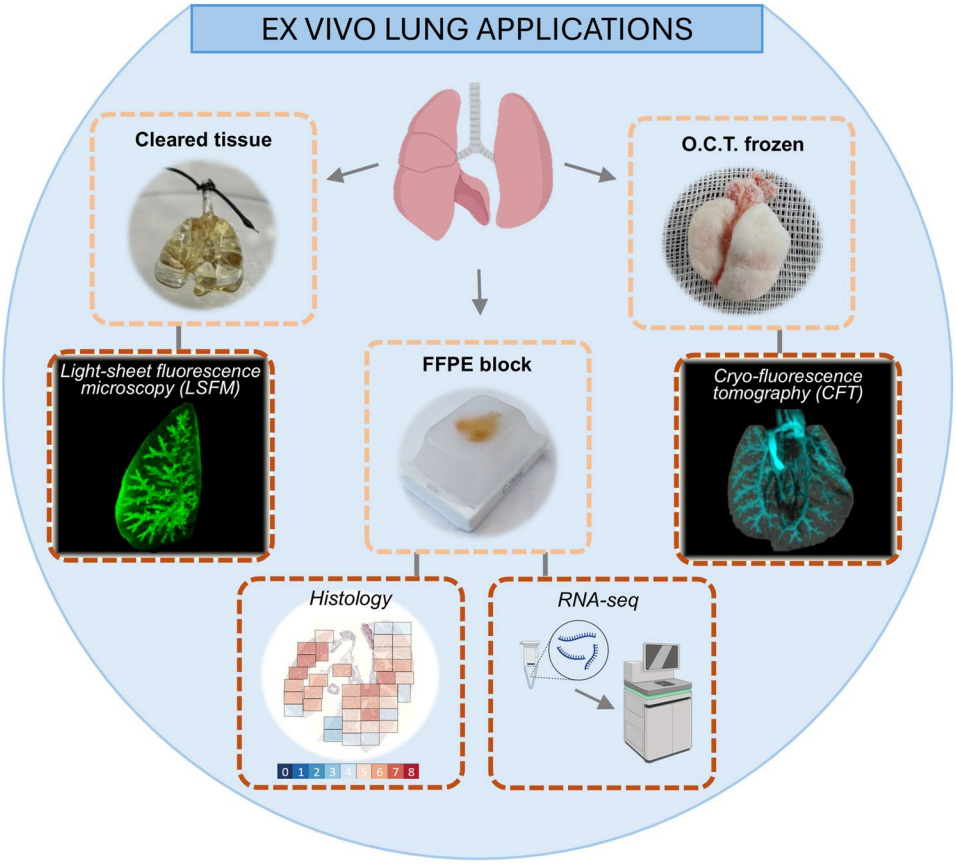

Fig. 2 Overview of ex vivo lung applications for structural and molecular analysis. After experimental endpoints, harvest lungs (center) can be processed using different methods depending on the type of analysis required. On the left branch, the tissue clearing and 3D imaging workflow is illustrated: lungs are rendered optically transparent using chemical clearing protocols, preserving morphology and enabling light-sheet fluorescence microscopy (LSFM), for high-resolution 3D visualization of entire organ. On the right branch, samples are embedded in Optimal Cutting Temperature (OCT) compound and cryo-preserved to maintain native fluorescence and molecular integrity. These samples are then processed using Cryo-fluorescence tomography (CFT), a technique that combines serial sectioning with fluorescence detection for high-resolution 3D imaging of large frozen tissue blocks. The bottom branch shows the Formalin-Fixed Paraffin-Embedded (FFPE) block processing pathway, a standard preservation method for long-term preservation. FFPE blocks are compatibility with multiple downstream analysis, including histology, for assessment of tissue architecture, inflammation, and fibrosis and RNA sequencing (RNA-seq), which provides genome-wide transcriptomic profiling of defined anatomical regions

protein interaction networks, and metabolic alterations at a systems level, omics approaches have great potential for biomarker discovery.

In pulmonary fibrosis, multi-omics strategies have provided important insights into disease mechanisms that may represent the necessary knowledge basis for the development of personalized therapeutic treatments. Using spatial transcriptomics in a bleomycin-induced mouse model of pulmonary fibrosis, Li et al. mapped cell-type-specific gene expression changes across different stages of disease progression and resolution, identifying key epithelial transitions and candidate self-resolving genes such as Prkca [43]. In a separate study, combined transcriptomic and metabolomic profiling uncovered two gene subnetworks closely linked to pulmonary function decline and showed that pharmacological inhibition of CB1R reduced fibrotic burden, thus supporting its therapeutic potential [44]. In a clinical setting, multi-omics integration enabled the identification of two molecular subtypes of IPF, one of which was characterized by a more severe disease condition and faster progression, underscoring the potential of multi-omics approaches to stratify patient risk and guide therapeutic decisions [45].

Despite these favorable features, omics approaches face several challenges. Lung tissue heterogeneity can dilute cell-specific signals, and bulk omics data often mask the contributions from rare or spatially restricted cell populations [46]. Additionally, the high dimensionality of omics datasets necessitates advanced bioinformatics and statistical analysis tools, and careful validation is required to avoid false discoveries or overfitting. Single-cell omics technologies, which enable the identification of disease-relevant cellular subpopulations at high resolution, can help to address some of these issues [46, 47]. To further improve spatial precision and data interpretability, omics results can be integrated with imaging and histological information. For instance, spatial transcriptomics platforms can localize gene expression within histologically defined tissue areas, while co-registration of in vivo micro-CT datasets with histological sections can provide anatomically aligned 3D references [48]. These workflows enhance sampling accuracy, enable spatial biomarker discovery, and support the development of predictive models combining morphological and molecular features.

Furthermore, multi-omics integration enhances systems-level understanding and biomarker identification [49], while machine learning and network-based tools improve complex data analysis, reducing false discoveries and improving reproducibility [50, 51]. In summary, omics technologies offer unparalleled depth of molecular analysis and are viewed as instrumental to the advancement of personalized medicine in pulmonary research. Proper integration with imaging, spatial mapping, and computational analysis represents a crucial step to maximize their translational relevance in pulmonary research.

In-vivo imaging

Non-invasive imaging enables longitudinal studies in intact animals, reducing biological variability, the number of animals required, and enhancing translational relevance. However, small-animal imaging poses specific challenges, including limited imaging volume and spatial-temporal resolution, a not always optimal signal-to-noise ratio, and ease of probe localization. For lung imaging, the most suitable technologies are micro-CT, optical imaging, MRI, and nuclear imaging. Among these, micro-CT is often predominant in preclinical lung imaging, not only due to its low maintenance cost and ease of use, but also because of its high spatial resolution and high air-tissue contrast. The widespread clinical application of High-Resolution Computed Tomography (HRCT) in pulmonary diagnostics further corroborates its high translational value. For these reasons, and given its capacity to provide whole-lung structural and functional information, micro-CT will receive somewhat greater emphasis compared to other imaging modalities described herein. The following sections outline the physical principles and type of structural, functional, and molecular information each imaging technology can provide, emphasizing how multimodal imaging can overcome the limitations of individual approaches, thus enabling a more comprehensive assessment of lung pathology.

Micro-Computed Tomography (Micro-CT)

Image acquisition and reconstruction

Micro-CT provides high-resolution, three-dimensional imaging of small animal anatomy by acquiring X-ray projections at equidistant angular steps and reconstructing volumetric datasets using algorithms such as filtered back-projection (FBP) or iterative reconstruction [52]. Reconstructed voxel intensities, expressed in Hounsfield Units (HU), allow quantitative evaluation of lung aeration and tissue density.

Respiratory motion is a major source of artifacts in thoracic imaging and requires the use of respiratory gating. Prospective gating synchronizes projection acquisition with a specific phase of the breathing cycle, ensuring regular angular sampling but prolonging acquisition time. Retrospective gating, in contrast, continuously acquires data and classifies projections post hoc according to the respiratory phase, reducing acquisition time but increasing the risk of angular under-sampling and reconstruction artifacts [53–56]. Modern systems support dual-phase (inspiration/expiration) reconstructions, enabling functional assessment of tidal volumes and ventilation distribution. In longitudinal and quantitative studies, standardized gating parameters, scanner

calibration, and phantom-based quality control is essential to ensure reproducible HU-based metrics across time and centers [52, 57, 58].

Figure 3 provides an overview of key structural and functional endpoints that can be derived from micro-CT analysis in preclinical lung research. With regard to structural/morphological information (*left*), densitometric analysis and airway segmentation allow the quantification of parenchymal abnormalities and airway remodeling. As for functional information (*right*), inspiratory-expiratory acquisitions and multi-volume imaging provide surrogate measures of regional ventilation and spirometry. Notably, the circular scheme underlines the notion that these readouts are not isolated outputs, but can be combined within the same imaging pipeline, thus enabling an integrated structure–function analysis.

Airways

Airway segmentation and morphological analysis in micro-CT provide essential structural information to precisely delineate airway architecture and remodeling in preclinical models. In clinical CT, high contrast between air-filled lumens and surrounding tissues enables robust automated segmentation [59], whereas in vivo micro-CT in small animals is somewhat limited by a suboptimal resolution and motion artifacts.

In vivo, breath-hold or iso-pressure inflation protocols allow accurate lumen-based and fast-marching airway segmentation [60, 61], whereas recent deep learning algorithms markedly strengthen the robustness of lung, heart, and airway segmentation for longitudinal studies, under free-breathing conditions in fibrotic lungs [62]. The resulting 3D reconstructions yield relevant structural metrics, such as airway diameter, length, and branching angle, enabling morphometric analysis across different disease models and strains [61, 63]. Notably, Lederlin et al. introduced the Peribronchial Density Index derived from gated in vivo micro-CT as a surrogate marker of airway remodeling, correlating with bronchial smooth muscle hypertrophy and hyper-responsiveness [64, 65]. These structural features, whether directly derived from segmentation or indirectly inferred, can inform computational fluid dynamics (CFD) models, linking airway

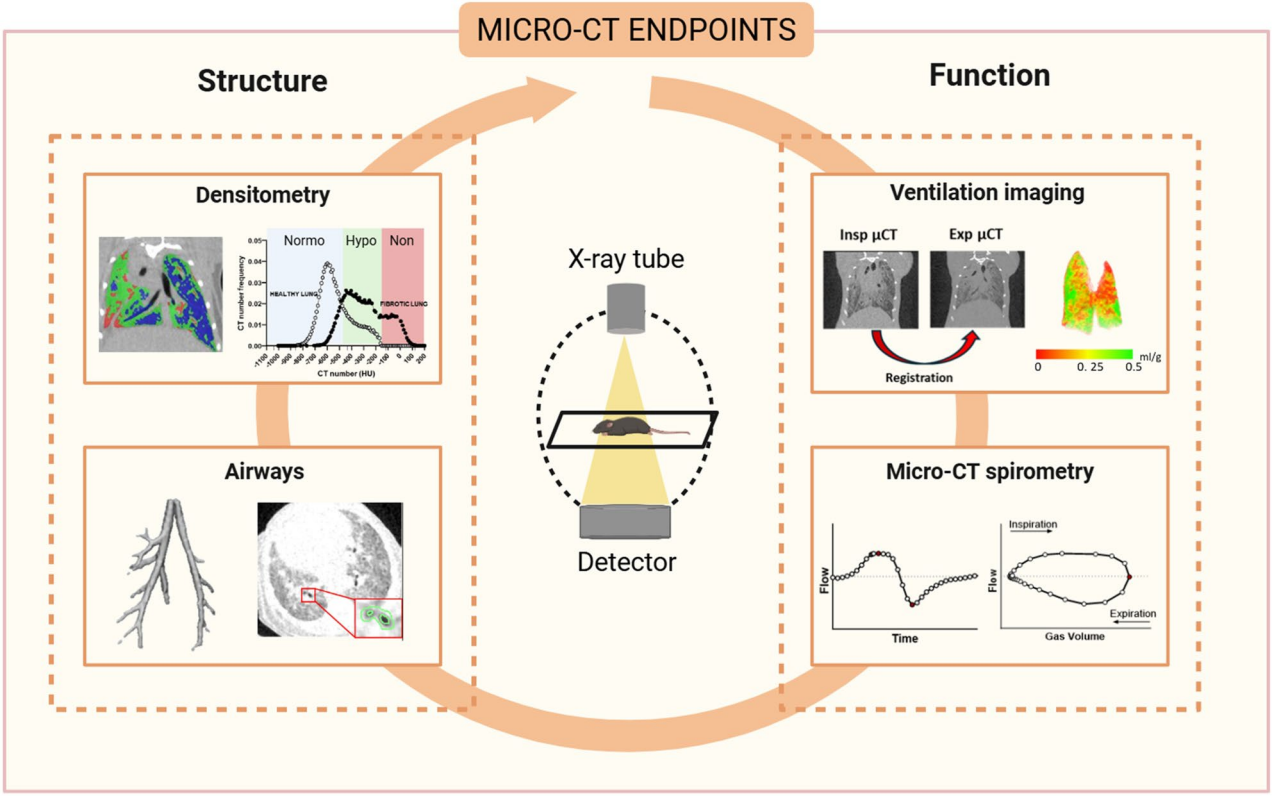

Fig. 3 Micro-CT endpoints for structural and functional lung analysis. The figure illustrates the key quantitative endpoints that can be derived from micro-CT imaging to assess both lung structure and function. The circular scheme emphasizes that these readouts are not isolated but can be integrated within the same imaging workflow, enabling comprehensive structure–function analysis. On the left, structural biomarkers are highlighted, including densitometry to evaluate parenchymal alterations, highlighting aeration compartments such as normo-aerated (blue), hypo-aerated (green), and non-aerated (red) regions, and airway segmentation to quantify airway remodeling. On the right, functional biomarkers are shown, derived from inspiratory-expiratory imaging or multi-volume acquisitions, allowing the assessment of regional ventilation and spirometry-like readouts, respectively

geometry to ventilation heterogeneity and particle deposition patterns in health and disease [66].

Densitometry

Micro-CT densitometry enables quantitative assessment of parenchymal abnormalities such as fibrosis and emphysema. Accurate lung segmentation is essential for this purpose and has traditionally relied on semi-automated methods, often requiring manual intervention [67, 68]. Recent deep-learning approaches have substantially improved automation and robustness, including CNN- and U-Net–based architectures for lung and disease-specific region detection [62, 69–71]. More recently, a generic deep-learning model trained on longitudinal micro-CT datasets has demonstrated robust, high-throughput lung segmentation across diverse disease models, supporting standardized analysis pipelines [72].

Segmented lungs are analyzed via histogram-based distributions of Hounsfield Units (HU), with shifts in peak values indicating pathology - higher HU in fibrosis, lower HU in emphysema. Derived metrics such as mean lung attenuation (MLA), median, skewness, and kurtosis further describe tissue heterogeneity [69, 73–75]. Longitudinal studies have demonstrated that micro-CT densitometry can sensitively monitor disease progression and therapeutic response [76, 77], and can even guide optimization of animal models by distinguishing resolving from non-resolving fibrotic patterns in vivo [76, 78]. While HU-based aeration thresholds are well established in clinical CT [79, 80], their application in preclinical imaging still lacks standardization. Some studies have proposed empirical thresholds for animal models [81–83], but some variability remains due to differences in animal species, disease models, and scanner calibration.

From a translational point of view, micro-CT densitometry closely mirrors clinical workflows and enables the extraction of analogous biomarkers, thus supporting the alignment of preclinical findings with clinical outcomes. Standardization of HU thresholds and segmentation strategies would enhance reproducibility and cross-species comparability, advancing preclinical-to-clinical translation.

Multi-volume acquisition for functional analysis

Micro-CT imaging at multiple lung volumes, i.e., by measuring lung volume and density at end-expiration and end-inspiration, enables non-invasive quantification of global lung function. From these measurements, tidal volume (Vt) and functional residual capacity (FRC) can be estimated as imaging analogs of spirometry, and have been validated in models of radiation- and bleomycin-induced lung injury [77, 84, 85]. These metrics serve as radiological surrogates of pulmonary function, offering spatial resolution and longitudinal monitoring. Recent reviews have further emphasized the value of CT-based ventilation assessment for characterizing regional impairment and disease heterogeneity, highlighting its translational potential from preclinical models to human lung disease [86].

Inspired by clinical applications [87–91], ventilation imaging based on density changes between respiratory phases has been adapted to preclinical models of fibrosis, enabling voxel-wise computation of specific gas volume differences (ΔSVg) as a surrogate indicator of regional ventilation [92]. These maps allow classification of lung regions as ventilated, hypoventilated, or non-ventilated (fibrotic), contributing to disease phenotyping, therapeutic treatment monitoring, and early detection of dysfunction [93, 94]. Multiphase micro-CT, which reconstructs approximately 30 respiratory phases, provides a dynamic functional assessment of lung function across the full breathing cycle, allowing derivation of gas volume, flow curves, and flow-volume loops [95]. In pulmonary fibrosis models, this method revealed obstructive and restrictive patterns over time and allowed the tracking of therapeutic treatment-associated effects on tidal volume and ventilation dynamics, well correlated with histological and other invasive readouts. Alternative low-dose approaches have also been explored. Retrospective gating of raw projections enables functional assessment without extra dose or acquisition time and has been applied to disease models such as Duchenne muscular dystrophy [96]. Similarly, a planar X-ray-based technique was found to produce results in very good agreement with micro-CT-derived volumes and outperformed plethysmography for sensitive, longitudinal lung function monitoring [97].

Advantages, challenges, and translational value

Micro-CT offers high spatial resolution and 3D anatomical coverage, and allows non-invasive, longitudinal imaging, providing quantitative metrics on lung structure and function. These capabilities make it a key experimental tool for studying lung remodeling, fibrotic progression, and therapeutic response under controlled preclinical conditions. Standardized protocols and quality control enhance reproducibility across multiple studies. Limitations include radiation exposure, motion-related artifacts, low soft tissue contrast without contrast agents, operation costs, and experienced technical personnel for image post-processing.

Despite these technical challenges, μCT-derived biomarkers such as mean lung attenuation, airway morphometry, and voxel-wise ventilation maps closely parallel HRCT metrics used in patients. Quantitative CT is increasingly applied in ILD and COPD to monitor disease burden and treatment efficacy [98–101], thus establishing a direct translational bridge between preclinical imaging readouts and clinical endpoints.

Optical imaging

Optical imaging (OI) comprises a set of non-invasive techniques that use visible and near-infrared (NIR) light to visualize and quantify biological processes in vivo. It offers high sensitivity and enables longitudinal studies in small animal models. In preclinical pulmonary research, the most common modalities are bioluminescence imaging (BLI) and fluorescence imaging (FLI), which differ by signal generation: BLI detects light produced by enzymatic reactions without external illumination, whereas FLI requires excitation of fluorescent agents followed by emission detection. Both use endogenous or exogenous contrast, and targeted or activatable probes enhance specificity through wavelength-shifted emission, thus enabling molecular imaging even in complex environments such as the lungs.

Bioluminescence Imaging (BLI)

Figure 4a illustrates the basic principles of BLI, which detects luciferase-mediated light emission. Genetically encoded and delivered via plasmid or lentivirus vectors, luciferase, a widely used reporter gene, catalyzes oxygen-dependent oxidation of its luciferin substrate with the concomitant production of a bioluminescent signal. This signal can be detected non-invasively and quantified using in-vivo imaging systems, without the need for external illumination, thereby minimizing background noise, while achieving femtomolar sensitivity. The representative mouse images in Fig. 4a show the spatial distribution of the signal, which reflects expression of the luciferase reporter under the control of a target gene promoter of interest in a given tissue.

In vivo gene delivery is a rather simple and effective method to achieve transient expression of a target gene or its reporter gene derivative in the lungs. BLI can monitor the activation of specific molecular pathways in the same animal over time because the transferred plasmid DNA contains luciferase under the control of an inducible gene promoter of interest, such as NF-kB, IL-8, or MMP1, that responds to particular treatments, stressors, and other experimental conditions. This can be particularly valuable for understanding the mode of interaction of a candidate drug with specific molecular targets in vivo [102–105].

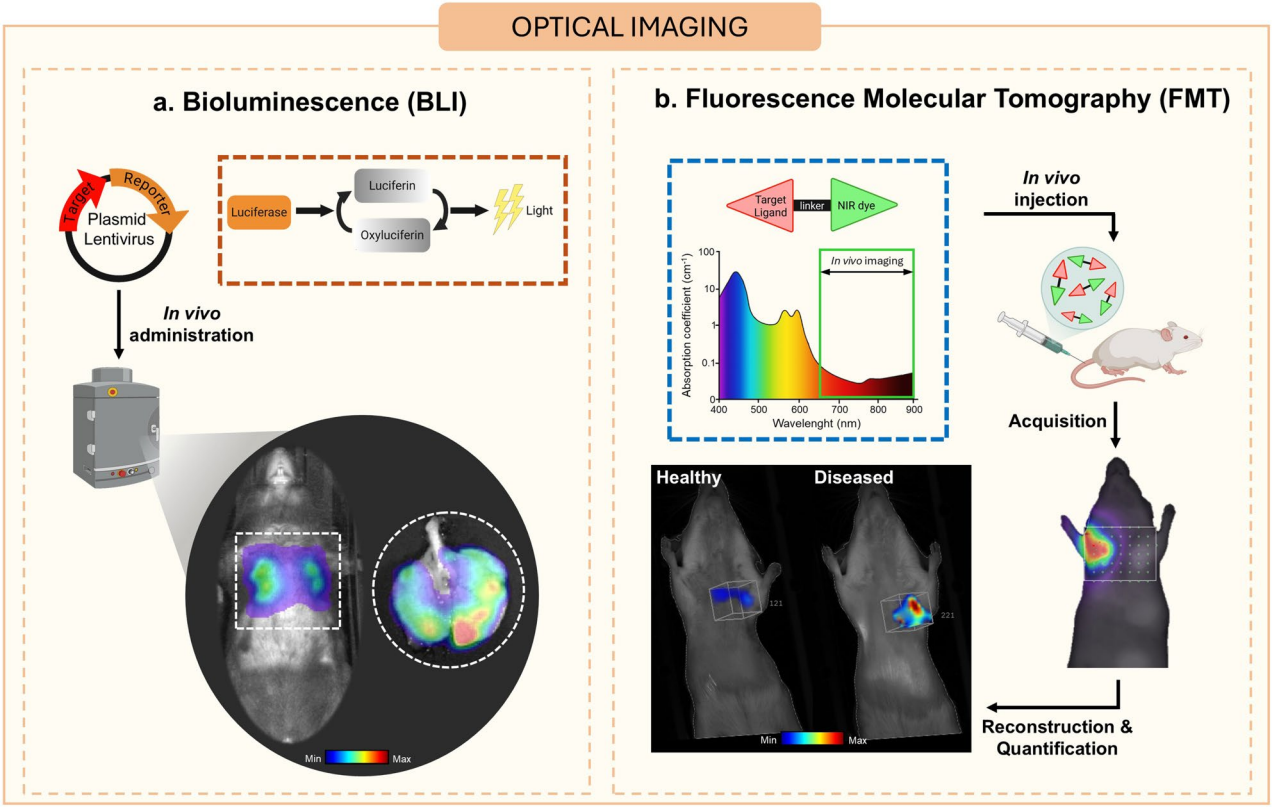

Fig. 4 Overview of two optical imaging techniques commonly used in preclinical research. **a** The bioluminescence (BLI) workflow relies on luciferase-mediated light emission following luciferin oxidation, and enables non-invasive visualization of reporter gene expression. The images illustrate the spatial distribution of bioluminescent signals in vivo. **b** The Fluorescence Molecular Tomography (FMT) approach relies on near-infrared (NIR) fluorescent probes for molecular targeting. After systemic administration, 3D tomographic reconstruction enables quantitative assessment of signal distribution. The emission spectrum highlights the optimal NIR window (650–900 nm), while representative images compare healthy and diseased animals, illustrating disease-related changes in probe uptake

Using Lenti or Adeno viral vectors to deliver genes directly into living organisms is another option for gene transfer, which usually allows a more stable and consistent expression of reporter genes. Although both methods are quite challenging, they allow a more consistent expression of reporter genes, especially in the case of ad hoc generated transgenic animals that can be utilized to produce stable and tissue-targeted reporter gene expression. The use of viral vectors is subject to biosafety regulations and requires certified containment, especially for infectious disease models, and the generation of transgenic animal models is rather time-consuming and expensive [106].

Cells genetically engineered to express a reporter gene can be injected into mice (e.g., intratracheally, endovenously, or through other routes) and their persistence, growth, or engraftment can be tracked longitudinally. This method is often employed for monitoring disease progression and response to treatment in lung tumors, infectious disease models, and cell therapy.

All the above approaches are widely used in pulmonary research for monitoring tumor progression, immune cell behavior, infection dynamics, and virus-mediated gene delivery, enabling real-time, non-invasive assessment of biological processes and transduction efficiency in the lungs [107, 108].

To overcome the intrinsic limitations of planar, 2D imaging, bioluminescence tomography (BLT) has been developed as a means to integrate surface photon emission data with anatomical priors (e.g., CT or MRI) as well as light propagation models in order to reconstruct 3D source distributions. While early studies used finite element-based methods to localize bioluminescent signals in tumor-bearing mice [109], more recent advances, such as multispectral differential reconstruction, have improved localization accuracy and anatomical fidelity [110].

Significant improvements in tissue penetration with reduced light scattering have been achieved with the use of red- and near-infrared luciferase systems such as AkaLuc/AkaLumine and NanoLuc, which provide an enhanced imaging sensitivity in fibrotic or inflamed lungs [111]. Dual-modality approaches, such as BLI combined with micro-CT or MRI, further enrich anatomical context details. For example, red-shifted BLI integrated with micro-CT has enabled simultaneous tracking of transplanted Mesenchymal Stem Cells (MSC) and structural lung changes in models of pulmonary fibrosis, showing that antifibrotic therapies increase MSC retention and efficacy [112]. BLI is also widely applied in infection models using luciferase-tagged pathogens, allowing real-time, semi-quantitative monitoring of lung infection dynamics and response to treatments [113–115].

While BLI yields quantitative data, typically reported as photon counts normalized with respect to acquisition parameters, its clinical translation is limited by the need for genetic reporters and substrate administration. Nonetheless, the development of hybrid reporter systems compatible with clinical modalities such as PET or SPECT may expand its translational potential in the future [116].

Fluorescence Imaging (FLI)

Figure 4b outlines Fluorescence Molecular Tomography (FMT), an in vivo imaging technique based on the excitation of internalized fluorophores and detection of emitted light at longer wavelengths. Near-infrared (NIR) dyes, often conjugated to target-specific antibodies or other ligands, are commonly used as fluorophores because of their deep tissue penetration capacity and low background autofluorescence, both of which are key requirements for lung imaging. Following injection, fluorescence signals are acquired using a sensitive, charge-coupled device (CCD) or complementary metal-oxide-semiconductor (CMOS) cameras and reconstructed into 3D tomographic images for quantitative analysis. The fluorophore absorption spectrum indicates the optimal NIR window (650–900 nm) for in vivo imaging. Comparative FMT images of healthy and diseased animals are shown in Fig. 4b (*lower panel*), which highlights differences in signal distribution and intensity that reflect underlying molecular or cellular alterations.

FLI supports multiplexed and activatable probe strategies, allowing the detection of specific molecular processes. In pulmonary fibrosis models, it has been applied to the visualization of oxidative stress by targeting reactive oxygen species (ROS), including hydrogen peroxide and hypochlorous acid, with the use of mitochondria-targeted NIR probes. These studies have correlated ROS dynamics with fibrotic severity and response to therapy, including signal reductions after nintedanib treatment [117, 118]. Nitric oxide (NO)-specific probes have similarly been used to monitor fibrotic progression and evaluate drug efficacy [119]. In models of lung inflammation and acute lung injury, activatable probes targeting inflammatory mediators have been employed to monitor neutrophil activation and immune cell recruitment with high specificity [120, 121]. In lung cancer research, imaging of tumor-associated enzymes and microenvironmental biomarkers has been used to delineate tumor burden and to monitor response to therapy [122, 123]. Fluorescently labeled antibodies and antibody fragments are increasingly employed for in vivo imaging due to their high target specificity and strong binding affinities, which enable the development of highly sensitive imaging agents [124, 125]. In pulmonary disease models, antibodies targeting extracellular matrix components have been used to monitor fibrotic remodeling, while tumor-associated antigens

and immune cell markers have been visualized in lung cancer studies [126, 127]. Despite challenges such as renal accumulation or potential alterations upon labeling, antibody- and nanobody-based imaging agents provide a versatile platform for in vivo phenotyping, target validation, and therapy monitoring [124].

Combination of FLI with anatomical imaging provides deeper insights into complex disease models. For instance, the fluorescence signal of indocyanine green (ICG) co-administered with bleomycin has been used in combination with micro-CT to longitudinally track fibrotic remodeling and emphysematous alterations, with persistent fluorescence in lung tissue positively correlating with disease severity [128].

While FLI provides high sensitivity and molecular specificity, particularly in the case of NIR fluorophores, it also faces some limitations, such as limited tissue penetration, scattering, and autofluorescence that can affect the accuracy of quantitative measurements in thoracic imaging. External excitation can introduce background noise, though this is significantly reduced in the NIR range. Fluorophore biodistribution and off-target effects also require careful consideration. Despite these challenges, FLI's compatibility with activatable and multiplexed probes makes it a powerful tool for imaging dynamic molecular events taking place in pulmonary disease. When combined with structural imaging modalities such as micro-CT, FLI enhances spatial localization and disease severity assessment. Although clinical translation is restrained by tissue optics and regulatory hurdles, ongoing advances in instrumentation and probe design continue to expand its range of applications in preclinical studies.

Advantages, challenges, and translational value

Optical imaging provides unmatched molecular sensitivity for tracking dynamic biological processes in vivo, offering mechanistic insights into pulmonary disease progression and therapeutic response. However, its clinical translation remains limited by tissue penetration, the need for exogenous probes, and stringent regulatory requirements. Only a few fluorescent probes have received medical approval, as their development and validation are complex and costly. A notable translational success is fluorescence-guided surgery, particularly using indocyanine green, which enables real-time tumor visualization and improves surgical precision [129, 130]. Although clinical use in pulmonary imaging is still limited, the success of NIR fluorescence in oncology highlights the potential for applying similar optical approaches in thoracic surgery and bronchoscopy [131]. These developments bridge molecular optical imaging from preclinical studies to clinical applications, reinforcing its translational relevance.

Nuclear imaging

Image acquisition

Nuclear imaging detects radiation emitted by small amounts of radioactive tracers administered to the animal, enabling the noninvasive visualization of physiological and molecular processes. In PET, positrons emitted by radiotracers such as 18F-Fluorodeoxyglucose (18F-FDG), which accumulate in tissues according to metabolic activity, annihilate with local electrons, producing pairs of gamma photons traveling in opposite directions, whose detection allows to reconstruct the tracer's distribution. SPECT, in contrast, uses gamma-emitting isotopes and detects single photons to localize tracer uptake. Both imaging modalities reveal the spatial and temporal dynamics of target-specific tracers or pharmaceutical (so called "theranostic") compounds. Due to the relatively low spatial resolution of PET, CT is often combined to provide anatomical reference, improve localization of radiolabeled probes, and enable attenuation as well as scattering correction [132], ultimately enhancing the accuracy and quantitation reliability of PET images. Figure 5a shows a total-body [^{18}F]-FDG-PET scan of a mouse, illustrating the typical biodistribution of the tracer, with high uptake in the heart and bladder, overlaid on a CT-based skeleton reconstruction as an anatomical reference. Most commonly, a low-dose CT scan acquired immediately before or after PET serves for both purposes. To further reduce radiation exposure, recent deep learning–based methods have been developed to synthetically generate CT-like images directly from PET data, allowing attenuation correction and anatomical co-registration without an actual CT acquisition [133,134]. Increasing usage of hybrid PET/CT systems has driven the development of PET/MRI platforms that combine molecular sensitivity with high soft tissue contrast [135]. Advances in Silicon Photomultipliers (SiPM)-based PET detectors, compatible with magnetic fields, have enabled even more flexible PET/MRI configurations, including compact PET inserts that are usable both inside and outside of the MRI bore. These enable simultaneous acquisition with enhanced flexibility in small animal imaging workflows, without compromising image quality or quantitation performance [136,137].

Molecular probes in pulmonary research

Recent advances have led to the development of a broad range of molecular probes allowing visualization of key biological processes involved in pulmonary diseases, including metabolic alterations, inflammation, immune cell recruitment, and tissue remodeling, in a noninvasive manner, with quantitative outputs that go beyond anatomical imaging captured by CT only.

Metabolic and hypoxia-sensitive tracers provide functional readouts of the pulmonary microenvironment.

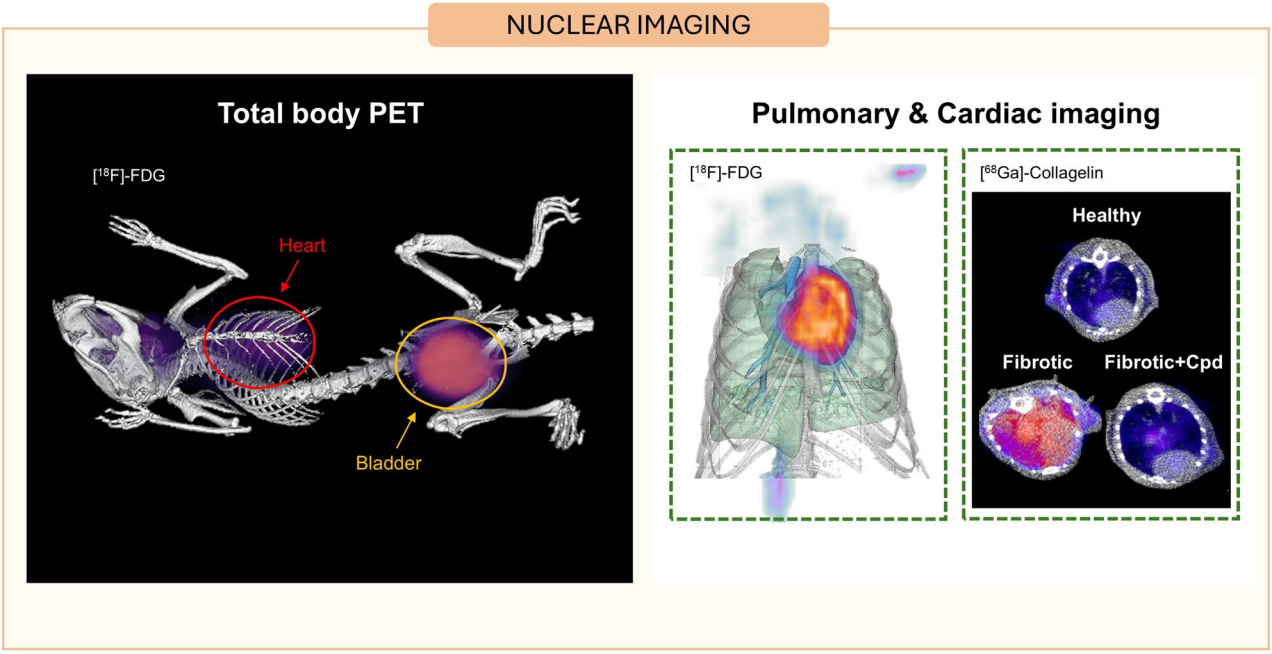

Fig. 5 Nuclear imaging applications in preclinical cardiopulmonary research. The left panel shows total-body Positron Emission Tomography (PET) using [18F]-Fluorodeoxyglucose ([18F]-FDG) in a mouse, with tracer uptake in metabolically active organs such as the heart and bladder. The right panel highlights organ-specific applications: [^{18}F]-FDG PET for combined pulmonary and cardiac imaging, and [^{68}Ga]-Collagelin PET to visualize collagen deposition in fibrotic lungs. Comparative axial slices illustrate signal differences among healthy, fibrotic, and drug-treated (fibrotic+Cpd) animals, demonstrating the utility of molecular tracers for assessing disease progression and therapeutic response

While [^{18}F]FDG remains a widely used marker of glucose metabolism and has been applied to idiopathic pulmonary fibrosis (IPF) due to TGF-β1–induced upregulation of glucose transporters in myofibroblasts [138], its limited specificity, namely, uptake by both inflammatory and fibrotic tissues, has raised concerns about its real diagnostic value [139]. As a result, hypoxia-sensitive tracers, such as [^{18}F]-Fluoromisonidazole (^{18}F-FMISO), have been explored to detect early hypoxic regions within fibrotic lungs, whose appearance may precede collagen deposition and drive fibrotic progression [140].

Probes targeting tissue remodeling allow assessment of extracellular matrix deposition and fibroblast activation. Collagen-specific PET tracers, such as [^{68}Ga]-Collagelin and [^{68}Ga]-CBP8, have shown promise for quantification of collagen deposition and monitoring the response to antifibrotic treatments [141]. Figure 5b shows how [^{68}Ga]-Collagelin PET distinguishes fibrotic, healthy, and drug-treated lungs in mouse models of pulmonary fibrosis, with a reduced tracer uptake indicating therapeutic efficacy. Translating this approach to humans, Montesi et al. demonstrated in a phase 2 trial that [^{68}Ga]-CBP8 PET effectively quantifies type I collagen in IPF patients, with a reduced tracer uptake after 12 weeks of bexotegrast treatment, a dual $\alpha v\beta_6/\alpha v\beta_1$ integrin inhibitor. These results, corroborated by Dynamic Contrast Enhanced (DCE)-MRI indicating improved perfusion and decreased extracellular volume, highlight the utility of molecular imaging for monitoring early response to treatment and lung remodeling [142]. Similarly, fibroblast activation protein (FAP) can be visualized using FAPI-labeled tracers such as [^{68}Ga]FAPI-04, which have displayed high uptake levels by fibrotic lung tissue in both preclinical models and in patients, providing important insights into disease progression and new potential therapeutic targets [143, 144]. In parallel, molecular imaging of integrin $\alpha v\beta_3$, a key regulator of TGF-β activation and myofibroblast differentiation, and somatostatin receptor 2 (SSTR2), expressed on epithelial and inflammatory cells, enables complementary, stage-specific characterization of disease. In mouse models of bleomycin-induced lung fibrosis, uptake of the integrin $\alpha v\beta_3$-targeted tracer [^{177}Lu]DOTA-RGD peaks during early inflammation, whereas the SSTR2-targeted tracer [^{177}Lu]DOTA-NOC accumulates at later fibrotic stages, supporting their use in disease stratification and timing of interventions [145].

Tracers targeting immune activation and inflammation have proven useful in capturing early disease dynamics predictive of fibrotic evolution. CCR2-targeted PET probes enable the detection of CCR2$^+$ cells during both the inflammatory and fibrotic phases of disease development in various mouse models [146], offering valuable insights into immune responses in different pathological settings. Notably, CCR2- or CXCR4-targeted tracers allow visualization of monocyte recruitment and immune cell infiltration during the inflammatory and fibrotic

phases [146–148]. CD206-targeted molecular imaging with the radiotracer [^{99m}Tc]-tilmanocept demonstrated accurate quantification of CD206^{+} macrophages in experimental lung fibrosis and, importantly, allows prediction of disease progression and response to antifibrotic treatments [149]. In parallel, enzymes involved in extracellular matrix remodeling have emerged as promising targets for molecular imaging; notably, LOXL2 has been visualized using the [^{111}In]-labeled antibody AB0023, with lung uptake correlating with fibrosis burden and treatment response, supporting its role as a theragnostic target [150]. Additional efforts have focused on imaging cysteine cathepsin proteases activity, a biomarker of macrophage activation and tissue remodeling, thus providing a complementary approach to monitor inflammation-driven fibrogenesis [151].

Advantages, challenges, and translational value

Nuclear imaging represents a powerful tool for preclinical pulmonary research, enabling non-invasive, quantitative, and longitudinal visualization of key biological processes such as inflammation and tissue remodeling. Its high sensitivity and molecular specificity make it ideal for early disease detection and monitoring therapy. When combined with anatomical imaging (e.g., CT or MRI), it provides spatial context information to functional signals. Importantly, by targeting disease-relevant pathways, many radiotracers have theragnostic capabilities, serving as both diagnostic and therapeutic compounds. Despite these advantages, nuclear imaging is limited by its relatively low spatial resolution, potential confounding effects caused by tracer uptake across different disease processes, and the need for specialized and very expensive infrastructures, as well as a radiochemistry facility. Nevertheless, its translational relevance is well established: targeted radiotracers such as [^{68}Ga]CBP8 and [^{68}Ga]FAPI have demonstrated strong cross-species consistency, correlating with fibrosis activity and treatment response in both animal models and patients with ILD and IPF [152, 153]. These tracers exemplify how preclinical nuclear imaging directly informs clinical biomarker development and therapeutic monitoring, reinforcing its role as a bridge between molecular mechanisms and patient-level imaging biomarkers.

Magnetic resonance imaging

Anatomical MRI

Proton MRI is emerging as a valuable alternative to CT in preclinical lung research, offering non-invasive and radiation-free imaging of pulmonary architecture. While CT provides superior spatial resolution, MRI benefits from a wider range of contrast mechanisms, including T2-weighted and diffusion-weighted imaging, which are often more sensitive to subtle tissue alterations [154–156]. However, lung anatomical MRI is technically challenging due to the low proton density of the air-filled parenchyma and magnetic susceptibility differences at air-tissue interfaces [157]. The latter is further exacerbated in preclinical research by the small volume of the lungs (~ 1 mL on average) and the high respiratory and cardiac rates of rodents [158]. These factors cause rapid signal decay and motion-related artifacts. Furthermore, to compensate for the very small lung volumes, preclinical MRI systems typically operate at high magnetic field strengths (≥ 4.5 T) to boost signal-to-noise ratio and achieve adequate spatial resolution. Unfortunately, these higher magnetic fields exacerbate susceptibility-induced artifacts and further shorten T2* relaxation times [159, 160]. Despite these constraints, early proton MRI studies demonstrated that parenchymal remodeling could still be detected, with simple proton-based metrics capturing fibrosis progression and short-TE radial acquisitions tracking emphysema-related airspace enlargement through changes in signal intensity and T2* [156, 161].

Recent advances, such as ultra-short echo time (UTE) and respiratory-gated sequences, are addressing these limitations. In particular, UTE sequences have significantly improved signal detection in the lung by initiating data acquisition during the free induction decay, with echo times as short as 10 μs [162–165]. These improved methods enable accurate detection and longitudinal monitoring of lung pathology, including orthotopic tumors and fibrotic lesions, with a strong concordance between MRI findings and histological validation data [166, 167], and have recently been applied to bleomycin-induced injury to characterize structural alterations and perfusion-related alterations [168, 169]. Moreover, the integration of molecular contrast agents such as hProCA32.collagen (a collagen-targeting protein contrast agent) allows for spatially resolved detection of fibrotic remodeling with specificity for collagen-I, a key component of the fibrotic matrix [170]. This approach facilitates both early-stage diagnosis and staging of fibrosis, as well as evaluation of the response to therapeutic treatments. In parallel, quantitative T2-mapping at 9.4 T has recently been proposed as a sensitive, non-contrast biomarker of inflammation- and fibrosis-related parenchymal changes across multiple ILD models, complementing UTE-based structural imaging and bridging toward clinically relevant parametric MRI [171].

Functional MRI

In contrast to anatomical MRI, hyperpolarized-gas MRI offers a unique opportunity to assess lung function by directly visualizing gas distribution, ventilation, and gas exchange within the pulmonary airspaces. ^{129}Xe is an inert noble gas that can be hyperpolarized via spin-exchange optical pumping, thereby increasing its MR

signal. Once inhaled, hyperpolarized (HP) ^{129}Xe provides high-contrast images of the ventilated regions of the lung, overcoming the intrinsic limitation of low proton density and enabling direct mapping of dynamic pulmonary function. Moreover, due to its partial solubility in tissues and blood, ^{129}Xe can also be used to probe gas exchange processes at the alveolar-capillary barrier. In preclinical studies, HP ^{129}Xe MRI has been successfully employed to detect regional ventilation defects across several models of lung disease, including emphysema, radiation-induced injury, pulmonary hypertension, and bronchopulmonary dysplasia [172–175]. Recent advances in acquisition protocols have enabled free-breathing imaging, reducing dependence on mechanical ventilation and minimizing technical artifacts [173]. In addition to ventilation imaging, HP ^{129}Xe MRI can also be used to characterize pulmonary microstructure, providing a direct readout of alveolar and acinar geometry [176]. These metrics reflect the microanatomy of distal airspaces and are sensitive to pathological changes such as alveolar destruction and airway enlargement, thus enabling early detection of pulmonary diseases such as emphysema well before macroscopic tissue damage occurs.

Advantages, challenges, and translational value

In summary, preclinical MRI is a powerful tool for studying lung disease, which allows for radiation-free anatomical and functional imaging in small animals. Proton MRI enables high-resolution assessment of structural changes, while HP gas MRI provides detailed insights into ventilation and gas exchange efficiency, supporting longitudinal studies and early detection of pathology. Despite these advantages, preclinical MRI remains technically demanding. In particular, its accessibility and ease of usage are limited by the need for on-site hyperpolarization equipment, gas handling systems, and custom MR sequences. Moreover, MRI provides no information on tissue or extracellular matrix composition, which limits its usefulness in contexts where fibrosis or inflammation is the primary pathology. Nonetheless, recent advances in ^{129}Xe MRI have demonstrated strong translational potential, with clinical studies showing its ability to quantify ventilation defects and microstructural changes in ILD, COPD, and IPF patients [177–179]. These developments support MRI's emerging role in identifying non-invasive, functional biomarkers that bridge animal models and human lung physiology.

Challenges and opportunities in translating preclinical imaging biomarkers

In vivo imaging biomarkers offer the most direct link between preclinical and clinical research because they provide non-invasive, quantitative, and longitudinal readouts. Yet, their translation into clinical practice remains difficult, as several biological and methodological factors limit their applicability beyond controlled preclinical settings.

A first barrier is the limited ability of animal models to reproduce the chronic, heterogeneous and comorbid nature of human lung disease. Bleomycin-induced fibrosis is self-limiting and highly inflammatory [180], while COPD and asthma models capture only selected clinical features and often miss small-airway involvement, comorbidities, and exacerbations [181–183]. Interspecies, strain- and sex-related physiological differences introduce further variability, complicating direct translation to humans [180]. PET imaging clearly illustrates this issue. Targeted probes, such as collagen-binding tracers (^{68}Ga-CBP8) and FAP inhibitors, show consistent cross-species performance, detecting early collagen changes and correlating with disease activity in both animal models and patients [144, 152, 184–186]. By contrast, general metabolic or hypoxia tracers (e.g., ^{18}F-FDG, ^{18}F-FMISO), despite strong preclinical signals, show limited predictive or treatment-response value in clinical IPF, suggesting that non-specific pathways are more sensitive to interspecies and microenvironmental differences [187, 188].

A second barrier is methodological variability, which remains high across preclinical imaging platforms. Micro-CT metrics are sensitive to scanner geometry and calibration; MRI performance depends on pulse sequence design; and nuclear imaging varies with reconstruction algorithms and quality-control procedures [189, 190]. Unlike the clinical field, where initiatives such as the Quantitative Imaging Biomarkers Alliance (QIBA) and American College of Radiology (ACR) accreditation programs provide structured, widely adopted standards [191–194], preclinical imaging still lacks harmonized acquisition protocols and reference calibration frameworks. Although recent efforts are moving in this direction [190, 195, 196], surveys continue to show substantial variability across laboratories and highlight the need for shared datasets and standardized operating procedures [197].

Looking forward, multimodal imaging offers a concrete opportunity to reduce this translational gap. By integrating structural, functional, and molecular readouts, and by enabling cross-validation across modalities, multimodal pipelines improve the biological relevance of animal models and inherently promote more consistent acquisition and analysis workflows. In this sense, multimodal in vivo biomarkers offer a practical strategy toward more reproducible, clinically meaningful preclinical pulmonary research.

Matching in vivo and *end stage* data

The integration of imaging and histology has become increasingly valuable in preclinical models, as it enables microscopic findings to be interpreted within the broader context of whole-organ structure and disease distribution. In mouse models of bleomycin-induced fibrosis, strong correlations have been observed between longitudinal micro-computed tomography (μCT) measurements and histological fibrosis scores, thus supporting the use of μCT as a non-invasive surrogate of histopathology [198]. Ventilation-sensitive biomarkers derived from μCT have also been shown to correlate well with histomorphometric outcomes, capturing both functional and structural aspects of lung remodeling [92]. Recent efforts using deep learning have further automated μCT-based quantification, yielding fibrosis estimates consistent with manual histological scoring [77]. Further advancing this integrative approach, Van Heest et al. developed a multimodal imaging workflow combining in vivo μCT, targeted ex vivo NIR fluorescence imaging, in vivo SPECT, and digital histopathology [199]. The authors demonstrated a high spatial correlation between imaging markers and histological endpoints, including Ashcroft scoring and collagen quantification.

Despite an indisputable methodological advancement, it should be noted that the above approaches typically evaluate global correlations and do not incorporate spatial co-registration between imaging and histology.

From a technical perspective, a broad framework for histology-to-imaging registration has been delineated, incorporating rigid and elastic transformations, cutting-plane optimization, and quantitative similarity metrics to address tissue deformation and cross-modal alignment challenges [200]. This conceptual framework was recently implemented through the use of fiducial 3D-printed phantoms co-embedded with hard-tissue specimens, enabling retrospective registration of histological sections into the anatomical context provided by pre-acquired μCT volumes [201]. This approach allowed to achieve high spatial precision and proved to be robust across different tissue types and staining protocols. Building upon this system, Vincenzi et al. introduced a semi-automated pipeline that enables direct spatial matching between in vivo μCT images and ex vivo histological sections in a bleomycin-induced mouse model of lung fibrosis [202]. Such a method aligns the μCT volume with the histological cutting plane, enabling spatially resolved comparisons between imaging-derived densitometry and histological Ashcroft scores. This approach improves the spatial accuracy of image-to-histology correlations, reduces operator bias, and facilitates multimodal integration of structural, functional, and molecular datasets.

The above-mentioned studies highlight the growing role of imaging-informed histology in overcoming sampling bias and improving the spatial and temporal resolution of disease assessment in preclinical respiratory research. By enabling the co-localization of structural, functional, and molecular readouts at high spatial fidelity, such integrative approaches allow for a more comprehensive characterization of disease heterogeneity, progression, and response to therapy. Moreover, tying up longitudinal imaging to histological data enhances the biological interpretability of non-invasive biomarkers and strengthens their potential for translation into clinical settings.

Discussion

The present review highlights the increasing focus in preclinical research on the development of integrative approaches capable of bridging the gap between end-stage and in vivo data in pulmonary disease models. The main aim of these approaches is to derive translational biomarkers capable of enhancing the predictive power, efficiency, and ethical standards of preclinical research. Although significant progress has been made across different technical modalities, growing attention is being directed toward cross-modal integration to better capture the complexity of lung pathophysiology, while strengthening the clinical relevance of preclinical findings.

A major barrier in the development of translatable biomarkers is the inherent discrepancy between clinical and preclinical approaches to disease assessment. In clinical practice, lung function is typically evaluated with the use of global measurements such as spirometry, which are widely accessible, standardized, and suitable for longitudinal monitoring. However, these methods lack the spatial resolution required to detect early or region-restricted pathology. Furthermore, clinical imaging data are often assessed qualitatively through visual inspection, a mode of operation that is favored in routine healthcare because of its ease of application, affordability, and ease of interpretation. This approach, however, limits the extraction of quantitative imaging biomarkers and exhibits a reduced sensitivity to capture subtle or dynamic structural changes. In addition, clinical imaging protocols are typically optimized for speed, patient safety, and overall workflow efficiency, which often come at the cost of spatial and temporal granularity. By contrast, preclinical models offer a controlled environment in which advanced techniques, such as high-resolution imaging, histology, and molecular profiling can be applied to capture disease mechanisms at high spatial and temporal resolution. However, many of the most informative preclinical readouts, particularly histological and molecular endpoints, are terminal and cannot be acquired longitudinally in the same subject. This mismatch in the type of data, spatial scale, timing, and invasiveness contributes

to the translational gap: i.e., biomarkers identified in preclinical studies may not be directly measurable, interpretable, or relevant in clinical settings. The development of integrative platforms and novel analytical technologies aims at bridging this gap, aligning preclinical readouts with clinically applicable endpoints.

Each preclinical modality makes its own, often unique contribution to the characterization of pulmonary diseases, reflecting a diverse range of structural, functional, and molecular readouts. Histological techniques remain a cornerstone for high-resolution endpoint validation. Classical 2D histopathology enables detailed cellular analysis and molecular labeling, while 3D volumetric histology preserves spatial relationships and allows for whole-organ phenotyping. Omics technologies complement these approaches by providing unbiased molecular details, enabling the discovery of disease-related genes and pathways and candidate biomarkers. Micro-computed tomography allows high-resolution, non-invasive, and longitudinal assessment of lung anatomy and function, with a strong alignment to clinical imaging endpoints. Nuclear imaging techniques, such as SPECT and PET, enable sensitive and quantitative tracking of key biological processes such as inflammation and tissue remodeling, and can be co-registered with anatomical modalities to provide a functional context. Optical imaging, including bioluminescence and fluorescence imaging, is a highly sensitive tool for tracking molecular and cellular events, particularly when combined with activatable or multiplexed probes. Functional MRI, including hyperpolarized gas imaging, provides direct insight into ventilation and microstructural changes within distal airspaces, albeit at the price of a significant technical complexity. However, no single modality can comprehensively capture the complex nature of pulmonary disease. Trade-offs exist in spatial resolution, molecular specificity, quantifiability, and invasiveness. For instance, while μCT provides excellent anatomical detail, it lacks molecular sensitivity. Conversely, optical and nuclear imaging display a high specificity but are limited by spatial resolution and/or tissue penetration. Conventional histology, though highly informative, is endpoint-based, prone to sampling bias, and often operator-subjective; 3D histology, on the other hand, improves coverage but introduces technical and computational challenges. To overcome the above limitations and fully exploit the translational potential of preclinical findings, there is a growing interest in integrative platforms that combine in vivo and ex vivo data across different modalities and scales. By aligning structural, functional, and molecular information, these multimodal pipelines enable spatial and temporal co-registration of complementary datasets, support biomarker validation, and improve biological interpretability through direct correlation with high-resolution histology. In particular, registration of in vivo μCT with ex vivo histology allows precise spatial matching; molecular imaging adds dynamic functional insights; and 3D histological datasets help bridge the resolution gap between organ- and cellular-level imaging. Altogether, these integrative frameworks reduce data interpretation bias, elucidate disease and therapy mechanisms of action, and facilitate the identification of clinically relevant, translatable biomarkers that better represent the complexity of human pulmonary diseases. These integrative approaches have shown strong potential for clarifying disease mechanisms, identifying early microstructural alterations, mapping inflammation and fibrosis, and capturing region-specific abnormalities that may precede global functional decline. This contributes to more precise diagnostic criteria and improves early detection of chronic and rare pulmonary disorders. When combined with high-resolution histology and molecular profiling, multimodal imaging also helps link structural and functional patterns to specific pathways, thereby supporting the identification of therapeutic targets, an aspect of particular importance in rare lung diseases, where quantitative biomarkers and mechanistic insight are essential to guide early diagnosis and to prioritize the development of new treatments [203].

The feasibility of fully integrated preclinical workflows depends on coordinated imaging, histological, omics, and computational steps, each with specific infrastructural and operational requirements. Micro-CT is widely accessible and cost-efficient, explaining its wide use in lung imaging; nuclear imaging and MRI require radiochemistry or magnetic resonance facilities and trained personnel; optical imaging is cost-efficient and easy to implement, but has limited depth sensitivity. End-stage analyses introduce additional needs, such as tissue-clearing and light-sheet setups, digital pathology platforms, and sequencing facilities. Integrative analyses, especially those involving machine learning, require curated datasets, standardized formats, and adequate computational resources. For this reason, feasibility depends not only on individual technologies but also on institutional capacity to coordinate acquisition, manage data consistently, and maintain quality-control procedures. Shared infrastructures and harmonized protocols are essential to support laboratories with different resource levels and to enhance cross-center reproducibility.

Ethical and regulatory considerations also remain central. Longitudinal micro-CT and PET imaging involve exposure to ionizing radiation, requiring optimized low-dose protocols and careful selection of time points to comply with the 3Rs principles [204, 205]. Multimodal studies increase procedural burden, making refined anesthesia, monitoring strategies, and transparent reporting essential. These constraints highlight the need for

rigorous experimental design and clear justification of imaging frequency, dose, and modality combinations.

Another challenge concerns methodological variability. Differences in acquisition parameters, scanner calibration, reconstruction algorithms, and quality-control practices limit reproducibility across laboratories. Standardized frameworks comparable to QIBA, EANM, or ACR accreditation are still emerging in the preclinical setting. As machine-learning tools become more prevalent, the lack of curated benchmarking datasets and interoperable data formats limits robust model development. To unlock the potential of AI-enabled biomarker discovery, the field will require standardized acquisition workflows, open repositories, and transparent reporting standards. Integration of diverse datasets is also greatly aided by advanced computational analyses. Machine learning and deep learning algorithms can interrogate high-dimensional, multi-parametric data to uncover relationships across modalities, identify disease subtypes, and predict progression as well as response to treatment. When applied to longitudinal studies enriched with molecular and histological endpoints, these models can facilitate the identification of biologically meaningful, imaging-derived features that are associated with specific pathological processes. Importantly, by enabling patient stratification, the above approaches also support the development of personalized medicine frameworks, identifying individual disease trajectories and guiding the selection of targeted therapeutic strategies. Importantly, the incorporation of explainable AI (XAI) techniques ensures that the predictive capabilities of deep learning models are accompanied by biological interpretability. By highlighting the features or regions that most influence model decisions, XAI approaches enhance transparency, support biomarker validation, and facilitate hypothesis generation.

Conclusion

The integration of complementary preclinical methodologies across spatial and functional scales, from the molecular and cellular level to tissue architecture and whole-organ imaging, represents a critical step toward the further development of clinically meaningful pulmonary research. The incorporation of advanced computational tools, including XAI, holds great promise for improving the robustness and the translatability of preclinical pulmonary research. In the long term, these multimodal and computationally enhanced approaches are expected to play a pivotal role in the advancement of personalized medicine by enabling the discovery of individualized imaging biomarkers and the optimization of therapeutic strategies based on specific pathophysiological profiles. Ultimately, the development of standardized, interoperable, and scalable multimodal pipelines will represent a key step forward to bridge the gap between preclinical models and human disease. Efforts in this direction will not only improve the discovery and validation of translatable biomarkers but will also promote the implementation of a more predictive and ethically responsible drug development in pulmonary pharmacology. Overall, these multimodal approaches can support earlier diagnosis and guide the development of new treatments by linking imaging findings with biological and clinical outcomes.

Gaps and future direction

Despite substantial progress, major challenges remain: a lack of standardized acquisition and calibration procedures, different metadata and animal randomization, limited interoperability of multimodal datasets, and few shared repositories for validation or AI development. Future efforts should prioritize harmonized imaging protocols, improved calibration methods, and more consistent experimental design. Equally important is the development of shared multimodal databases that link imaging, histology, and omics data to support multicenter validation and strengthen biomarker robustness. Finally, further work is needed to identify which imaging-derived features best predict disease onset, progression, or treatment response, supporting earlier diagnosis and more targeted therapeutic strategies.

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Authors' contributions

FP, MB, EF, and FFS conceptualized the study. FP, MB, and EF prepared figures. FP, MB, EF, and FFS drafted the manuscript. FP, MB, EF, AA and FFS edited and revised the manuscript. All authors approved the final version of the manuscript.

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Data availability

Not applicable.

Declarations

Ethics approval and consent to participate

FFS and EF are employees of Chiesi Farmaceutici S.p.A., that supported the research work. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for publication

Not applicable.

Competing interests
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