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Delineation of the healthy rabbit lung by immunohistochemistry – a technical note

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\textbf{ABSTRACT}

Investigation and studies of pulmonary diseases and injuries require pre-clinical animal models. The rabbit lung model is widely used and allows for a diverse set of readouts. Among them, histology and immunohistochemistry are of invaluable merit because qualitative and quantitative information about tissue morphology and composition can be easily obtained. In this technical note, we performed several histological and immunohistochemical stainings in the rabbit healthy naïve lung tissue. Overnight formalin fixation with subsequent paraffin embedding was compared to cryopreservation with a subsequent 10-minute formalin fixation prior to staining. Antigen retrieval (AR) for paraffin embedded sections proved to enhance the corresponding signals compared to analogous staining without AR. Advantages and disadvantages of chromogenic versus immunofluorescence stainings were discussed. In addition, several morphological structures, such as the intrapulmonary bronchus with its mucosal folds, the pulmonary artery, the alveoli and the lymph nodes, were stained with various stainings at the same site in order to give a comprehensive picture of their composition. Besides Haematoxylin and Eosin and Elastica van Gieson staining, collagen I, collagen III, fibronectin, α-SMA, ki-67 and protease-activated receptor-2 (PAR-2) immunohistochemistry was performed. Collagen I, collagen III and fibronectin expression was positive at the outer rim of the pulmonary arteries, while the inner rim was collagen III positive. Moreover, the fibronectin staining in the intrapulmonary bronchus showed an opposite trend when compared to the collagen III staining. The alveoli exhibited PAR-2 expression, while PAR-2 was not expressed in lymph nodes of the healthy rabbit lung.

1. Introduction

Pulmonary diseases, such as bronchopulmonary dysplasia, require pre-clinical animal models to be elucidated and studied in detail (Salaets \textit{et al.}, 2020). Pathological structures demand for proper histological and immunohistochemical staining of the lung tissue (Andrikakou \textit{et al.}, 2016). Among others, the rabbit lung model is a valuable animal model to investigate pulmonary diseases and lung injuries (Das \textit{et al.}, 2020). Above all, reliable baseline values and reference images are needed in order to compare the pathological situation to the normal healthy situation in naïve tissue of the rabbit lung. The rationale for the study presented here lies in the necessity of detailed images of the rabbit lung tissue, including bronchioles, alveoli, pulmonary vessels and more – with distinct staining for extracellular matrix (ECM) components, among others. Besides collagen I and III, fibronectin is an essential component of the ECM (Halper and Kjaer, 2014; Kadler \textit{et al.}, 2008). In addition, the assessment of the vascular morphology and smooth muscle actin expressing cells, proliferating cells as well as connective tissue composition are important readouts in the context of lung diseases and injuries (Salaets \textit{et al.}, 2020). Moreover, inflammation related markers and their protein expression in the lung are important tools to investigate devastating lung diseases such as COVID-19 (Helmy \textit{et al.}, 2020).

In this technical note, we cover basic aspects of paraffin tissue sections fixed with formaldehyde compared to cryopreservation; an additional antigen retrieval (AR) step compared to immunohistochemical staining without AR in the case of paraffin sections, as well as chromogenic versus fluorescence staining, respectively. Images of differently

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stained sections of the same morphological structure are presented adjacent to each other and from the same tissue site. We have assessed Haematoxylin&Eosin (HE) and Elastica van Gieson (EvG) staining in the healthy rabbit lung tissue, collagen I, collagen III, fibronectin, alpha smooth muscle actin (α-SMA) and protease-activated receptor-2 (PAR-2) staining, the latter being an inflammation related protein on the cell surface, potentially interesting in studies of lung injuries, pulmonary diseases, fibrosis (Richeldi et al., 2017) and COVID-19 (Shanmugaraj et al., 2020). In addition, we chose specific morphological structures, such as the mucosal folds of the intrapulmonary bronchus, the intra-alveolar septa and a lymph node and compared their different stainings to elucidate the local composition of these structures in a qualitative, semi-quantitative and quantitative way.

We believe that the data presented here can support researchers who intend to use the rabbit lung model in choosing an appropriate staining to delineate a typical morphological structure – a specific target under view. In addition, the results shown here could help other researchers who use the rabbit lung model to compare their results by providing a set of baseline or reference images. Finally, the images provided for the rabbit lung here may also be used for comparison to other animal species or to the human lung.

2. Materials and Methods

2.1. Animal work and lung extraction

A fresh female New Zealand White rabbit cadaver from a study of calvarial bone defects was obtained, which was approved by the Animal Ethics Committee of the local authorities (Canton Zurich 2H 108/2012 and 115/2015) (Ghayor and Weber, 2018; Siegenthaler et al., 2020). The lung was entirely extracted from the rabbit body without pressure fixation, shortly stored on ice and immediately afterwards processed for histology as described below. The tissue sections were randomly selected; no selection of central or peripheral segments was performed.

2.2. Histology and Immunohistochemistry

Different lung pieces were taken and halved for either paraffin embedding or cryopreservation. The pieces for paraffin embedding were fixed in formalin for 24 h, then dehydrated, paraffin-embedded and sectioned into 5-μm-thick slices. Paraffin embedded sections were deparaffinized with xylene and rehydrated prior to any histological staining.

The organ pieces for cryopreservation were embedded in Tissue-Tek® O.C.T. (Sakura, Alphen aan den Rijn, The Netherlands, Europe), then frozen according to commonly established procedure and cryosectioned into 5-μm-thick slices. After thawing, the sections were fixed with formalin for 10 min, then washed with 1xTBS and stained with IHC procedures (see below).

Haematoxylin&Eosin (HE) and Elastic van Gieson (EvG) staining was performed according to commonly established protocols. For immunohistochemistry, paraffin sections underwent an antigen retrieval (AR) step in 10 mM citrate buffer (pH 6.0) with 0.05 % Tween-20 for 20 min at 95 °C (for technical reason and control management, for selected stainings no AR was performed, in order to visualize the staining without AR and discuss the reason for this step). If needed, depending on the epitope to stain, paraffin and cryosections were permeabilized with 0.5% Triton X-100 in 1xTBS for 10 min and subsequently washed three times with 1xTBS. Next, sections were blocked in 5% donkey serum and 1% BSA in 1xTBS for 1 h at room temperature. Afterwards, sections were incubated with mouse anti-collagen I antibody (ab90395; Abcam, Lucerne, Switzerland, 1 : 200 dilution) or mouse anti-collagen III antibody (AF5810; Acris, Wettingen, Switzerland, 1 : 200 dilution) or mouse anti-α-SMA antibody (A2547; Sigma-Aldrich, Buchs, Switzerland, 1 : 500 dilution) or mouse anti-fibronectin antibody (F0791; Sigma-Aldrich, Buchs, Switzerland, 1 : 200 dilution) or mouse anti-ki67 antibody (NB62-22112; Novus Biologicals, 1 : 500 dilution) or mouse anti-PAR-2 antibody (Santa Cruz Biotechnology, sc-13504 (SAM11), 1:250 dilution) on Refine-kit (anti-Rabbit-Polymer) and histofine-Mouse Polymer (1:50 dilution) diluted in 3% BSA in 1xTBS overnight at 4 °C. For laboratory validation of PAR-2 antibody, see Supporting Information Figure SI 1. As a negative control, Normal Mouse Serum Control (08-6599, Invitrogen, no dilution) was used and sections were incubated with it overnight at 4 °C, too.

For some markers, fluorescent immunohistochemistry was performed (collagen I and III, fibronectin and α-SMA), while for some and others, chromogenic immunohistochemistry was performed (collagen I and III, fibronectin, ki67 and PAR-2). For fluorescent immunohistochemistry, primary antibody solution was removed and samples were washed with 1xTBS before incubation with secondary donkey antimouse Alexa-488 antibody (A-21202; Invitrogen, Basel, Switzerland, 1:500 dilution) and 10 μg/ml 4’6-diamidino-2-phenylindole dilactate (DAPI) (Sigma-Aldrich, Switzerland) diluted in 3% BSA in 1xTBS for 1 h at room temperature. Afterwards, slides were washed in 1xTBS and mounted using Dako Fluoresance Mounting Medium (Agilent, Basel, Switzerland).

For chromogenic immunohistochemistry samples were blocked with 3% hydrogen peroxide solution in water, 10 min at room temperature and subsequently washed 3x with 1xTBS, the primary antibody detection was performed using a biotinylated anti-mouse IgG secondary antibody and streptavidin-horseradish peroxidase (HRP) (ZytoChem Plus HRP Kit Mouse; Zytomed Systems, Muttenz, Switzerland), followed by colorimetric detection using DAB substrate (DAB Substrate Kit High Contrast; Zytomed Systems, Germany) according to the manufacturer’s protocol. Afterwards, slides were washed in tap water and mounted using Paramount Aqueous Mounting Medium (Agilent).

Whole tissue sections were imaged on a slide scanner (Pannoramic 250 Flash II, 3Dhistech, Budapest, Hungary), visualized with CaseViewer-Software v.2.1 where snapshots of fields of view were taken, or imaged with a Leica 6000 light microscope (Leica, Basel, Switzerland).

For quantitative readouts, Fiji Image J 1.52a was used.

3. Results and Discussion

3.1. Formalin fixation compared to cryopreservation

A crucial decision before staining histological sections is choosing the best tissue preservation and embedding technique in order to obtain optimal conservation of tissue morphology and structure. In other words, the choice how to fix and embed a certain tissue is very important in order to be able to show the desired target structure and proper antibody-antigen staining. Fixation of a tissue leads to the inactivation and stabilisation of enzymes, but it should avoid their denaturation (Murray, 1992). Fixation also leads to the stabilization of the tissue structures and the cells so that they are protected against mechanical stress during tissue sectioning and further processing. However, such a protection has to be paid by the price of changing the chemical composition of tissue and cellular structures as well as crosslinking proteins. Among others, cells get permeable – the natural barrier realized as cell membrane gets partially destroyed and the original cytoplasm changes to a network of proteins. Depending on the fixative used, this permeabilisation occurs to different degrees, as shown for immunohistochemistry after fixation by formalin, paraformaldehyde-lysine-periodate, bounin or acetic formalin (Salguero et al., 2001). Consequently, if antigens are located not only on the surface of cells, but also within the cells, permeabilisation of the membrane might be an aim during immunohistochemical questions to reach the epitopes of the desired antigens.

In our technical study, we compared two fixation and embedding options that are widely used. In the healthy rabbit lung model, we prepared formalin fixed and paraffin embedded sections, and compared...
Fig. 1. Collagen I immunohistochemical staining with DAB as chromogen (brown colour) for paraffin sections with AR (left) and for cryosections (right). Corresponding inserts (red dashed line) depict area used for images at the next lower level with higher magnification. Below: semi-quantitative readout with scores (1-3) for image contrast and degree of sputtered brown colour (n = 15). Definition of scores is given in the Supporting Information Figure SI 2.
them to cryosections of cryopreserved lung tissue. While cryosections were expected to retain antigens better than formalin-fixated paraffin sections, critical comparison of selected morphological structures in the rabbit lung showed some advantages of the paraffin embedded sections over the cryosections.

Immunohistochemical staining for collagen I (Fig. 1) with DAB as chromogen revealed that at lower magnifications, morphologies of bronchi, arteries and veins had a higher contrast to the staining intensity of the surrounding tissues, such as alveoli, intra-alveolar septa, alveolar sacs and ducts, in the cryosections compared to the paraffin sections. This was based on the fact that the brown colour originating from DAB exhibited a high background in the paraffin sections, while it was more specific in the cryosections. At higher magnification, however, morphological details in cryosections were generally less visible than in paraffin sections because the brown colour was sputtered between cells to a higher degree than observed in the paraffin sections, recognizable only at this high magnification (Fig. 1 bottom row and semi-quantitative scoring). Hence, depending on the focus and staining distribution, various magnifications gain different importance. At high magnification, details of the alveolar septa are better recognizable in cryosections because of a better depth of field, although the same structure was better preserved during the paraffin embedding process.

Immunohistochemical staining for ki67, however, revealed distinctively the proliferating cells in both, the paraffin embedded as well as the cryosections, respectively (Fig. 2). Independent of the magnification, in both types of sections, the dark brown cells were clearly visible, depicting ki67 positive and thus proliferating cells; no advantage of either fixation technique was observed here.

For immunofluorescence detection and fibronectin staining, colour intensity in cryosections was higher compared to paraffin sections at higher magnifications, with higher sharpness that was scored semi-quantitatively and lower colour slurring as assessed quantitatively by the distance to the basic structure.

The same was found for α-SMA immunofluorescence staining (Fig. 4). As cryopreservation with a short formalin fixation step preserved antigens to a greater extent than a long formalin fixation did, the intensities in cryosections were much higher. However, particularly at high magnification, a slurry of intensive green colour superimposed the blue DAPI nuclei staining to such an extent, that specific cell nuclei were not distinguishable anymore. We are aware that the high extent of staining is dependent on various factors, including image acquisition and antibody dilution besides the duration of formalin fixation. Nevertheless, although much less in intensity, α-SMA between cells were better visible with clearer structure boundaries in the paraffin sections, as assessed semi-quantitatively.

3.2. Antigen retrieval in paraffin embedded sections

Although excellent preservation of morphological structures is warranted within formalin-fixated and paraffin embedded sections, formalin fixation may lead to some loss of the immunoreactivity of certain antigens. Therefore a method for antigen retrieval has been developed as early as 1991 (Shi et al., 1991). This technique was originally based on heating the paraffin embedded sections, thus retrieving the immunoreactivity of the antigens. Since then, several antigen retrieval methods have been steadily improved (Shi et al., 2006) and many different sub-techniques are currently at hand (Shi et al., 2011).

When we compared tissue samples of the healthy rabbit lung for immunofluorescent fibronectin staining in paraffin sections, with antigen retrieval and without, we found an increase in signal for the respective antibody staining, as for example well seen at the margin of the pulmonary artery, but also clearly distinguishable when secondary
Fig. 3. Immunohistochemical fibronectin staining with immunofluorescence detection. Fibronectin (light green) and cell nuclei (DAPI staining, blue) are visualized for paraffin sections with AR (left) and for cryosections (right). Corresponding inserts (red dashed line) depict area used for images at the next lower level with higher magnification. Below left: semi-quantitative readout with scores (1-3) for accuracy and sharpness (n = 5). Definition of scores is given in the Supporting Information Figure SI 3. Below right: quantitative readout for distance of slurred colour to basic morphological structure (n = 5), defined in the Supporting Information Figure SI 4.
Fig. 4. Immunohistochemical α-SMA staining with immunofluorescence detection. α-SMA (light green) and cell nuclei (DAPI staining, blue) can be seen in paraffin sections with AR (left) and for cryosections (right). Corresponding inserts (red dashed line) depict area used for images at the next lower level with higher magnification. Below: semi-quantitative readout with scores (1-3) for accuracy and specific staining (n = 5). Definition of scores is given in the Supporting Information Figure SI 5.
Fig. 5. Comparison of fibronectin immunofluorescence staining with antigen retrieval (AR) or without AR and Isotype negative controls in paraffin sections. Intensity of green colour was assessed with Image J ($n = 15$). Corresponding regions of interest used for quantification in bronchus and vessel walls are given in Supporting Information SI Figure 6 for fibronectin and isotype negative control, respectively.
bronchi are compared. Signal intensity was significantly enhanced by AR compared with sections treated without AR (Fig. 5).

Using mouse isotype fluorescence immunohistochemistry as negative control, the difference between samples treated with antigen retrieval compared to no AR was also obvious. Hence, if the rabbit lung is under view it might be worthwhile to include an AR step during immunohistochemical staining in order to enhance the corresponding signals by unmasking the epitopes of the antigens under view.

3.3. Comparison of immunofluorescence and bright field imaging

In order to investigate if a decision for bright field imaging, exemplified with the brown colour from DAB chromogen, or rather immunofluorescence imaging might depict the rabbit lung morphology better, we compared collagen I and fibronectin stained sections (Fig. 6).

While in the two collagen I stained sections the morphology of distinct structures could be evaluated better with chromogenic staining and bright field imaging, the situation in the two fibronectin stained sections was the other way round – here, the immunofluorescence image showed the brink with fibronectin positive tissue in a much higher contrast to the surrounding tissue than did the chromogenic stained images in brightfield, where there was a lot of background staining, and the different intensities of brown colour could not be distinguished very well. Depending on the structure under view, such as whole areas or only spots, a suitable choice has to be taken about the mode of staining. In addition, technical problems like fast optical colour loss typical for fluorescent sections or inadequate scanning by digital microscopic tools have to be taken into account.

3.4. Anatomy of the rabbit lung – an overview

Fig. 7A shows a fibronectin stained paraffin embedded cross-section of the whole healthy rabbit lung. Several tissue structures, such as the pulmonary arteries and veins, the secondary bronchi, a lymphatic nodule, the alveoli, the inter-alveolar septa, the alveolar ducts and sacs as well as the terminal bronchioles with their mucosal folds are clearly visible. In addition, a Haematoxylin&Eosin (HE) stained section (Fig. 7B) reveals the adventitia and the smooth muscle surrounding the intrapulmonary bronchus with its mucosal folds. It has to be emphasized that the commonly used HE staining is very useful for a fast staining because it is easy to perform and cost-effective in comparison to the more sophisticated immunohistochemical approach and it allows to clearly distinguish the main morphological structures well – as shown for the healthy rabbit lung tissue here.

3.5. Expression of different markers in the rabbit lung tissue

Immunohistochemistry for different markers was assessed, with serial sections (possibly immediately taken one behind each other) in order to be able to compare the same specific structures at the same site. For example, a typical pulmonary artery was stained for collagen I and III (Fig. 8A). While collagen I expression was primarily pronounced at the outer rim of the vessel wall, collagen III was rather weak in signal intensity, but homogenously distributed throughout the artery wall. Fibronectin was co-localized with collagen I, which was very nicely shown in the immunofluorescence images. The localization and distribution of fibronectin in the extracellular matrix is important to be assessed because an aberrant fibronectin matrix is indicating a switch intra-murally to activate the receptor, resulting in an increase of pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-α (Tripathi et al., 2014). Therefore, high levels of PAR-2 expression might indicate an inflammatory area. For example, lung inflammation has been reported to be induced by serine proteases that were activated by the asthmagen Alternaria alternata in Balb/c mice (Boitano et al., 2011). In addition, applied to human bronchial epithelial cells, Alternaria alternata activated PAR-2 under in vitro conditions, which was accompanied by and reflected in changes of the intracellular calcium ion concentration (Boitano et al., 2011). In our images taken here (Fig. 8A), there was no PAR-2 positive staining in the pulmonary artery, which is, however, not
Fig. 7. Overview of healthy rabbit lung section immunohistochemically stained for fibronectin with AR (A) with corresponding tissue structures. Arrows depict specific substructures. HE stained paraffin section (B) of the healthy rabbit lung with detailed description of the substructures.
Fig. 8. Selected lung tissue structures immunohistochemically stained for a series of different markers as well as Haematoxylin&Eosin and Elastica van Gieson for elastin at different magnifications in paraffin sections: Pulmonary tissue with respiratory bronchiole, alveoli and pulmonary artery (A); intrapulmonary bronchus with mucosal folds, pulmonary artery and lymphatic nodule (red dashed circle) (B) and detail of intrapulmonary bronchus with mucosal folds (C).

Key: Col = Collagen, Fn = Fibronectin, α-SMA = alpha smooth muscle actin, ki67 = proliferation marker ki67, PAR-2 = protease activated receptor-2 and HE = Haematoxylin&Eosin, AR = Antigen retrieval. If not otherwise stated, all stainings were performed with AR.
surprising – as we used healthy rabbit lung tissue, without any expected inflammation. Nevertheless, our PAR-2 immunohistochemical staining of the pulmonary artery might act as a reference staining of healthy tissue for future other studies, particularly dealing with inflammation in lung vessels. Inflamed vasculature was recently mentioned as an epiphenomenon of COVID-19 infections (Helmy et al., 2020; Varga et al., 2020), but is also relevant for other pulmonary diseases that are accompanied by inflammation or aberrant angiogenesis (Walsh and Pearson, 2001).

In Fig. 8B, we also visualized pulmonary arteries, but in addition to this, also alveoli, inter-alveolar septa and lymphatic nodules are shown at higher magnification. The staining for collagen I and III (upper row in Fig. 8B) confirmed collagen I positive regions at the rim of the arteries and the alveoli; with the latter showing intensive green-coloured spots, not homogeneously distributed. On the other hand, collagen III was found at the inner and the outer brink of the artery and seaming the bronchus tissue. Like collagen I, collagen III exhibited distinctively stained spots in the alveoli.

As for the fibronectin stained sections, it confirmed the findings discussed for Fig. 8A, especially in the immunofluorescence image; a clearly fibronectin positive rim on the surface of the artery was visible. The terminal bronchiole fibronectin staining looked like the opposite of the collagen III stained section – wherever it was positive for collagen III, there was no fibronectin, and wherever it was fibronectin-positive, it was negative with respect to collagen III expression. It seemed that collagen III and fibronectin complement each other in the connective tissue composition, making up the bronchus. α-SMA staining was positive throughout the vessel wall of the pulmonary artery. Airway smooth muscle content is a parameter which is often assessed in lung studies (Gie et al., 2020), so a detailed view of α-SMA positive regions is worthwhile to include here. While only the inner area of the sectioned terminal bronchiole was positive, it was α-SMA positive within the fibronectin area, localized at the smooth muscle tissue of the vessel wall. This was also confirmed with images in Fig. 8C at high magnification.

Interestingly, lymphatic nodules were well recognizable in an immunofluorescence image of Isotype Negative Control with AR (red dashed circle, Fig. 8B), depicting a blue area (DAPI) with a high cell density, which was confirmed with a dense area of proliferating cells (ki67 in Fig. 8B). This lymph node looked like pliable and adaptable around the artery. The area of the lymphatic nodule was the only area in this tissue extract where PAR-2 staining was completely absent, while it was at least weakly stained in terminal bronchiole and alveoli tissues. As for elastin staining, the lymph node was elastin negative; however, around the lymph node, there were some few spots that were positive for elastin.

Having a closer look at the mucosal folds of a intrapulmonary bronchus in Fig. 8C, the alveolar tissue was more prominently PAR-2 positive compared to the mucosal folds – they were stained very weakly in light brown, with a few proliferating cells (ki67 positive).

In Fig. 9, collagen I, collagen III, fibronectin, ki67 and α-SMA expression were assessed in cryosections. The wall of the primary bronchi were stained positive for collagen I at the outer rim and throughout the wall for collagen III. Fibronectin expression was co-localized with collagen I. As for α-SMA, partial co-localization with collagen I and fibronectin was observed, while it delineated the rim of intrapulmonary bronchi (Fig. 9B). At higher magnification (Fig. 9C), the artery wall showed different areas with typical protein expressions: collagen I positive was the tunica adventitia, while both, the tunica adventitia as well as the tunica intima were positive for collagen III and fibronectin. Furthermore, α-SMA expression was found to be distributed throughout the artery wall (tunica media) and additionally, in the walls of veins, the smooth muscles and the lamina propria of the primary bronchi. As for the excretory ducts of the bronchial glands, collagen I and III expression were similar and to a great extent co-localized with fibronectin, while α-SMA expression was adjacent to the area positive for collagen I, III and fibronectin. Proliferating cells positive for ki67 were evenly distributed over the bronchial gland tissue.

In Figs. 9D and 9E, hyaline cartilage plates are depicted, with the
typical cobblestone-like morphology of chondrocytes. These cartilage plates were only positive for collagen I and III at the surrounding rim, co-localized with a weak fibronectin signal – but no α-SMA. There was only a very restricted and small area of α-SMA positive tissue, localized between the hyaline cartilage plate and the wall of the primary bronchus, indicating some smooth muscle.

3.6. Particular structures in the rabbit lung

In Fig. 10, we present the pulmonary bronchus in detail, for an HE and EvG stained sections, and for PAR-2, fibronectin, collagen I and α-SMA immunohistochemically stained sections. Interestingly, the inflammation-related protein PAR-2 was expressed in the alveoli surrounding the pulmonary bronchus, but not in the mucosal folds neither in the smooth muscle of the bronchus. Only the adventitia showed regions of brown staining, depicting a small rim at the outer part of the pulmonary bronchus. It has been shown earlier that PAR-2 triggers proinflammatory pathways in lung epithelial cells A549 that occur in the alveoli (Heuberger et al., 2019), in accordance with our finding of PAR-2 expression in the alveoli. As for fibronectin, the immunofluorescence image shows nicely that the lamina propria as well as the adventitia were positive for this ECM component, co-localized with collagen I; lack of
fibronectin, but strongly positive \( \alpha \)-SMA signal was characteristic for the smooth muscle tissue within the wall of the bronchus. Moreover, the lamina propria was strongly elastin positive, while the adventitia did only show a weak elastin expression. The morphology and distribution of the cells (violet colour) can also be very well seen in the HE stained sections, particularly the high cell density characteristic for the mucosal folds is exhibited very well, as well as the flat and long cell nuclei are recognizable in smooth muscle.

In addition, we focused on the alveoli (Fig. 11A) and a lymph node (Fig. 11B). The alveoli were characterized by a spotty collagen I (probably by erythrocytes) and fibronectin staining. Compared to collagen I, the spots positive for fibronectin were considerably less in numbers, standing in contrast to the rather fibronectin-rich lung alveolar epithelium of fetal rabbit lungs (Snyder et al., 1987). Moreover, alveoli had some few regions of \( \alpha \)-SMA positive expression, delineating small and very small blood vessels. They had a quite high cell density and a lot of ki67 positive proliferating cells. In addition, as mentioned in the previous paragraph, the alveoli exhibited PAR-2 expression – with an evenly distributed brown colour from DAB staining throughout the alveolar wall. PAR-2 protein staining might be a future biological marker for inflammation during lung injuries, where usually IL-6, TNF-\( \alpha \) and IL-1\( \beta \) are used to denote the severity of the inflammation (Frank et al., 2006). In contrast, lymph nodes were PAR-2 negative (Fig. 11B). Their main characteristic is a very cell-dense area with a high percentage of proliferating ki67 positive cells, which was confirmed by the HE stained section, showing very cell-dense areas in the region of the lymph node. Moreover, non-consecutive sections for HE and ki67 showed cell-dense areas at different zones around the artery, implying that the corresponding lymph node was partly surrounding this artery (Table 1).

### 3.7. Recommendations

The combination of our baseline images and the comparison of different techniques is summarized in Tables 2 and 3. While Table 2 refers to the decision about which fixation technique could be favoured for subsequent high quality imaging of a certain lung structure, Table 3 gives a recommendation for the choice between chromogenic staining with brightfield imaging versus immunofluorescence staining.

### 3.8. Novelty and limitations

As for the comparison of paraffin sections with cryosections, several other publications have addressed it. Using human tonsil, Hira and co-workers found that tissue morphology in paraffin sections was superior than in cryosections (Hira et al., 2019). In their study, they did not look at different magnifications as reported here. We judge this to be an important detail because sometimes paraffin sections were better at higher magnifications, while cryosections were more convincing at lower magnifications, depending on the antibody. Moreover, another study that compares paraffin to cryosections for the pig lung model reports that the bronchus wall shrinks as a consequence of differently fixated sections, with a significantly smaller bronchus wall in cryosections compared with paraffin sections. However, this study used only H&E staining. We did not find any rabbit lung model with images of sections stained for so many antibodies as provided in our study; in addition, subsequent sections of the same structure with a series of different antibodies in the healthy rabbit lung is also novel.

In addition, PAR-2 stained sections of the healthy rabbit lung have not been shown in the literature before. There is a report on an endotoxin-induced acute lung injury in the rabbit model (sepsis), where lung tissue immunohistochemical staining was restricted to PAR-1 staining of the bronchus, the alveoli and the pulmonary vessels of endotoxin-treated rabbits and of control rabbits – however, no PAR-2 staining of these tissues was presented (Jesmin et al., 2004). In another experimental rabbit study, PAR-2 expression was analyzed by real-time PCR after induction of asthma, but no lung tissue, neither with nor without astma, was immunohistochemically stained for PAR-2 (D’Agostino et al., 2007). Nevertheless, there is an experimental rabbit study where PAR-2 was stained in the coronary and arterial tissue (Shi et al., 2020). Although the target structure was not the pulmonary artery as reported here, the PAR-2 stainings provided in the corresponding study confirmed our findings; they found coronary and arterial control tissue (without arteriosclerosis) to be PAR-2 negative, corroborating our findings of PAR-2 negative pulmonary arteries.

To the best of our knowledge, immunohistochemical staining for PAR-2 in the healthy rabbit lung tissue and substructures was not yet performed, particularly not yet for alveoli, mucosal folds of the...
Fig. 10. The pulmonary bronchus – ultrastructural localization of PAR-2, fibronectin, elastin, collagen I and α-SMA expression. Key: PAR-2 = protease activated receptor-2, Fn = Fibronectin, HE = Haematoxylin&Eosin, Col I = Collagen I, α-SMA = alpha smooth muscle actin. All stainings were performed on paraffin embedded sections and with AR.
We judge our images of PAR-2 stained immunohistochemical sections as novel. There are also some limitations to our study. We focused on healthy rabbit lung tissue, but did not address the pathological side. Considering the huge amount of different pulmonary diseases that would have to be covered and addressed, however, it would be beyond the scope of the study to provide another as elaborated set of images for specific lung diseases as provided for the healthy lung.

3.9. Conclusive remarks on immunohistochemistry of the rabbit lung tissue

The morphology of the rabbit lung tissue with its components of extracellular matrix can be very well visualized using immunohistochemical approaches besides the simple and traditional HE staining. We have shown advantages and disadvantages of cryopreservation compared to formalin fixation and paraffin embedding, differences after an antigen retrieval step and were able to elucidate main proteins making up the pulmonary bronchi, the alveoli, the arteries and the lymph nodes in the natural, naive rabbit lung tissue. The alveoli were PAR-2 positive, but only the adventitia of the pulmonary bronchus was positive, while the rest did not express PAR-2. Especially the lymph node
Recommendation for chromogenic versus fluorescence staining in paraffin sections, with regard to magnification. Key: chro = chromogenic; IF = immunofluorescence; Coll = collagen; Fn = fibronectin; α-SMA = alpha-smooth muscle actin.

Table 2

| Component Staining | bronchus | alveolus | artery, vein | Intra-alveolar septa | Intra-alveolar sacs and ducts | Lymph node |
|---------------------|----------|----------|--------------|----------------------|-------------------------------|------------|
| Col I, DAB          | cryo, low| para, high| cryo, low    | para, high           | para, high                    | cryo, low  |
| Col I, IF           | cryo, low| para, high| cryo, low    | para, high           | para, high                    | cryo, low  |
| Col III, DAB        | cryo, low| para, high| cryo, low    | para, high           | para, high                    | cryo, low  |
| Fn, DAB             | para, all| para, high| para, all    | para, high           | para, high                    | para, all  |
| Fn, IF              | cryo, low| para, high| cryo, low    | para, high           | para, high                    | cryo, low  |
| α-SMA, IF           | para, all| para, high| para, all    | para, high           | para, high                    | para, all  |
| ki67, DAB           | both, all| both, all  | both, all    | both, all            | both, all                     | both, all  |

did not show any PAR-2 positive regions. Our overview of histological and immunohistochemical staining may serve as baseline imaging for pre-clinical studies using the rabbit lung model. Images of diseased lung tissue can be compared to the here presented images of healthy rabbit lung tissue in the future.

Author statement

GMB assisted and performed IHC stainings, imaged all sections and composed all figures. OE performed cryopreservation and cryosectioning, established IHC protocols (except for PAR-2), performed IHC stainings and imaged the samples with the slide scanner. DMH gave input on PAR-2 staining. MC and PG supervised the study. JB provided funding, wrote the manuscript and supervised the study.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actahist.2020.151648.
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