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Review

Imaging the lung: the old ways and the new

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Summary. Our understanding of lung biology can be greatly enhanced by studying embryonic and postnatal lung development, and the perturbations which occur during disease. Imaging techniques provide a unique insight into these processes. A wide variety of imaging techniques have been used to study the lungs at various stages of development and disease, ranging from histological stains to more novel techniques such as single plane illumination microscopy (SPIM), intravital microscopy (IVM), and micro-computed tomography (micro-CT). Each of these tools can be used to elicit different information about the lungs and each has its own unique advantages and disadvantages for pulmonary research. In this review we assess some of the most commonly-used and novel imaging techniques available for lung research today.

Key words: Brightfield microscopy, Fluorescence microscopy, Intravital microscopy, Confocal microscopy, Electron microscopy, Computed tomography

Introduction

Beyond the nose and mouth, the trachea is the most proximal region of the respiratory system, this divides into two main bronchi, which lead into the left and right lungs. The bronchi in turn divide to form bronchioles increasing the number of airways. Distal to the

lungs. The bronchi in turn divide to form bronchioles increasing the number of airways. Distal to the *Offprint requests to:* Charlotte H. Dean: Inflammation, Repair and Development Section, National Heart and Lung Institute, Sir Alexander bronchioles, lie the alveolar units which are specially adapted for gas exchange (Metzger et al., 2008; Warburton et al., 2010). Lung development begins during embryogenesis, and continues postnatally in both mice and humans (Warburton et al., 2010). Lung development can be divided into four stages based on histological appearance: the pseudoglandular, canalicular, saccular, and alveolar stage (Warburton et al., 2010). In mice, the lungs originate by evagination of the anterior foregut endoderm, the primary buds undergo a series of of highly stereotypical rounds of branching to generate a ramified network of tubes (Metzger et al., 2008; Short et al., 2013). Once the airways have been generated, the gas-exchanging region of the lungs is formed from the distal ends of the small airways by thinning of interstitial tissue and widening of airspaces, eventually giving rise to mature alveoli. More detailed descriptions of lung development and its impact on adult lung disease can be found in the following reviews (Shi et al., 2007; Warburton et al., 2010; Herriges and Morrisey, 2014).

At the end of development, the adult lungs are remarkable structures, with a large surface area optimised for gas exchange. Adult lungs, for the large part, are in a quiescent state with relatively little cell turnover (Volckaert and De Langhe, 2014). However, numerous studies have suggested that lungs are able to regenerate following damage or injury (Hsia et al., 1994; Voswinckel et al., 2004; Butler et al., 2012; Eisenhauer et al., 2013). This field of research becomes critically important in combating the rising incidence of chronic lung disease, with diseases such as chronic obstructive pulmonary disease (COPD) predicted to become the third leading cause of death by 2020 (Vlahos and Bozinovski, 2014). Chronic lung diseases cover a broad

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spectrum, including COPD, interstitial lung disease (ILD), asthma, and lung cancer. These arise from a multitude of environmental and genetic factors, and interfere with breathing, greatly affect quality of life, frequently proving fatal (Shi et al., 2007; Warburton et al., 2010; Volckaert and De Langhe, 2014). Imaging technologies have a vital role to play in enabling us to understand the biological processes occurring within the lung throughout the lifespan and in homeostasis and disease.

Microscopes have been pivotal to furthering our understanding of the world around and within us. Although it is highly disputed precisely when the first microscope was invented, as early as the 17th century Robert Hooke published *Micrographia*, which contained detailed illustrations of the microscopic world; showing that microscopes are a powerful tool for studying this unique world. The microscope was then refined by Antonie van Leeuwenhoek later in the 17th century, which improved the ability of contemporary microscopes to observe structures in greater detail. Van Leeuwenhoek made many instrumental scientific discoveries such as bacteria and blood cells, and is commonly revered to as "The father of Microbiology".

Microscopes have since become astonishingly powerful and diverse tools used readily in the biological community to understand the microscopic world. Modern microscopy allows us to investigate subcellular organisation and structures within the cell using electron microscopy, or to study rapid cellular interactions and changes in real time from *in vitro* cell cultures to whole organisms. In this review we will assess the advantages and pitfalls of current imaging techniques available to study the lungs, and highlight some of the key techniques used to analyse the lungs at given stages.

Brightfield microscopy

Histological stains

Histological stains of tissue sections have formed the backbone of imaging for many decades. They are an ideal tool for highlighting distinct histological features present in the tissue, and give detailed information on the structural organisation depending on the type of staining used. The true advantage of these staining techniques lies in their flexibility to provide a wide range of information from embryonic development to adulthood, in both homeostasis and disease. Some of the common histological stains are discussed below.

Haematoxylin and eosin

Haematoxylin and eosin (H&E) is one of the most widely used and recognised tissue histological stains; even today, it remains a key method for histological examination. H&E consists of two principle components: haematoxylin and eosin. Haematoxylin binds to negatively charged compounds such as nucleic acids and is used to stain the nucleus blue. Eosin is an acidic dye which stains cytoplasmic structures a red/pink colour. Lipid containing structures, such as lipid vacuoles appear white as they are dissolved during tissue processing. A standard H&E staining procedure can be found in the methods paper by Cardiff et al. (Jackson et al., 2001).

H&E staining can be performed on any stage of lung tissue from the earliest point of development, Embryonic day (E) 9.5 in mouse (Warburton et al., 2010). H&E can be used to highlight the characteristic lung architecture present at different stages of development, from the dense embryonic glandular-like tissue to the differentiated thin alveolar structure in adults (Warburton et al., 2010) (Fig. 1A-D). H&E staining is also a powerful tool for studying diseases in both animal models and human patients. Classification and staging of diseases such as lung cancer can be assessed by analysis of H&E staining to characterise their progression (Travis, 2012). Histologically, small cell lung carcinomas (SCLC) appear as small rounded cells with very little cytoplasm, with the tumour growing typically as diffuse sheets (Travis, 2012). However non-SCLCs (NSCLC) such as large cell carcinoma or large cell neuroendocrine carcinoma tend to be larger with a lower nuclear/cytoplasmic ration; detailed classification of SCLC and NSCLC is reviewed in by Travis (Travis, 2012). Using H&E, the progression of lung cancers can be characterised to assess the tumour growth from small nodules to malignant carcinomas with disrupted tissue organisation and invasion into surrounding tissue (Platt et al., 2014).

The lungs are constantly exposed to a wide range of microbes such as bacteria, viruses, and allergens, which can lead to disease. During an allergic response, following repeated house dust mite (HDM) exposure the mouse lung responds with thickening of bronchiolar epithelial cells and recruitment of immune cells (Fig. 1E). Similarly, during viral infections, infiltration of immune cells into the alveolar spaces, as well as around the vascular and bronchiolar walls occurs (Fig. 1F). H&E facilitates assessment of morphological changes occurring in lung disease and following infection, allowing us to characterise disease responses.

However, there are limitations to the use of H&E, primarily because it stains all cells within a tissue section. It is not possible to be certain which cell types are present or absent within a tissue section; we can only infer this information based upon their shape and location within the tissue together with histology/ pathology expertise. Furthermore, we cannot gain detailed information about other structures within the lung tissue, such as the extracellular matrix (ECM) using this technique.

Wright-Giemsa

A modified version of H&E staining, Wright-Giemsa staining, is frequently used to assess immune cell

populations. Wright-Giemsa staining allows us to identify specific immune cell populations to assess immunological changes during disease. Wright-Giemsa stain, is a mixture of eosin and methylene blue (which binds to nucleic acids such as DNA) (Strober, 2001); thus, it uses similar principles to H&E staining. These dyes differentially stain immune cells based upon their preference for the dyes (a standard procedure for staining is outlined by Strober (Strober, 2001).

One method of assessing response to disease is to study immune cell recruitment in the bronchoalveolar lavage fluid (BALF), following cytospin. Alveolar macrophages are the major population of immune cells, which in naive mice accounts for almost all immune cells in the BALF (Saglani et al., 2013). However, during disease other immune cells are recruited in response to infection (Fig. 1G). During early infection, innate immune cells such as neutrophils are recruited (Gualano et al., 2008; Tate et al., 2008; Miao et al., 2010). As the infection progresses, adaptive immune cells such as lymphocytes are recruited (Miao et al., 2010). During allergic diseases like asthma an influx of eosinophillic cells into the BALF is observed (Saglani et al., 2013; Snelgrove et al., 2014). As such we are able to gain insight into the immune cell population present in the lungs, and assess their contribution to disease progression.

Although we gain an overall understanding of the inflammatory cell populations present from Wright-Giemsa staining, we are unable to differentiate further subtypes. For example, the lymphocyte population contains B cells and T cells which play different roles during disease (Gasteiger and Rudensky, 2014).

Miller's elastin stain

Other histological dyes highlight specific components within lung tissue. One example is Miller's elastin stain which is composed of three amino-triarylmethanes dyes: Victoria blue 4R, new fuchsin, and crystal violet (collectively known as Weigert's resorcin-fuchsin). This staining marks elastin fibres with a blue/black colour (Proctor and Horobin, 1988). Co-staining with Curtis' stain can also be performed to stain collagen fibres red (Leach, 1946).

Miller's staining provides information on the organisation of the elastin component of the ECM. Elastin is particularly important during lung development to guide cells, as well as for postnatal lung function as it enables elastic stretch and recoil that is necessary for breathing (Warburton et al., 2010; Branchfield et al., 2016). In the adult lung, elastin fibres are found around the upper airways, vasculature, and in the lung parenchyma (Fig. 1H,I). However, in diseases such as emphysema, elastin organisation becomes disrupted and the fibrils are not well organised forming loose and weblike structures (Finlay et al., 1996; Deslee et al., 2009). Using Miller's stain, disruption of elastin organisation can be assessed to study changes in the

elastin structure occurring during disease.

Immunohistochemistry

Immunohistochemistry (IHC) has proved to be a useful tool for identifying specific cell types. The principle of IHC uses antibodies raised against the protein of interest to specifically identify the protein in cells or tissue sections. A second antibody congujated to an enzyme such as horseradish peroxidise (HRP) or alkaline phosphatise (AP) is then used recognise and bind to the primary antibody. The staining intensity can also be amplified by use of systems such as the Avidin-Biotin Complex molecule (ABC) (Hsu et al., 1981). Visualisation is achieved using a chromogen detection system, commonly 3,3'-diaminobenzidine (DAB) for HRP and fast red/fast blue for AP to detect the bound antibody-antigen complex (Katikireddy and O'Sullivan, 2011). At the sites of the antibody binding to the protein of interest, enzymatic activity catalyses oxidation of chromogen to form insoluble precipitates which can then be observed. Antibodies can be raised as either monoclonal or polyclonal. Monoclonal antibodies bind to a specific epitope on the target, whereas polyclonal antibodies bind to different epitopes on one antigen which makes them more antigen-specific and more stable in different experimental conditions (Bratthauer, 2010; Oliver and Jamur, 2010).

In the lung, IHC staining has been used extensively to identify different cell populations within a tissue using antibodies for proteins uniquely present in a specific cell type. For example, pro-surfactant protein C (Pro-SPC) is a marker of alveolar type II cells and club cell secretory protein 10 (CC10) for club cells (Fig. 2A,B). Using these markers, it is possible decipher whether or not there is any disruption to specific cell populations or to cell differentiation. For example, mice with lung epithelial specific $\beta 1$ integrin deficiency show increased numbers of cuboidal alveolar type II cells and fewer flattened squamous alveolar type I cells compared to control mice (Plosa et al., 2014). Thus, using antibody based IHC techniques it is possible to identify specific cells types to discern their localisation, as well as any changes occurring during disease such as cancer (Ordóñez, 1999, 2013).

Although enzymatic methods only require a light microscope and allow us to identify localisation of a cell of interest, they are generally used just for single antibody detection. Co-staining is possible, by sequential staining using HRP and then AP enzymatic activity allowing detection of two different proteins (van der Loos, 2008), however this method is technically challenging and time consuming. To assess localisation of two proteins in the same cells/section, immunofluorescence remains the technique of choice.

Fluorescence microscopy

Antibody based fluorescence imaging, to identify

specific proteins, was introduced by Coons and collaborators in the early 1940s (Coons et al., 1942; Coons and Kaplan, 1950) and shares similar principles to IHC staining. However, the antibodies are conjugated to a fluorochrome as opposed to an enzyme. A major advantage of fluorescence imaging over IHC is double immunostaining to identify two or more proteins simultaneously in the same sample. This is possible because different fluorochromes are excited and emit light at different wavelengths. Provided that there is no, or limited, overlap of excitation and emission spectra between fluorophores, it is possible to visualise different proteins in the same sample. This technique has been widely used to identify different cell types in the embryonic and adult human lung, and to understand how protein distribution varies at different stages of embryogenesis or in genetically modified animal models (Ten Have-Opbroek, 1975, 1979). Immunofluorescence has also become relevant in clinical practice e.g to diagnose autoimmune diseases with lung involvement, such as Wegener's granulomatosis and Goodpasture syndrome (Van Der Woude et al., 1985).

The main limitations of immunofluorescence are related to antibody specificity, cross-reactivity and high background that can be misinterpreted as true protein localisation. Other potential issues are autofluorescence



Fig. 1. Histological stains of murine lungs. **A-F.** H&E staining of the lung structure in embryonic and adult mice. **A.** In early lung development at E14.5 the lung airways are formed by branching morphogenesis. **B.** The lungs then begin to thin to form the immature alveoli at E16.5. **C.** During postnatal alveolarisation stage at P7 alveolar septation enables rapid increase of the surface area. **D.** Adult 10 week old mature lungs have thin alveolar walls specialised for gas exchange. **E.** During allergic disease induced by house dust mite (HDM), epithelial cells surrounding the airway thicken, and there is recruitment of immune cells surrounding the airways and blood vessels. **F.** Following influenza viral challenge, lung architecture is altered with oedema and immune cell influx in to the alveoli and interstitial space. **G.** Wright-Giemsa stain of bronchoalveolar lavage fluid (BALF) from mice treated with lipopolysaccharide (LPS) showing macrophages (black arrow), neutrophils (red arrow), eosinophils (blue arrow), and lymphocytes (green arrow). **H, I.** Miller's staining showing elastin deposition as a dark blue/black colour in the lungs. Scale bars: A-F, H, 120 μm; I, 25 μm.

that can be present in the tissue and photobleaching where the intensity of fluorescence is lost or bleached by exposure to light during imaging.

Confocal and widefield microscopy

Widefield microscopes acquire images with conventional optics, and without a pinhole. Therefore, the whole specimen is illuminated, the microscope then images light emitted from the whole specimen resulting in some regions appearing out-of-focus (Fig. 2C). This problem becomes emphasised with increasing sample thickness, resulting in more out-of-focus light, limiting the image resolution. The advent of confocal microscopy in 1955 provided a solution to the increasing requirement for greater image detail and resolution (Minsky, 1988; Inoué, 2006). Confocal microscopy uses a pinhole to reduce out-of-focus information and therefore provide a sharper image at each point in three dimensions (Shaw, 2006). This enables imaging of tissue with greater detail (Fig. 2D). The consequence of such 'optical sectioning' is to facilitate three-dimensional imaging of the specimen by acquiring several "slices" and reconstructing them in the x, y and z planes. These advantages of confocal microscopy have been crucial for understanding the spatial relationship between different proteins and cell types in murine lung development (Yates et al., 2010a; Lazarus et al., 2011; Plosa et al., 2014). Similarly, such high resolution microscopy has facilitated studies of lung stem cell populations and the regenerative properties of alveolar cells (Lee et al., 2014; Jain et al., 2015). Progress has been made in the high resolution imaging and reconstruction of thicker sections of lung, such as with murine and human precision-cut lung slices (Uhl et al., 2015).

The speed and efficiency of widefield microscopy has resulted in its continued popularity over confocal microscopy, for imaging where increased resolution in the z-axis is not necessary. The introduction of deconvolution techniques which allow for 'optical sectioning' following imaging, have overcome some of the disadvantages of widefield vs confocal microscopy. Convolution describes the complex mathematical models that govern the relationship between the specimen and the acquired image (Agard et al., 1989). Each point in the sample is equivalent to a blurred point in the image, and the appearance of each blurred point in the image is described by the point spread function (PSF) (Shaw, 2006). Deconvolution uses an algorithm with a theoretical point spread function (PSF) to eliminate out of focus light and reverse the imaging process. Because widefield microscopes are able to acquire images with greater speed than confocal microscopes, and due to the power of modern deconvolution software, deconvolved images from widefield can have a higher resolution than unprocessed images from confocal microscopes (Fig. 2E,F). However, widefield focal planes are still limited and therefore confocal microscopy remains the technique of choice for imaging thicker samples or identification of sub-cellular components, such as mitochondria.

Single plane illumination microscopy

Single plane illumination microscopy (SPIM), also referred to as light sheet microscopy, is a relatively new, rapidly evolving imaging tool for analysis of dynamic cellular processes. The speed of imaging and the ability to construct 3D images makes this an ideal tool for studying highly dynamic processes such as cell migration during embryonic development. More detailed descriptions of SPIM are covered in other reviews (Santi, 2011; Weber and Huisken, 2011).

To exploit the unique properties of SPIM, such as imaging specimens in multiple axes and prolonged realtime imaging, specific mounting strategies are required. Specimens are mounted vertically in a cylindrical enclosure made from solid fluorinated ethylene propylene (FEP) or agarose, which have a similar refractive index to water to allow for optimal imaging quality (Kaufmann et al., 2012). During SPIM, specimens are illuminated by a single thin sheet of light which illuminates the specimen only in a single plane (Weber and Huisken, 2011). This method of illumination holds many advantages over confocal imaging, which is also capable of imaging a single plane of the specimen. Primarily, SPIM only illuminates the plane of interest, whereas during confocal imaging the whole specimen is illuminated. As a result, there is less out of focus light using SPIM allowing for greater contrast. Furthermore, confocal imaging requires the image to be acquired sequentially by lines, whereas SPIM obtains the whole image at once. SPIM is therefore well suited for realtime high speed imaging. Moreover, the single plane of illumination reduces photobleaching and phototoxicity (Jemielita et al., 2013), as only the region of interest is excited, thereby allowing for prolonged imaging time. These conditions are advantageous in, for example, imaging sensitive embryos whilst limiting disruption of embryo morphology.

One of the greatest advantages of light sheet microscopy is the ability to perform simultaneous imaging with multiple views (Santi, 2011). During imaging the specimen is mounted on a platform which is able to rotate on its axis. Once the specimen has been imaged on one plane it can be rotated and imaged again at another angle. This overcomes issues of light penetration, where light struggles to penetrate more complex and dense tissues, reducing resolution. However, by rotating and imaging samples from multiple angles, post-processing can generate images with greater contrast. This gives SPIM a unique advantage over traditional confocal imaging where increasing tissue complexity greatly limits the resolution and contrast of the image. In addition to rotation of the specimen, the focal plane of the image can be controlled by the microscope to acquire images as a z-stack allowing for visualisation of specimens in all three axes.



Fig. 2. Identification of specific protein localisation by immunostaining. DAB immunohistochemistry showing localisation of **(A)** alveolar type II cells in the lung parenchyma using Pro-SPC as a marker (orange triangles), and **(B)** club cells in the upper airway marked by CC10 antibody. **C.** Widefield immunofluorescence image of lungs stained for E-cadherin shows localisation around basolateral cell membranes. However, as the whole section is illuminated some areas appear in focus, whilst other regions are out of focus. **D.** Confocal image of E-cadherin in lungs, where out of focus light is reduced, results in greater image resolution. **E, F.** Lung slices imaged using widefield microscopy show poor resolution from out-of-focus light before deconvolution. Post-processing using deconvolution algorithms reduces out of focus background staining, enhancing image resolution. Scale bars: A-C, 110 μ m; D, 120 μ m; E, F, 100 μ m.

Thus far, SPIM has mainly been utilised to study embryonic development of zebrafish and *Drosophila*, where the whole developing embryo can be imaged to study cell dynamics and migration (Keller et al., 2010; Jemielita et al., 2013). Studying mouse embryonic development is more difficult, owing to larger species size and increased tissue density. Recently, SPIM has been used to study embryonic mice at E6.5 and E8.5. By using multiple angles of illuminations Udan et al were able to image with greater resolution and penetration into the tissue (Udan et al., 2014).

Recent studies have used SPIM to study the adult mouse brain and lung (Mertz and Kim, 2010; Ertürk et al., 2014). However, due to large sample volumes and dense tissue structure, specimens must undergo "clearing" prior to imaging. This is the process by which organic solvents are used to render the tissue transparent to enable light to penetrate deeper into the tissue (Ertürk et al., 2014). However, clearing requires the sample to be fixed and undergo harsh chemical treatment; it is therefore not possible to study live specimens with this technique in adult mice. SPIM is a developing technology and in the coming years refined imaging and algorithms are likely to enable imaging of more complex samples using this technique.

Electron microscopy

Light microscopy allows us to study events in cells or tissues. However, as we increase magnification we eventually reach a resolution limit at 200 nm as the resolving power becomes limited by the wavelength of light. Electron microscopy uses the wave properties of electrons which have a much smaller wavelength compared to visible light, thus it is able to resolve up to 0.05 nm (Erni et al., 2009). There are two main types of electron microscopes: transmission electron microscope (TEM) and scanning electron microscope (SEM) (Vernon-Parry, 2000; Ross, 2015; Van Aert et al., 2016). TEM electron microscopy is used to provide highresolution images of sub-cellular structures such as the organisation of organelles and cell junctions. SEM is useful for imaging details on the surface across a field of cells on the cell surface, such as cilia.

One example of the usefulness of electron microscopy in lung imaging is that it allows us to study organisation of structures such as the ECM in greater detail than light microscopy. In COPD there is severe disruption of the ECM in the lung tissue which results in elastin destruction (Finlay et al., 1996; Wells et al., 2015). SEM can be used to visualise the disruption to the normal ECM architecture such as the fragmented and web-like appearance of elastin in the alveoli of lungs with COPD (Finlay et al., 1996).

TEM is also used clinically in the diagnosis of primary ciliary dyskinesia (PCD) (Papon et al., 2010; Lucas et al., 2014). PCD is a genetic disorder which results in impaired mucociliary clearance and is often, though not always, associated with disrupted motile cilia organisation and ciliary beating (Lucas et al., 2014), resulting in increased frequency and severity of respiratory infection. Using TEM, the cilia and the organisation of the microtubules within them can be visualised (Papon et al., 2010; Lucas et al., 2014).

For electron microscopy, specimens are placed in a vacuum during electron microscopy to avoid scattering of the electron beam by air which will disrupt imaging (Ross, 2015). Furthermore, TEM requires extremely thin sections of less than 100 nm to allow electron beams to pass through the specimen with good resolution (Ross, 2015), further hindering live imaging. However, recent developments in environment SEM (ESEM) allow for specimens to be imaged in water or gas without prior preparation to maintain the native state of the sample (Donald, 2003); this technique is particularly useful when studying specimens which become unstable when dehydrated. Using ESEM it is possible to image live cells of up to 10 µm thick (Peckys and de Jonge, 2011), allowing greater image resolution compared to light microscopy in live cells. Although this method cannot yet be used to image lungs, it is possible to apply this technique to *in vitro* cell culture models of the lung.

Computed tomography

Computed tomography (CT) is a powerful imaging technique originally developed for use in a clinical setting but has now become widely used in pulmonary research both in humans and *in vivo* models. CT imaging uses a rotating source that takes X-ray images of the patient from multiple angles. By moving the X-ray source along the desired axis of the subject's body, multiple X-ray projections are acquired from the region of interest. The multiple images are then reconstructed using a tomographic algorithm to generate a 3D stack (Goldman, 2007). As the reconstructed 3D image is composed of voxels (i.e. a pixel in 3 dimensional space), this allows retrospective 're-slicing' of the original 3D stack such that the original image can be viewed from a different orientation compared to the orientation of the original imaging process (Goldman, 2007). Unlike traditional 2D X-ray images, however, CT imaging shows much better contrast between soft tissues as the image is combined from X-ray transmission through multiple angles, rather than from one plane. CT is therefore widely used for diagnostic purposes in pulmonary medicine.

In addition to clinical uses of CT, microscopic CT (micro-CT) has found widespread use in pulmonary research involving small animals to image non-invasively in 3 dimensions, and enable retrospective image manipulation/reconstruction, including volumetric measurements. The term micro-CT in this context generally refers to laboratory bench-top systems that can image at a resolution of up to 1 μ m, although it is sometimes used to refer to systems with much smaller voxel size (more appropriately termed nano-CT) (Ritman, 2011). Compared to more longstanding

histo/cytological techniques, the use of micro-CT allows researchers to study lung architecture and derive accurate 3D reconstruction of organ anatomy (Johnson, 2007; Namati et al., 2010; Rodt et al., 2010). From this, many parameters such as whole lung volume can be studied. These advantages have led to the increasing use of micro-CT by biomedical researchers and the increasing availability of commercially available benchtop systems. The ability of micro-CT to provide 3Danatomical information is particularly useful for laboratories using small animal models in their research, both in disease modelling and phenotype characterization. Micro-CT can provide accurate measurement of how macroscopic dimensions of airways and lungs respond to pharmacological interventions, pathology, or genetic manipulation (Badea et al., 2004; Holm et al., 2015). Non-terminal CT imaging can also be used to track the progression of tumours in small-animal models over-time (Namati et al., 2010). Furthermore, micro-CT can be applied to study the vasculature of the respiratory system with the use of appropriate contrast agents (Das et al., 2016). In the small animal, the need to minimize the disruption of breathing is more challenging than in humans, due to the high respiratory rate and communication barriers, but can be achieved using image correctional techniques in combination with carefully applied general anaesthesia and analgesia (Namati et al., 2010).

Intravital imaging

Intravital microscopy (IVM) allows live imaging of the pulmonary circulation and its corresponding cellular repertoire in vivo. In this system, the thoracic cavity of an anaesthetised and artificially ventilated rodent is fitted with a 'thoracic suction window', through which single or multi-photon microscopy can be conducted (Looney et al., 2011). With this system, tissue penetration to a depth of 125 µM can be achieved, with limited ventilation-induced disruption in image capture. This technique has been able to visualise immune cell responses in real-time, such as the differentiation of perfusion velocities and morphology between naïve and activated T-cells (Looney et al., 2011), the recruitment and swarming of neutrophils during lung injury (Looney et al., 2011), and macrophage-dendritic cell interactions (Fiole et al., 2014). IVM has revealed real-time physiology of lung injury, such as from leakage of intravenous dextran from the vasculature into the extravascular compartment following intratracheal LPS administration (Looney et al., 2011). Finally, this method of microscopy has improved our understanding of tumour progression in the lung, such as by showing the generation, adherence, migration and phagocytosis of tumour microparticles from parent circulating tumour cells which had lodged in the lung microvasculature (Headley et al., 2016). Therefore, techniques to visualise



Fig. 3. Fluorescence staining of PCLS. Brightfield (left) and fluorescent confocal images (right) of human precision cut lung slices (PCLS). Human lung specimens were inflated by point injection of agarose directly into the tissue. 400 μm PCLS were cut using a vibratome (Precisionary Instruments Inc, North Carolina, USA). Here both brightfield and fluorescent images were obtained following fixation with 4% paraformaldehyde, and labelling with phalloidin for F-actin, and DAPI for nuclei. Images were obtained using a Zeiss LSM 510 with 20x objective. Scale bars: 100 μm.

live circulating cell interactions in the lung have shed light on lung injury, pathogenic infection and tumour development. Although this model crucially allows for imaging during both ventilation and with a circulating blood supply, it has still not been possible to apply these

techniques in a spontaneous breathing animal, and therefore conclusions about physiology must be acknowledged in the context of artificial ventilation.

Applications of imaging techniques

Although imaging techniques have progressed in sophistication, resolution and specificity, attempts are being made to move towards techniques that capture dynamic processes in the lung with greater biological relevance, and away from imaging of fixed specimens of lung.

In vitro cell culture

In vitro systems have been at the forefront of biological research for many years. Culturing cell lines such as the alveolar adenocarcinoma A549 cell, or primary cells such as endothelial (Comhair et al., 2012) and alveolar type II cells (Corti et al., 1996; Gonzalez and Dobbs, 2013) from the lungs allows the study of cell behaviour, pharmacology and high throughput analysis. However, *in vitro* cell culture models do not fully recapitulate *in vivo* physiology. Organotypic and airliquid interface (ALI) cultures aim to address this issue and recapitulate an *in vivo*-like phenotype in an *in vitro* system; these systems promote cells to adopt *in vivo*-like characteristics such as the columnar pseudostratified epithelium in the respiratory tract (Lam et al., 2011), and differentiation into specific cell types (Lam et al., 2011;

Xu et al., 2014). Using this model, injury responses e.g. from exposure to cigarette smoke, a key driver of COPD (Balkissoon et al., 2011), can be analysed to better recapitulate the cell damage and morphological changes which occur (Lam et al., 2011; Azzopardi et al., 2015). The versatility of *in vitro* systems makes them ideal for studying protein localisation and interaction at the cellular level following either antibody staining or fluorescent dyes. Live cell imaging is possible to elucidate cell migration, proliferation, and morphology. As such, *in vitro* cell culture systems are ideal for imaging cell dynamics in a simplified system, with the limitation that they cannot fully recapitulate *in vivo* responses.

Ex vivo lung culture

Ex vivo culture models act as bridges between wellcontrolled and flexible in vitro models and more physiologically relevant in vivo models. Culture of whole embryonic lungs or precision cut lung slices (PCLS) from adult lungs are useful models for the assessing cellular behaviour in a physiological environment comprised of the diverse cell types seen in vivo. The primary advantages of these culture techniques over cultured primary cells are the maintenance of a realistic 3D cellular repertoire, a preserved microenvironment, and intact cell-cell and cell-ECM interactions (Davidovich et al., 2013; Shamir and Ewald, 2014). Explant cultures of embryonic lungs have been extensively utilised to study embryonic lung development as this techniques allows temporal imaging of tissue morphogenesis such as branching morphogenesis (Dean et al., 2005; Yates et al., 2010b, 2013); these process are difficult to study using fixed tissues

Table 1. Table of notable imaging techniques highlighting the key advantages, disadvantages, and applications.

Technique	Visibility	Advantages	Disadvantages/ experimental consideration	Applications
Brightfield microscopy	Tissue architecture, cellular morphology of the lungs	Low capital cost, well established histology protocols	Poor subcellular resolution Most histology stains are not compatible with live tissues	Histological stains Explant culture <i>In vitro</i> cell culture
Fluorescence microscopy	Tissue architecture, cell types/ morphology, some organelles of the lungs, location/ expression of proteins	A large range of commercially available flourescent antibodies, probes and dyes, including those compatible with live imaging of cells or tissues	Photobleaching after imaging and overtime Phototoxicity may be an issue in live imaging Tissue autoflourescence may confound real staining	SPIM In vitro cell culture Ex vivo lung cultures
Confocal microscopy	Tissue architecture, cell types/ morphology, some organelles of the lungs, location/ expression of proteins	Focus on a single plane of the image, thus allowing greater resolution and contrast of flourescent markers Construction of 3D stacks from multiple images	High cost of equipment Faster photobleaching flourescent markers compare to basic flourescent microscopes	<i>In vitro</i> cell culture PCLS Intravital
Electron microscopy	Sub-organelle organisation	Very high resolution, can clearly resolve lung structures at subcellular level (e.g. airway cilia)	Extensive and destructive processing of samples renders live imaging impossible, except for ESEM	Subcellular organisation of fine cell strcuture
Computed Tomography	Tissue architecture, gross anatomy	Non-invasive, imaging in live patients/animals. Non-destructive imaging in speciments. Shows high contrast between lung tissue and air	Very high cost of equipment, require specialist personnel to operate, Radiation exposure	Whole lung 3D imaging

alone. PCLS on the other hand are useful for studying adult organs, where a large number of slices can be generated from a single organ. Importantly, this facilitates consistency between experiments, whilst preserving scarce resources and limiting animal use. Precision cut slices from lung tissue are being increasingly used in research (Ressmeyer et al., 2010; Sanderson, 2011).

Ex vivo culture techniques hold many advantages over in vivo models; first, they overcome difficulties encountered in maintaining the viability of the whole organ. Typically explants cultures are viable in culture for 12-96 hours, and lung slices for up to 6 days (Liberati et al., 2014; Uhl et al., 2015), during this time lung physiology and response to stimuli can be imaged in detail. Second, ex vivo cultures allow improved light penetration of the tissue for imaging with greater contrast and resolution. For example, PCLS enable direct visualisation of airway physiology, particularly with respect to allergic or asthmatic responses (Martin et al., 1996). Third, ex vivo cultures can be manipulated in a number of ways to observe dynamic processes in response to gene manipultion, pharmacological agents or recombinant proteins in a living tissue (Martin et al., 1996; Dean et al., 2005; del Moral et al., 2006; Yates et al., 2010b). Progress in fluorescent and confocal microscopy has further opened up the lung allowing dynamic observation of cellular behaviour, such as alveolar epithelial remodelling in repair following COPD-like injury (Uhl et al., 2015), macrophage-ECM interactions and live visualisation of intracellular structures such as focal adhesion complexes (Burgstaller et al., 2015).

Both lung explant cultures and PCLS provide replicable and realistic research models of the lung that are accessible for pharmacological intervention and imaging (Fig. 3). These systems permit dynamic observation of near-physiological cell behaviour in the living lung. However, these applications do not completely replicate the living lungs. First, the lack of perfusion limits the investigation of immune or circulating cell populations, beyond the very short term (a few hours). Second, it is currently not possible to accurately 'ventilate' PCLS to model breathing. Finally, the need to keep the tissues viable in the explant precludes the use of some antibodies that only function in fixed samples, to identify specific cell types in the tissue. This can be overcome in part by using explant tissues derived from transgenic animals where a flourescent construct, such as green fluorescent protein (GFP), is placed under the promoter of a gene that is only expressed in particular tissue compartment or cell type (Schnatwinkel and Niswander, 2013).

Conclusions

The range of imaging techniques used in respiratory research and pulmonary medicine is rapidly increasing and becoming more sophisticated. The development of imaging technologies alongside an expanding range of *in vitro*, *ex vivo* and *in vivo* model systems allows us to study the mechanisms of lung development in ever greater detail (see Table 1 for a summary of techniques). In addition, imaging is increasingly playing a part in the clinical diagnosis of disease. Imaging methods, both the old and new alike, are likely to continue providing significant insight into our knowledge of the lung, from development to old age in both health and disease. A key goal is to adapt these continuously evolving imaging technologies to benefit the diagnosis and treatment of disease in patients.

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337

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