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„Comparison of fixation methods and immunohistochemical staining methods on Luciferase-positive murine lung tissue“

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1. Abstract English

The evaluation of tissue is an important tool in various fields of research and medicine. The study of morphological alteration in the lung is for example essential to rate the degree of emphysema and also the histologic type and progress of lung cancer.

To optimize the fixation of lung tissue, we performed three different methods for lung fixation: Inflation fixation, intratracheal instillation and no intratracheal fixation. Afterwards, the alveoli diameters were compared and used as a parameter to rate the qualification of each fixation method for our experiments. As the best method for lung fixation intratracheal instillation was identified. The lung anatomy was most of all preserved, the alveoli walls were thinwalled and had a small diameter.

After successfully identifying the ideal fixation technique, we started an immunohistochemistry (IHC) approach to detect Firefly-Luciferase after its non-viral gene transfer to the lung.

We wanted to evaluate the success of this gene-transfer by detecting the expressed protein Firefly-Luciferase with a polyclonal and monoclonal antibody directed against it. The experiments with the polyclonal antibody were performed following an established protocol. The staining results with and without an Isotype control were more satisfying than the results obtained with the monoclonal antibody after applying different antigen retrieval methods.

In summary, it was found that intratracheal instillation was the ideal method for the fixation of murine lungs. Concerning the IHC we successfully proved the expression of Firefly-Luciferase with the polyclonal antibody. The goal to establish a protocol for the monoclonal antibody could not be fulfilled.

2. Zusammenfassung Deutsch

Die histologische Untersuchung von Gewebeproben ist ein wichtiges Werkzeug in verschiedenen Bereichen der Forschung und Medizin. Die Beurteilung von morphologischen Veränderungen in der Lunge ist beispielsweise essentiell, um das Fortschreiten eines Emphysems zu bewerten, oder um den histologischen Typus und das Voranschreiten von Krebs zu beurteilen.

Um die Fixierung der während unserer Forschung verwendeten Lungen zu optimieren, wurden drei verschiedene Fixierungsmethoden vorgenommen: Inflationsfixation, intratracheale Instillation und keine intratracheale Fixation. Danach wurden die Durchmesser der Alveolen verglichen und als Parameter für die Beurteilung der Eignung der jeweiligen Fixationsmethode verwendet. Hierbei stellte sich die intratracheale Instillation als Methode für die Fixation von Mäuselungen als die am besten geeignete heraus. Die natürliche Lungenanatomie war weitgehend erhalten, die Wände der Alveolen waren dünnwandig und hatten einen kleinen Durchmesser.

Nach erfolgreichem Evaluieren der idealen Fixierungsmethode begannen wir mit dem Versuch, mithilfe von IHC die Expression von Luciferase nach nicht-viralem Gentransfer nachzuweisen.

Wir versuchten, den Erfolg jenes Gentransfers nachzuweisen, indem wir das expremierte Protein Firefly-Luciferase mit einem polyklonalen und monoklonalen Antikörper, welche jenes Enzym als Antigen erkennen, mittels IHC Staining anfärbten. Die Experimente mit dem polyklonalen Antikörper wurden mithilfe eines bereits etablierten Protokolls durchgeführt.

Hierbei erzielten wir sowohl mit als auch ohne Isotypkontrolle befriedigendere Resultate als mit dem monoklonalen Antikörper, der nach Durchführung verschiedener Antigen-Demaskierungsprozesse auf dem Gewebe angewendet wurde.

Zusammenfassend kann man über die Ergebnisse aller drei behandelten Themen sagen, dass die ideale Fixierungsmethode für murine Lungen intratracheale Instillation ist. Die Expression von Firefly-Luciferase konnte mit dem polyklonalen Antikörper erfolgreich nachgewiesen werden. Das Ziel, ein Protokoll für den monoklonalen Antikörper zu entwickeln, konnte nicht erreicht werden.

3. List of Abbreviations

MMCT	Macromolecular Cancer Therapeutics
2nd	Secondary antibody
A.d.	Aqua destillata
AB	Antibody
ABC-HRP	Avidin-Biotin-Complex Horse-Radish-Peroxidase
BLI	Bioluminescence
DAB	3,3'-Diaminobenzidinetetrahydrochloride
DNA	Deoxynucleic Acid
EtOH	Ethanol
Fab	Antigen-binding fragment
Fc	Crystalline fragment
g	Gramm
HE	Hematoxyllin-Eosin
hv	Hypervariable regions
Ig	Immunoglobuline
IHC	Immunohistochemistry
infla.	Inflation fixation
intratr.	Intratracheal instillation
L	Liter
LPEI	Linear Polyethylenimine
Lu	Lung
Luc.	Luciferase
M	Molar
m.point	Measurement point
ml	Milliliter
mM	Millimolar
MOM	Mouse on Mouse

N	Normal
neg.	Negative
nm	Nanometer
no fix	No intratracheal fixation
PAMAM	Poly(amidoamine)
pH	Potential of hydrogen
pos.	Positive
RLU	Relative light units
s	Seconds
μl	Microliter
μm	Micrometer

4. Introduction

4.1 Lung-Anatomy

The blood is supplied to the lungs by two different circulatory systems, the bronchial and the pulmonary one. The bronchial circulation originates from the intercostal blood vessels and the aorta. By the bronchial circulation, oxygenated blood is provided to bronchi, large blood vessels, hilar lymph nodes and visceral pleura.

The bronchial circulation comes to an end at the terminal bronchioles. In this area it joins with the venules and the capillaries of the pulmonary circulation. The poorly oxygenated blood is transported by the pulmonary circulation from the right side of the heart to the alveoli. Here, the carbon dioxide is exchanged for oxygen.

The capillaries of the pulmonary empty into pulmonary veins, where oxygen-enriched blood is being transported back to the heart. (Piper M. Treuting, Suzanne Dintzis, Denny Liggitt and Charles W. Frevert (2012). Comparative Anatomy and Histology-A Mouse and Human Atlas)

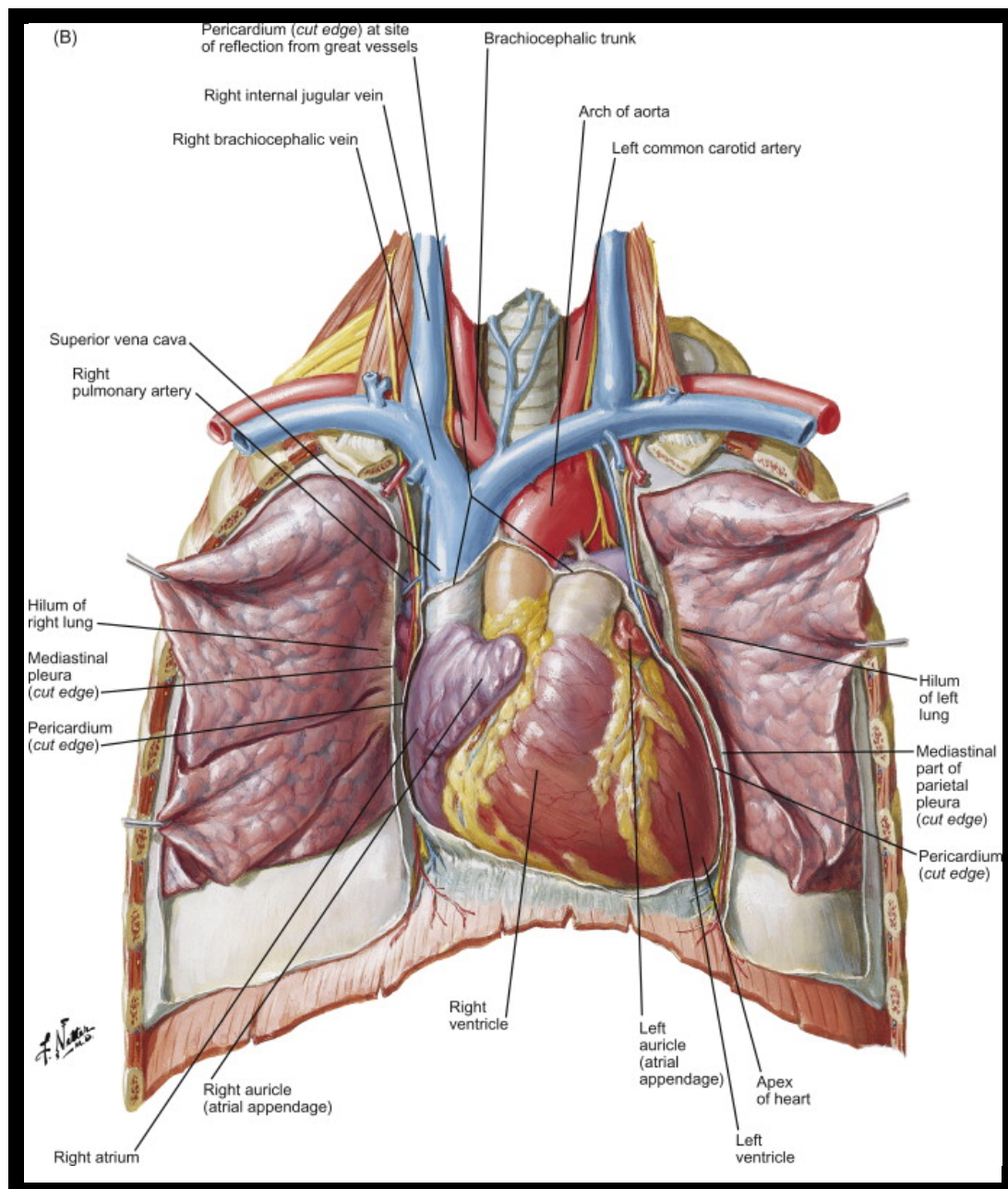


Figure 1-Overview over the regional anatomy of heart and lung

Source: Piper M. Treuting, Suzanne Dintzis, Denny Liggitt and Charles W. Frevert (2012)-Comparative Anatomy and Histology-A Mouse and Human Atlas

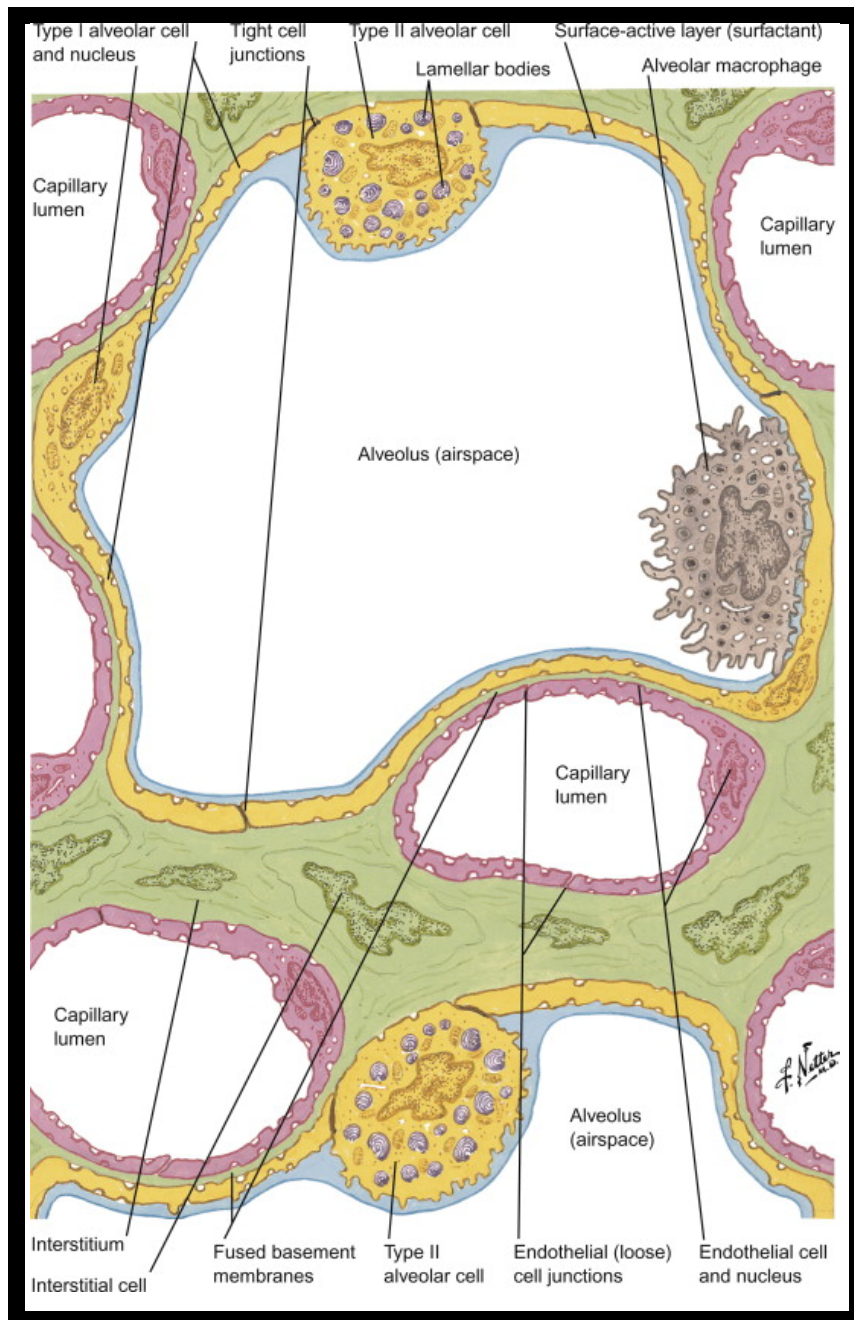


Figure 2-Overview of the lung anatomy

Source: Piper M. Treuting, Suzanne Dintzis, Denny Liggitt and Charles W. Frevert (2012)-Comparative Anatomy and Histology-A Mouse and Human Atlas

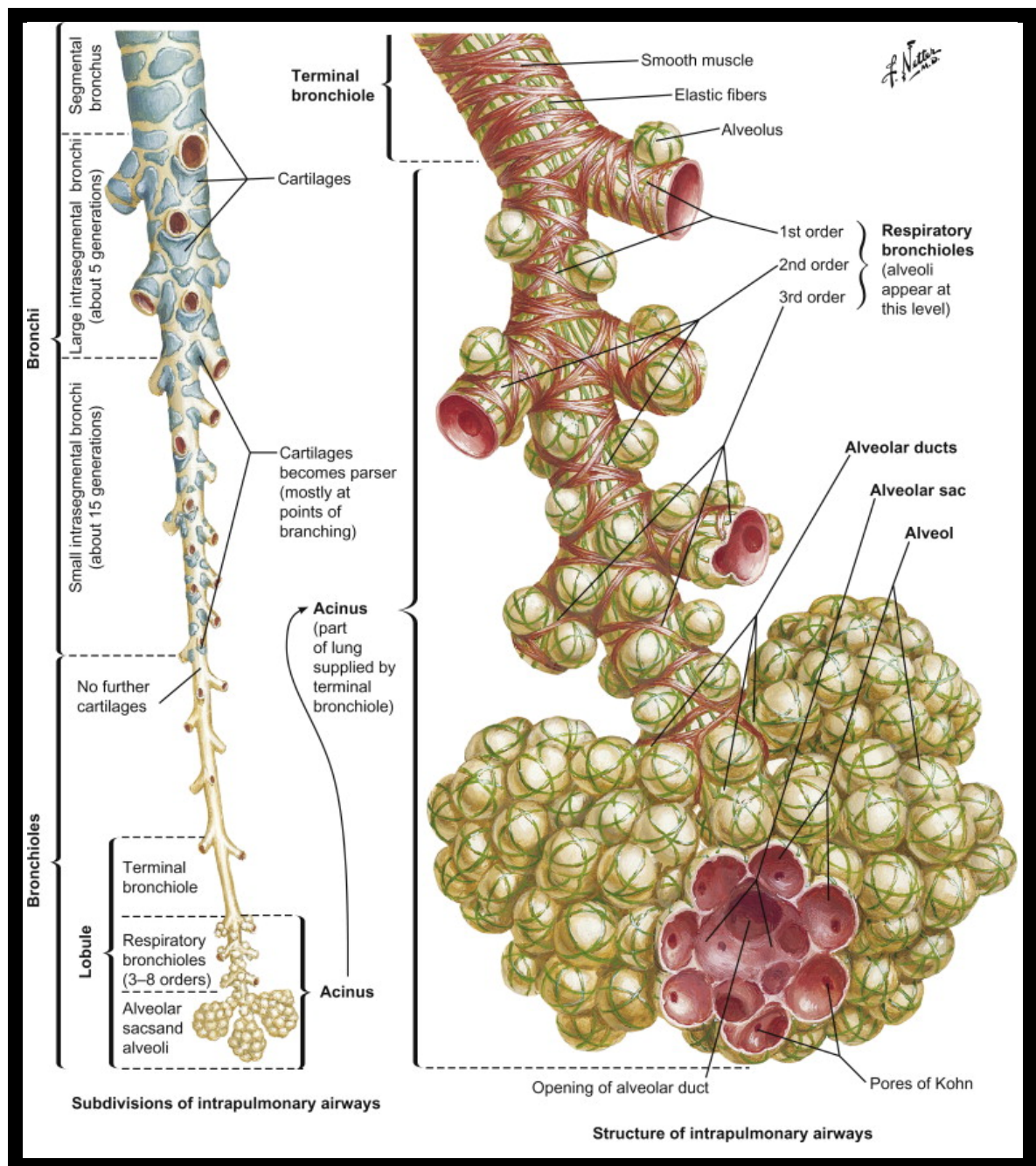


Figure 3-Detailed view of conductive airways and the respiratory zone in the human lung

Source: Piper M. Treuting, Suzanne Dintzis, Denny Liggitt and Charles W. Frevert (2012)-Comparative Anatomy and Histology-A Mouse and Human Atlas

4.2. Respiratory Zone

The respiratory bronchiole is the first segment of the zone for respiration. In mice, there are only few to no respiratory bronchioles, which is why the transition of the respiratory bronchioles goes directly into the ducts of the alveoli. The ductus alveolus is the second part of the respiratory zone. Its wall is built up by bundles of smooth muscle and alveoli.

The third segment, which was the most important one for the evaluation of the ideal fixation technique during the experiments, are the alveolar sacs which have walls formed completely by alveolar septa. The collateral ventilation is provided by alveolar pores (Pores of Kohn). The equal pressure across adjacent alveoli and the prevention of atelectasis is made possible because of these pores.

The functional and structural element of the respiratory zone is the alveolus. In the alveolar wall's fine epithelial layer of squamous cells, you can on the one hand find type 1 pneumocytes or squamous alveolar cells which are infiltrated with type 2 pneumocytes that are cuboidal. (Piper M. Treuting, Suzanne Dintzis, Denny Liggitt and Charles W. Frevert (2012)-Comparative Anatomy and Histology-A Mouse and Human Atlas)

These two different cell types have various functions: Type 1 pneumocytes are important for gas exchange, while type 2 pneumocytes produce surfactant. Surfactant plays an indispensable role for the lung, because the surface tension of the fluid lining the alveoli decreases through its detergent-like effect. (Fehrenbach, 2001)

The measurement of the mean diameter of the alveoli is not only of importance for the evaluation of the ideal fixation technique, it is also essential to rate the degree of emphysema. The mean distance alveolar walls increases during the aggravation of the disease. (Thurlbeck, 1967), (Measurements, 1966)

The prevention of collapse, deflation and disruption of the original lung structures is relevant during lung fixation. Fixation artifacts could also lead to the presumption of thickened alveolar walls or hypercellularity, which should therefore be avoided. (Van Kuppevelt et al., 2000)

Thus, a correct morphological analysis is only possible, if a fixation method and an appropriate embedding procedure, that obtains the native lung structure before fixation, is performed. (Braber, Verheijden, Henricks, Kraneveld, & Folkerts, 2010)

The lungs during my experiments were fixed with a 4% Formaldehyde-solution. This fixative changes the protein structure with the intention to prevent natural processes that occur in unfixed tissue like elution, degradation or other modifications. (Braber et al., 2010)

4.3. Formalin Fixation

During fixation, on the one hand, the location of the antigen, either in the nucleus, cytoplasm or membrane should be preserved. On the other hand also the secondary and tertiary structure of the antigen should not be altered too much during the process, since they are a target for antibodies used for the detection of the antigens.

The advantage of formalin-fixation is that the tissue morphology is preserved, while the disadvantage is the alteration of three-dimensional protein structures which can modify the antigen-epitopes. This influences the ability of the antigen's epitope to react with the antibody's paratope. Thus, for IHC an antigen retrieval is necessary for tissue that is used for antibody staining. (Dako, 2013)

Formalin is a reactive, electrophilic species. Multiple groups of biological macromolecules react with it in a cross-linking fashion. The cross-linking can be described as a formation of CH₂ bridges (Methylene-bridges). This first stage of the reaction is reversible. (Thavarajah, Mudimbaimannar, Rao, Ranganathan, & Elizabeth, 2012)

4.4. Hematoxylin-Eosin (HE) Staining-Explanation

Hematoxylin and Eosin stains are essential for the recognition of various tissue types and changes in tissue morphology that are important for the contemporary cancer diagnosis.

The nucleic acids get stained by Hematoxylin in a deep-blue purple color by forming a complex. This reaction is complex and has not been fully understood. Eosin non-specifically stains proteins. The cytoplasm and extracellular matrix are stained in varying degrees of pink staining by Eosin and the nuclei are stained blue by Hematoxylin in typical tissue.

The staining pattern of Hematoxylin is diagnostically very important, since the stained nuclei can show the different specific patterns of heterochromatin condensation of varying cell-types and cancer-cells.

During the experiments Hematoxylin was also essential as a counterstaining agent as an addition to the cytoplasmic IHC-staining of Luciferase.(Fischer, Jacobson, Rose, & Zeller, 2008)

4.5. Immuno-Histo-Chemistry (IHC)

A monoclonal and a polyclonal antibody were used for the IHC stainings. The basic structure of antibodies and how monoclonal and polyclonal antibodies differ from one-another will be described here.

Immunoglobulines (Ig) are a major component of the adaptive immune system. There are five major classes of these proteins: IgA, IgE, IgD, IgG and IgM, while IgG is the quantitatively most frequent one in plasma or serum.

The basic constitution of all immunoglobulines is identical: They are built of two identical heavy chains (H) and two light chains (L), which are also identical. The class and subclass of the immunoglobulins is determined by different antigenic and structural properties of the H chains. The L chains can be either of kappa (κ) or lambda (λ) type. The connection between L to H are covalent interchain disulfide bridges, which participate in the tertiary structure and increase the stability in the immunoglobuline molecule.

In the IgG molecule, the two identical heavy chains are called gamma (γ) chains, whereas the two light chains are either κ or λ type. (Dako, 2013)

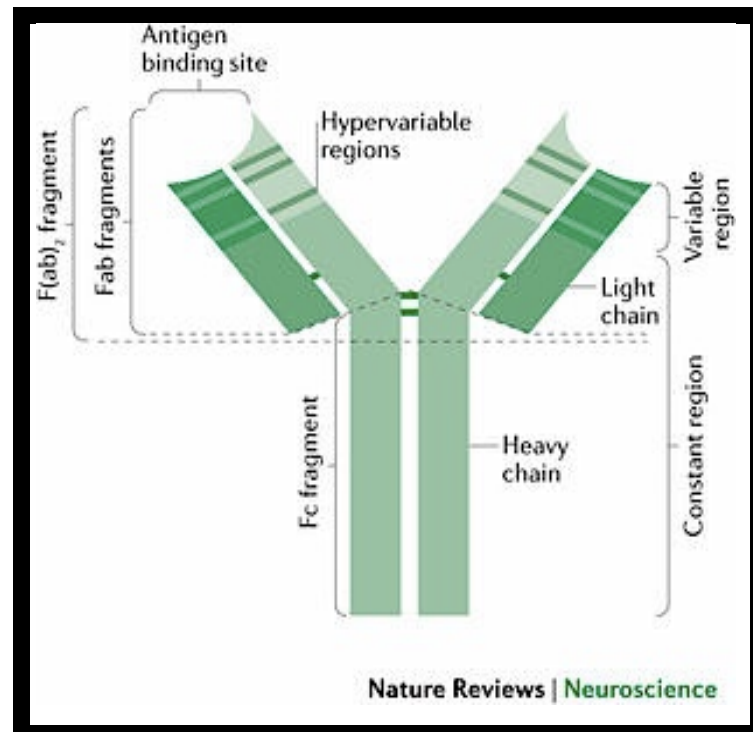


Figure 4-Structure of antibodies Source: (Crisp, Kullmann, & Vincent, 2016)

There are variable (V) and constant (C) regions in the light and heavy chains,

which are linked by disulfide bridges, that can be either present in or between the chains. A proteolytic digestion of the IgG molecule with Papain leads to splitting it into a crystalline fragment (Fc) and two antigen-binding fragments (Fab). The variable domains of the light and heavy chains also contain several hypervariable regions (HV). Once the hypervariable regions come in close proximity to the epitope of the antigen, which is the antigenic determinant, the idiotypic determinant of the antibody will react with the antigen in a reversible reaction. Every clone of an antibody expresses its own idio type. (Dako, 2013)

4.6. Differences between polyclonal and monoclonal antibodies

Polyclonal Antibody	Monoclonal Antibody
Heterogenous mixture of antibodies	Homogenous population of immunoglobulin
Directed against different epitopes of the same antigen	Directed against a single epitope
Production in various B-cell clones leads to immunohistochemical dissimilarities and cause slightly different specificities and affinities	Production in a single B-cell clone of the same animal in ascites leads to immunohistochemical similarities and constant specificity and affinity
Exact concentration measurable and known	Concentration can only be estimated because of the production in ascites
More robust due to their multiclonality, recognises multiple epitopes, therefore has less specificity and increased chance for cross-reactivity	Less variation between productions lots, less variability due to immunological state of the animal. The sustained production is independent of the animals lifetime due to the use of hybridoma cells
Inexpensive in production	Expensive in production

Table 1-Comparison of monoclonal and polyclonal antibodies (Dako, 2013)

The monoclonal antibody that was used was produced in ascites fluid.

4.7. Challenges in IHC

Even though a rapid reaction between antibody and antigen rapidly takes place under ideal conditions, there are several hurdles for a reliable, specific reaction in immunohistochemistry such as unstable temperature, various length of tissue fixation or also varying concentration of the antibody. (Dako, 2013)

There is an inverse relationship between the duration of incubation and the antibody-concentration. The required incubation time needed for optimal results is shorter for higher antibody-titer. But practically it is useful to first determine a suitable incubation time before defining the optimal dilution for the antibody varying within up to 24 hours. The most common incubation time for antibodies is 10-30 minutes.

For a sufficiently strong reaction during short incubation times, the concentration and affinity of the antibody must be high and the reaction milieu has to be optimized regarding pH and diluent ions for example.

The disadvantage of shorter incubation time is that a higher concentration needs to be used, which makes a 24-hour incubation with the possibility of a higher dilution more economic.

The antigen-antibody reaction is also influenced by incubation temperature. The equilibrium is reached more quickly at 37°C in comparison to room temperature. Unfortunately with a higher temperature, that allows greater dilutions and/or also a shortened time of incubation, the consistency of incubation time gets even less reliable.

Frequently, the temperature of 4°C is used during overnight incubations. At all temperatures, using a humidity chamber is recommended. (Dako, 2013)

Another challenge in IHC is the possibility of antibody cross-reactivity, which means that a reaction between an antibody and several antigens or a reaction between the antigen and several different antibodies occurs. (Dako, 2013)

For optimal results it takes a lot of steps and reagents while performing an immunohistochemic staining on specimen. The secondary antibody, that was used during the experiments is coupled with HRP.

It is necessary to block the endogenous peroxidase, that is mainly present in granulocytes and erythrocytes. This endogenous peroxidase can react with the chromogen (DAB- 3,3'-Diaminobenzidine) and produce the same staining as the peroxidase coupled with the secondary antibody. Therefore unspecific staining will lead to false positive results. (Bussolati & Radulescu, 2011)

4.8. Firefly Luciferase

Both antibodies, that were used during the experiments are directed against Firefly-Luciferase. Firefly-Luciferase is an enzyme, that functions as a reporter gene for Bioluminescence (BLI) and therefore is a useful tool for diagnostics in the living animal or for evaluating the expression of a delivered gene in several tissues. (Michalet et al., 2005)

For the research at the laboratory for MMCT (MacroMolecularCancerTherapeutics), Firefly-Luciferase is used as a tool to study the expression and distribution of genes transferred via synthetic gene delivery systems like linear polyethylenimine (LPEI), which are administered intratracheally (i.t.) or intravenously (i.v.). as a complex with plasmid DNA. (Geyer et al., 2017) , (Taschauer et al., 2016)

The expression of the enzyme can be quantified and evaluated via the enzymatic reaction of Luciferase. The substrate of Firefly-Luciferase, Luciferin, is oxidized in the presence of ATP. The emitted photons can be detected as a bioluminescence signal. (Lyons, Patrick, & Brindle, 2013)

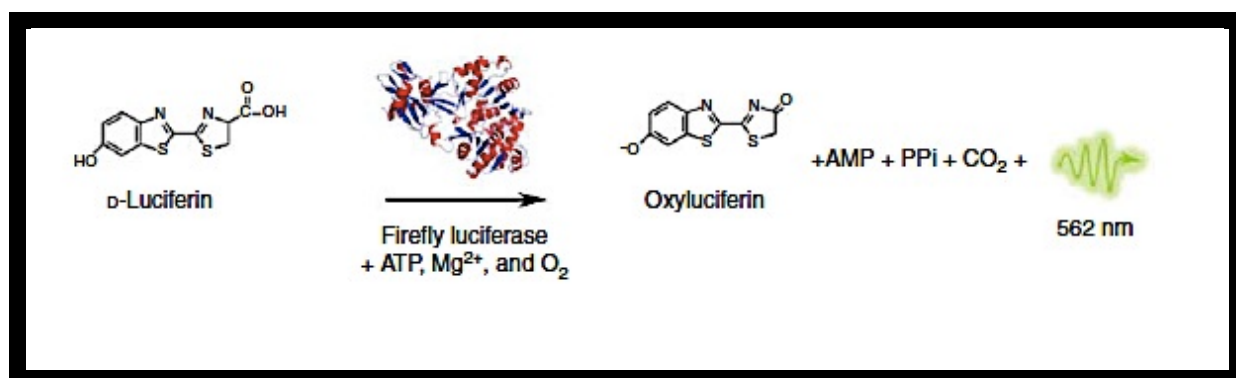


Figure 5 - Mechanism of the reaction of Firefly Luciferase

Source: (Lyons et al., 2013)

Bioluminescence occurs in living organisms. A chemical reaction produces an electronically excited substance, that emits light during its decay to the ground level. The substrate of Firefly-Luciferase is called Luciferin, which is converted into Oxyluciferin, when oxygen, ATP (Adenosine triphosphate) and a metallic cation like Magnesium ²⁺ is present.

The light emission occurs, when the enzyme converts its substrate Luciferin. Thus, no excitation is needed for the emission of photons after the reaction of Luciferase, which is an advantage in bioimaging. The emission spectrum of the Oxidoreductase Firefly-Luciferase is typical at 550-570 nm, whereas the peak is at 562 nm at pH 7.5-7.8. (Marques & Esteves Da Silva, 2009)

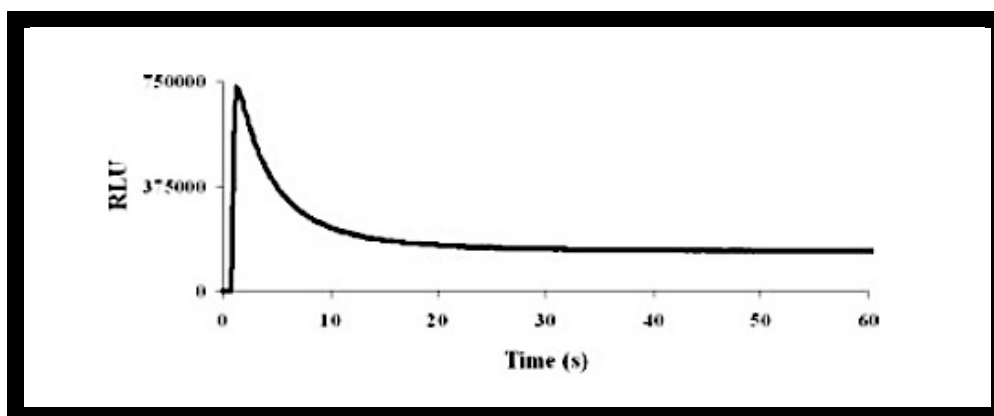


Figure 6 - Light production of the Luciferase in vitro with high concentration of substrate. First, there is a rapid increase in the light output followed by the decrease of light emission in vitro. RLU: Relative Light Unit

Source: (Marques & Esteves Da Silva, 2009)

To introduce the luc gene into the desired organism, an expression vector is needed, for example a plasmid. Once the gene is delivered to the cell, it gets translated into functional Luciferase protein. (Marques & Esteves Da Silva, 2009)

In the research of the department of MMCT, Firefly-Luciferase is used as a tool to evaluate the success of the gene-transfer with cationic polymers. Gene-transfer is used to study the function and regulation of genes, help establish various disease models and to explore the potential of various therapeutic applications to several diseases.

Some advantages of non-viral gene-transfer are the following: The risk for insertional mutagenesis, toxicity and immunogenic response is decreased compared to viral gene-transfer. Even cell or tissue specificity is achievable by chemically modifying the chemical or biological vectors. The disadvantages of non-viral gene-transfer are the decreased efficiency and duration of gene-expression. Genes delivered via non-viral gene-transfer often only cause a short-lived gene expression.

One of the most effective known cationic polymers for transfection is PEI. The efficiency of transfection and toxicity of PEI is dependent on the molecular weight, the charge-ratio of DNA to polymer and the configuration of the molecule. The molecular weight also has influence on the toxicity of the transfecting agents: A high molecular weight of more than 25,000 Dalton causes toxic effects on cells, while medium to low molecular weight of 5000-25000 Dalton shows a higher efficacy and decreased toxicity. Cationic polymers and negatively charged DNA have electrostatic interactions and therefore form a complex. This formed complex facilitates the cell uptake and protects the DNA. After being administered systemically, the polyplexes aggregate to form larger complexes, which leads to an accumulation in tissues such as lung and liver. Branched PEI is toxic and has a low transfection efficiency compared to the linear PEI derivatives, but it forms stable nanoparticles suitable for gene-delivery through aerosol administered intratracheally into the lungs, which qualifies it for the treatment of airway epithelial cells and lung cancers. (Al-Dosari & Gao, 2009)

The BLI of Luciferase is popular for diagnostics, since the decreased sensitivity due to background signals, that occur in Fluorescence-Imaging, is not as present at the wavelengths of the light emission by Luciferase. (Contag & Bachmann, 2002) , (Jacques, 2013)

During the experiments, the aim was to evaluate the location and quantity of the enzyme expression after non-viral gene-transfer in murine lung tissue by means of a polyclonal and a monoclonal antibody staining procedure.

The experiments during the diploma thesis were about three different topics:

1. Comparison of different fixation methods of mouse-lungs
2. Evaluation of Firefly-Luciferase expression in murine lung tissue with a polyclonal antibody
3. Optimization of a new protocol for monoclonal antibody staining of Firefly-Luciferase

5. Aims of the Thesis

The first aim was to evaluate the ideal fixation technique for murine lungs to ensure reliable results during the investigation of the lungs after IHC in the other two topics of the thesis.

After successfully finding the ideal fixation technique, we wanted to prove Luciferase-expression in murine lung tissue after non-viral gene-transfer. The first IHC-experiments were performed with a polyclonal antibody following an established protocol.

The experiments were then continued on the lungs, that showed the best staining results after IHC with the polyclonal antibody, but this time with a new monoclonal antibody with the aim to establish a reliable protocol for the new antibody.

The goals during the experiments with the monoclonal antibody were to find the ideal antigen-retrieval technique, -buffer and incubation times for the reagents used during IHC with the not yet established antibody.

6. Materials and Methods

All animal experiments were conducted by Mag. Antonia Geyer (Veterinarian). All procedures were approved by local ethics committee and are in accordance with the Austrian law for the protection of animals in the EU directive 2010/63/EU for animal experiments.

Catalogs of used reagents, IHC kits, devices and animals:

Reagent	Product detail
Acetic acid glacial	Manufacturer: VWR Prolabo Chemicals Product number: 20104.334 Batch: 14A160513
Ammonium Solution 28-30%	Manufacturer: Sigma-Aldrich Product number: 221228-1L-A Lot: SZBD3180V
BSA (Bovine Serum Albumine)	Manufacturer: Sigma Product number: A1933
DAB (3-3'-Diaminobenzidine tetrahydrochloride) 10mg substrate per tablet	Manufacturer: Sigma-Aldrich Product number: D5905-50TAB Lot: SLBS4779
EDTA (Ethylenediaminetetraaceticacid) Disodiumsalt dihydrate	Manufacturer: VWR Prolabo Product number: 443885J Batch: 2X004158
Entellan	Manufacturer: Merck Product number: 1.07961.0100
Eosin Y Solution	Manufacturer: Sigma-Aldrich Product number: 318906-500ml Lot number: MKBQ9365V
Ethanol 100% (Ethanol >99,8% with 1% MEK	Manufacturer: Roth Product number: K928.2

HBS (Hepes buffered saline)	Manufacturer: Xantec Product number: B HBS10-500 Lot number: HBST100217.a
Hematoxylin solution Harris	Manufacturer: Roth Product number: X903.3 Charge: 037253899
Hydrochloric Acid fuming 37%	Manufacturer: Sigma-Aldrich Product number: 30721-2,5L-GL Lot: SZEBE110V
Hydrogenperoxide 35%	Manufacturer: Roth Product number: 9683.1
KCl (Pottasium-Chloride)	Manufacturer: AppliChem Panreac Product number: A2939,1000
KH ₂ PO ₄ Pottasium phosphate monobasic	Manufacturer: Sigma Aldrich Product number: P5655-500G Lot number: SLBJ7258V
Na ₂ HPO ₄ .12H ₂ O Di-Sodium hydrogen phosphate dodecahydrate	Manufacturer: AppliChem Panreac Product number: A3906,1000
NaCl (Sodium-Chloride)	Manufacturer: Roth Product number: P029.3
NaOH (Sodiumhydroxide)	Manufacturer: PanRec AppliChem Product number: A6829,1000
Paraffinum (Parplast)	Manufacturer: Sigma Product number:mP3558-1KG Lot: SLBN3818V
Paraformaldehyde	Manufacturer: AppliChem Panreac Product number: A3813,1000
PBS (Dulbecco's Phosphate Buffered Saline)	Manufacturer: Sigma Product number: D8537-1L Lot: RNBF102

Silicone Oil	Manufacturer: VWR Chemicals Prolabo Product number: 84542.290 Batch: 13K190500
Tri-Sodium-Citrate	Manufacturer: VWR Chemicals Prolabo Product number: 27833.237 Batch: 13/170003
Tris: Tris(hydroxymethyl)aminomethane	Manufacturer: Sigma-Aldrich CAS: 77861 LOT: 3374C433
Triton-X	Manufacturer: Sigma-Aldrich Product number: X100-1L
Tween 20	Manufacturer: Sigma-Aldrich Product number: P228-500ml Lot number: MKBS1669V
Xylenes	Manufacturer: Sigma-Aldrich Product number: 534056-4L Lot: BCBP3972V

Table 2-Catalog of chemical reagents used during the experiments

Mouse	Description
29	Type: Balb/c = Balb/cByJRj Treatment: none
53	Type: Balb/c = Balb/cByJRj Treatment: none
67	Treatment: 250ul (=50ug pDNA) pDNA-dendriPLEXES
121	Type: MDA-MB-231 Treatment: PGKEGFPLuc, 3mio cells/mouse

127	Type: SCID = CB-17/lcr- Prkdc ^{scid} /RJ Treatment: none
143	Balb/c = Balb/cByJRj Treatment: none
211	Type: SCID = CB-17/lcr- Prkdc ^{scid} /RJ Treatment: none
240	Treatment: 1x10 ⁵ cells of 4T1- iRFP720

Table 3-Catalog of animals used during experiments

Antibody	Description and Product detail
Goat anti Firefly Anti-Firefly Luciferase Antibody	Polyclonal antibody for detection of Luciferase Product: ab181640 Lot #: GR226575-1 Manufacturer: AbCam
Luci-21 1-107	Monoclonal antibody for detection of Luciferase Lot #: RH2257531 Catalog number: MA1-16880 Manufacturer: Thermo Fisher
Mouse IgG κ Isotype Control (Isotype of the polyclonal antibody)	Clone: P3.6.2.8.1 Product: Lot number: 4305295 Manufacturer: Thermo Fisher
Purified mouse IgG1 κ -Isotype (Isotype for the monoclonal antibody)	Material: #557273 Clone: MOPC-31C Isotype: Mouse (BALB/c) IgG κ Manufacturer: BD Biosciences

Table 4-Catalog of antibodies used during IHC-experiments

Device	Description and Details
Bottles for buffers/DAB	Manufacturer: VWR Product number: 215-1596
Caroussel Embedder	Manufacturer: Slee Type: MTP SN: K130063
Computer	HP Compaq Elite 8300 Convertible Microtower Product number: QV993AV Serial number: CZC4100J8F OS: Windows 2 SP1
Cooling Plate	Manufacturer: Thomas-Medical Product number: T26-3003-10
Crystallizing dish	Manufacturer: Schott Duran No.: HX08.1
Embedder	Manufacturer: Slee Prod.: MPS/P1
Filter for Hematoxyllin	Manufacturer: GE Healthcare Life Sciences Whatman Diameter: 70mm Cat. number: 1003-070
Filter for syringe Sterile Syringe Filter 0,45 µm Cellulose Acetate	Manufacturer: VWR Product number: 28145-481 Lot: 12874550
Freezing-Cooling-Combination -20°C and +4°C	Manufacturer: Alletric Class: SN-T (5) Ap-Type: 6 6064 4 Serial number: 82.929.125.2

Heating oven	Manufacturer: Heraeus Typ: UT 6060 Product number: 51000164
Heating Plate and magnetic stirring	Manufacturer: Heidolph Type: MR 3001 K No.: 504-10100.00
Histology Marker (Elite PAP Pen)	Manufacturer: Cedarlane Labs Product number: CLSG80125
Laminar Air Flow	Manufacturer: Thermo Scientific Type: KS 12
Microscope KCC-REM-OLY -022400002	Manufacturer: Olympus Model: BX53F 100-120/220-240V 1.7/0.8A 50/60 Hz SN: 3J41795 20/309
Microtom	Manufacturer: SLEE Medical Type: CUT 5062 SN: B13 0120
pH-Meter	Manufacturer: ETV Intertec 2001759 InoLab pH 7110 Serial number: 14031607
Pipette 100-1000 µl	Eppendorf Research plus M21671C
Pipette 2-20 µl	Eppendorf Research plus N29925F

Pipette 20- 200 µl	Eppendorf Research plus M32204C
Software for microscopical analysis	Cell Sens Standard
Superfrost +	Manufacturer: Thermo Scientific Ref.: J1800AMNZ
Superfrost Microscope slides	Manufacturer: Roth Product number: 1879
Syringe 50ml (60ml) Luer	Manufacturer: Henke Sass Wolf Ref.: 4850001000 Lot number: 16 I 02 8 B

Table 5-Catalog of devices used during the experiments

Kit used for IHC	Product details and contents
M.O.M. Kit for detecting mouse primary antibodies on mouse tissue Peroxidase	Used for IHC with monoclonal antibody Product #: PK-2200 Contents: Protein concentrate, Mouse IgG blocking reagent, biotinylated-anti mouse IgG reagent, ABC reagent A and B
VECTASTAIN® ABC-HRP Kit (Peroxidase, Goat IgG)	Used for IHC with polyclonal antibody Catalog Number: PK-4005 Contents: Rabbit normal serum (blocking reagent), Anti-Goat IgG biotinylated antibody, ABC reagent A and B

Table 6-Catalog of IHC-Staining kits used during IHC experiment

6.1. Evaluation of Alveoli Diameters after performing different Fixation Methods

For the evaluation of the alveolar diameters, we tried different fixation methods on 6 different animals, where 2 mice were used for each fixation method.

Animal 1 Mouse 53	Animal 2 Mouse 143	Animal 3 Mouse 29	Animal 4 Mouse 127	Animal 5 Mouse 121	Animal 6 Mouse 249
No intra-tracheal fixation	No intra-tracheal fixation	Inflation fixation, 5 minutes Formalin with a syringe pump at a rate of 200ml/hour	Inflation fixation, Syringe pump at a rate of 200ml/hour, 50ml in 14 minutes, 24 minutes formalin	Intra-tracheal Instillation of 4% Formalin/ 10% buffered Formalin for ten minutes	Intra-tracheal instillation of 4% Formalin/ 10% buffered Formalin for ten minutes

Table 7-Mice used during the experiments for the evaluation of alveoli diametres

Before fixation, the mice were sacrificed with an overdose of anaesthesia.

6.2. Tissue Processing of Specimen

This following description of tissue processing, dehydration, paraffinization including embedding and cutting also applies for the other organs used for IHC.

After removal of the abdominal organs, the lungs were preserved in 70% Ethanol and kept in the fridge at 4°C before they were fixed with 4% Formalin for 22 hours at room temperature. To avoid deformation of the organs, the ratio between Formalin-Solution and organ was 1:20 for optimal fixation results. After the 22 hours the Formalin was substituted by 70% Ethanol and stored at 4°C again until further processing

6.2.1. Formaline-Solution 4% Formaldehyde w/v in HBS pH 7-7.5

Reagents	Equipment
Paraformaldehyde	Filter equipment
HBS	Heating plate
0.1M NaOH	Magnetic stirrer
	pH-Meter

Table 8-Reagents and equipment used for the preparation of 4% Formaline solution

8g of Formaldehyde were suspended in 500ml HBS in a bottle. To ensure complete dissolution, the HBS was heated to 60°C on a heating plate and stirred with a magnetic stirrer. Afterwards, 1.5 L of HBS were added to obtain a 2 L solution. The pH was measured with the pH-meter and adjusted to 7-7.5 with 0.1M NaOH. Once the pH was adjusted, the 4% Formalin was filtered and kept at 4°C in the cooling room.

6.2.2. Dehydration and Paraffinization of Specimen

To enable histological evaluation and preservation of biological material, the tissue specimen have to be dehydratized and paraffinized. This was performed with the carousel embedder.

The machine was programmed for an overnight run due to the long duration of the dehydration and waxing process. Before starting, the specimen are usually cut to fit histology cassettes. In this case, one cassette was used for one specimen, since it is common to embed the lung including the heart in one paraffin block for histology.



Figure 7-Histology cassettes and metal moulds used for the experiments



Figure 8-Embedder used for tissue processing

The cassettes were labeled with experiment and mouse number, date of embedding and organ name. The embedder has 12 stations. To ensure a proper dehydration of the tissue before paraffinisation, the tissue undergoes dehydration in an ascending row of various percentages of alcohol before reaching Xylene and Paraffin.

Overall, it takes up to 18.5 hours until this process is done:

Station	Reagent	Duration
I.	70% Ethanol	flexible
II.	70% Ethanol	1 hour
III.	96% Ethanol	1 hour
IV.	96% Ethanol	1 hour
V.	96% Ethanol	1 hour
VI.	100% Ethanol	1 hour
VII.	100% Ethanol	1 hour
VIII.	100% Ethanol	1 hour and 30 minutes
IX.	Xylene	1 hour and 30 minutes
X.	Xylene	1 hour and 30 minutes
XI.	Paraffinum 65°C	1 hour and 30 minutes
XII.	Paraffinum 65°C	1 hour and 30 minutes

Table 9 - Incubation times for the dehydration and paraffinisation of specimen

6.3. Embedding of the Organs

Next to dehydration and paraffinization, the specimen were ready to be embedded in wax (Paraplast). Before starting, the paraffin in the embedder had to be completely liquified after heating it to 58°C. The metal moulds and the organs in the cassettes that were used for the specimen were heated also.

The lid of the cassette was removed, the organs taken out and put into the metal mould (both shown in figure 6). Then, a little bit of liquid paraffin was poured into the metal mould, pressed tightly on it's base and put onto the cooling plate for about 30 seconds to make sure the organ stayed in the right place during embedding.

After that, the cassette was put onto the metal mould and an excess amount of paraffin was poured on it. The metal mould with the cassette and organ in it was then cooled on the plate for at least 20 minutes to ensure complete hardening of the paraffin.

When the paraffin block was solidified, it was popped out of the mould. The excess paraffin was scratched off using a microscope slide. Next, the cassette was labeled using the labeling machine and filed into the histology drawers before the cutting of the organs.

6.4. Cutting of the Specimen

Before cutting, the embedded organs were cooled on the cooling plate set to -23°C for at least 20 minutes. During this time, two big crystallizing dishes filled up with distilled water were prepared. One of them was heated up on the heating plate up to maximum 60°C.

The microscopic slides for the specimen of interest were prepared. Superfrost slides for HE-stainings and superfrost plus slides for IHC stainings were used during the experiments.

Afterwards, the block with the organ was clamped into the Microtom. At first, the thickness for the cut was set on 20 Micrometer. Following, cutting was performed several times to uncover the specimen from the thin layer of paraffin. After that, the thickness was set to 2-4 µm, whereas the ideal was 2-3 µm. For ideal staining results, the slides of specimen were always cut as thin as possible.

After carefully cutting the organ, microscopic preparation needles were used to transfer the slice to the cold water in the first crystallizing dish. Then, the labelled microscopic slide was used to transfer it into the hot water of the second one, whereas the cut was supposed to be unfolded and straight.

Then the slice was transferred onto the microscopic slide for further staining and investigation.

The microscopic slides were left to dry at room temperature over night before HE or IHC staining.

6.5. Deparaffinization of the Slides

To make the tissue accessible for further staining techniques, the wax in the specimen had to be removed. First, the slides were kept in a heating oven at 55°C for about 2 hours. Afterwards, a rehydration of the tissue was performed. This procedure is similar to the dehydration of the tissue but in the other direction and with differing incubation times. The tissue was then treated with Xylene and the descending Xylene and Ethanol row using histology cuvettes:

1. Xylene	2. 100% Ethanol	3. 96% Ethanol	4. 70% Ethanol
3 x 5 minutes	3 x 30 seconds	3 x 30 seconds	3 x 30 seconds

Table 10-Incubation times and reagents for deparaffinization of specimen

After the tissue was freed from paraffine and rehydrated, the sliced organs were now ready for staining methods and further investigation. Until this point, the methods were the same for the specimen, but from here on, the techniques start to differ inbetween the 3 different topics, that we were working on during the thesis.

6.6. HE-Staining for the Evaluation of Alveoli-Diameters

HE staining was used for the evaluation of the ideal fixation technique and also as a control for the IHC-sections. The process of HE-Staining and mounting was the same during all three topics.

Four different solutions and two cuvettes of 70% Ethanol are needed for Hematoxyllin-Eosin stainings:

Hematoxyllin	HCl-EtOH	Ammonium-H ₂ O	Eosin
3 min 30 sec	1 sec	20sec	10 min 30 sec
160ml	175ml	250ml	25ml
Harris	EtOH 100%	A.d.	Eosin Y solution
Hematoxylin	2.5ml	10 drops of a	200ml
solution	Hydrochloric Acid	32% Ammonium-	A.d.
	fuming 37%	solution	0.125ml
80ml A.d.	72.5ml A.d.		Glacial acetic acid

Table 11 - Reagents and incubation times for HE staining

After preparing the solutions and filtering the Hematoxylin prior to each staining procedure, the rehydratized slides with the specimen on them were dipped into Hematoxyllin first. In general, the incubation time with Hematoxylin is between 3-4 minutes. The slides were left in Hematoxyllin for 4 minutes because this helped achieve the best results.

After this, the slides were rinsed off with cold tap water. Then they were dipped into the HCl-EtOH solution just for one second, rinsed with tap water once again and then left in Ammonium-water-solution for 20 seconds. Before 10 minutes and 30 seconds incubation with Eosin-solution, the slides were rinsed with tap water again. Following the incubation with Eosin, the slides were immersed in 70% Ethanol twice for only one second and the HE-staining was finished.

The slides were then dehydrated in an ascending alcohol row and mounted using Entellan.

1. 70% Ethanol	2. 96% Ethanol	3. 100% Ethanol	4. Xylene
3 x 1 second	3 x 1 second	3 x 1 second	3 x 2 minutes

Table 12 - Incubation times and reagents for dehydration of stained slides before mounting the with Entellan

To mount the slides with Entellan, a single slide placed in the the Xylene bath was taken out with forceps and two drops of Entellan mounting medium were dripped on one slide. Afterwards the specimen were covered with a mirosopic coverglass and the mounted slides were kept under the hood to dry overnight before being able to evaluate the stained sections with the microscope.

6.7. Measurement of Alveoli using the Microscope

When the slides were completely dry, the sections were analyzed with a microscope using the software on the computer, that enabled the measurement of the mean diameter of the alveoli.

First, the diametres were measured using the 10x magnification of the microscope. Five pictures were taken from different areas of the organ of each slice. Then, five representative areas for the alveoli diameters were selected and evaluated with the measuring function of the program.

The mean diameter of the alveoli was calculated based the measured diameters. The alveoli, that were selected, were supposed to be thin walled with no or little signs of rupture or collapsed lung structure. Unfortunately with most of the used specimen, this was not possible due to lot of collapsed alveoli and fixation artefacts, which is why the most common alveoli size in the organ was measured in some cases.

6.8. Immunohistochemical Staining

For the other two topics, two different antibodies were used, a monoclonal and a polyclonal one:

Polyclonal Antibody Goat vs. Firefly

Product name: Anti-Firefly Luciferase Antibody
Product: ab181640
Lot number: GR226575-1
Manufacturer: AbCam

Table 13 - Product details of the polyclonal antibody used during the IHC experiments, Source: Datasheet Abcam, for further information see appendix

For the first experiments, 2nd and buffer control only were made. For the following ones, Isotype-Control of the polyclonal antibody was made instead of the buffer control.

Mouse IgG κ Isotype Control

Clone: P3.6.2.8.1

Product (REF): 14-4714-82

Lot number: 4305295

Manufacturer: Thermo Fisher

Table 14 - Product details of the Isotype control antibody used during the IHC experiments with the polyclonal antibody, Source: Datasheet Thermo Fisher, for further information see appendix

Firefly Luciferase Monoclonal Antibody Luci-21 1-107

Catalog number: MA1-16880

Lot number: RH2257531

Manufacturer: Thermo Fisher

Table 15 - Product details of the monoclonal antibody used during the IHC experiments. Source: Datasheet Thermo Fisher. For further information see appendix

The challenge with the monoclonal antibody is, as explained in the introduction, that the exact concentration is unknown. This becomes a problem, when the Isotype-control, that is needed, is supposed to have the same concentration as the primary antibody. The isotype that was used was the following:

Isotype Purified mouse IgG1 κ -Isotype

Material #557273

Clone MOPC-31C

Isotype Mouse (BALB/c) IgG κ

Manufacturer: BD Biosciences

Table 16 - Details for the Isotype control antibody that was used during the IHC stainings with the monoclonal antibody. Source: BD Biosciences datasheet. For further information see appendix

6.8.1. IHC Staining with the polyclonal Antibody

First, the methods for the polyclonal antibody will be described, since these were the first experiments. After trying different organs, the experiments were continued with the lung of mouse 67 as a Luciferase-positive and the lung of mouse 53 as a Luc-negative control, whereas the first experiments were made on the lung of mouse as a negative control.

As mentioned above, the first experiments with the polyclonal antibody were done without the Isotype-Control, but with a buffer control instead. The established protocol was used for this antibody. This protocol was evaluated and established by Manuela Simlinger during her diploma thesis.

6.8.1.1. Slide Scheme for the Experiments without Isotype Control

To be able to differ specific from non specific staining in IHC, two controls without primary antibody are needed. Before each staining, a slide-scheme was prepared and the microscopic slides were labeled as shown. The following table shows how the specimen were placed on the slides, while two sections of the same organ were put on one slide.

Luc.-pos. Mouse 67 Lu2 Date	Luc.-pos. Mouse 67 Lu2 Date	Luc.-neg. Mouse 211 Lu2 Date	Luc.-neg. Mouse 211 Lu2 Date
AB 1:1000	2nd. Control	AB 1:1000	2nd. Control
AB 1:1000	Buffer-Control	AB 1:1000	Buffer-Control

Table 17-Slide scheme for IHC stainings with the polyclonal antibody during the experiments with a 2nd and a buffer control

AB: Antibody (Goat anti Firefly) in different dilution

Buffer Control: The specimen were only incubated with BSA/PBS during the incubation of 1st and 2nd antibody

2nd Control: Only the 2nd Antibody was applied on the organ. During the incubation of the first antibody and Isotype, these organs were incubated with 2% BSA/PBS.

6.8.1.2. Slide Scheme for the Experiments with Isotype Control

To bring the antibody's specificity to proof more precisely, also a staining experiment with the polyclonal antibody using its Isotype instead of a buffer control was performed, using the lung of mouse 240 as a Luciferase-negative control.

Luc.-pos. Mouse 67 Lu2 Date	Luc.-pos. Mouse 67 Lu2 Date	Luc.-neg. Mouse 240 Lu2 Date	Luc.-neg. Mouse 240 Lu2 Date
AB 1:1000	2nd. Control	AB 1:1000	2nd. Control
AB 1:1000	Isotype-Control	AB 1:1000	Isotype-Control

Table 18 - Slide scheme for IHC stainings with the polyclonal antibody during the experiments with a 2nd and an Isotype control

AB: Antibody (Goat anti Firefly) in different dilutions (1:100 and 1:1000)

Isotype Control: The Isotype control was applied and handled just like the primary antibody

2nd Control: No primary antibody, but only the secondary antibody was applied on the organ. During the incubation of the first antibody and Isotype, these organs were incubated with 2% BSA/PBS.

6.8.1.3. Antigen Retrieval

As explained in the introduction, before antibody-staining, the tissue has to go through a demasking process. Otherways the antigen, in this case Luciferase, can not be recognized by the antibody.

To do this, the deparaffinized, rehydratized slides were taken out of the 70% Ethanol and dipped into distilled water for 30 seconds before the antigen-retrieval with the waterbath was performed.

In a previous work during experiments in MMCT, the best outcome in IHC staining with the polyclonal antibody was accomplished after demasking the antigens with a retrieval solution at pH 8:

Tris-EDTA-Buffer pH 8	10mM Tris Base, 1mM EDTA solution, 0.05% Tween 20
Tris: Tris(hydroxymethyl)aminomethane)	1.21 g
EDTA (Ethylenediaminetetra- aceticacid), Disodium salt dihydrate	0.37 g
Distilled water	1 L
Tween-20	0.5 ml

Table 19 - Reagents used for preparation of Tris-EDTA buffer pH8

370mg of the EDTA were put into a 1 L bottle and added to 1 L of Millipore water before putting the bottle on the plate using a magnetic stirrer for complete dissolution of the EDTA. After this, the pH was measured using the pH-meter. To increase the pH, 1N NaOH that was added dropwise with a pasteur pipette. To lower it, 1N HCl was used performing the same technique, in both cases the magnetic stirrer was on and the pH was constantly measured with the pH meter. Afterwards the buffer was stored in the cooling chamber at 4°C.

To demask the antigens, the waterbath method was performed for this experiment. The cuvettes were filled up completely, the slides were put in them and the buffer was heated to its boiling point by putting the cuvettes in silicone oil.

At about 98°C the buffer started boiling. The heating of the slides was continued for exactly 20 minutes, then cooled down for 5 minutes on the plate. Afterwards, the cuvettes with the slides were left to cool out at room temperature for another 30 minutes before proceeding.

6.8.1.4. Endoperoxidase Blocking

The blocking was performed by incubating the slides in a 3% solution of Hydrogenperoxide in PBS in a histology cuvette for 6 minutes, then the slides were washed three times with PBS and left in PBS while preparing the next steps.

3% H ₂ O ₂ /PBS	
H ₂ O ₂	21.43ml H ₂ O ₂ 35%
PBS	225ml

Table 20 - Reagents used for the preparation of a 3% H₂O₂/PBS solution that is used for blocking of endogenous peroxidase

PBS (Phosphate buffered Saline) 10x

pH 7.4

NaCl	80.06g
KCl	2.01g
Na ₂ HPO ₄ .12H ₂ O	19.34g
KH ₂ PO ₄	2.45g
Aqua dest.	10 Litres

Table 21 - Reagents used for the preparation of a PBS solution that is used for washing steps and for the preparation of other solutions used during IHC experiments

The solid reagents were weighed and solved in 9 Litres of Millipore water. The pH was adjusted with 10M NaOH or 1M HCl using the pH meter. Afterwards the rest of the volume was added before measuring and adjusting the pH again, before the PBS solution was stored at room temperature.

6.8.1.5. Blocking Procedure

To reduce non-specific background staining, the blocking was performed with the blocking reagent in the kit, whereas one drop of the blocking serum was added to 3333µl of 2% BSA/PBS:

2 % BSA/PBS

400 mg BSA (Bovine Serum Albumine)
20 ml PBS

Table 22- Table 16-Reagents used for the preparation of a 2% BSA/PBS solution that is used for preparing the antibody and blocking solutions

After the reagents were mixed, filtering of the solution using a 50 ml Syringe with a sterile filter in the laminar air flow was performed.

Before the blocking, a humidity chamber was prepared. To create a humid milieu for the slides, 4 pieces of paper towels wetted with distilled water were used. Then, plastic beams to place the slides on were put into the chamber.

The specimen were encircled with a special histology marker and placed horizontally on the beams. Then the blocking serum was put on the sections using a pipette applying about 50 µl on each organ. The slides were incubated with the blocking serum for 30 minutes. The blocking serum was then removed carefully with a pipette.

6.8.1.6. Primary Antibody

After removing the blocking serum, the specimen were incubated with the primary antibody. The dilution used was 1:1000, whereas the primary antibody was diluted with the 2% BSA/PBS. The antibody solution stored at -20°C was thawed and then 2 µl of the primary antibody were added to 1998 µl BSA/PBS.

The primary antibody was applied on all sections besides the control sections, which were incubated with the 2% BSA/PBS instead for the experiments without an Isotype control. During the experiments with an Isotype control, the solution of it was applied at the same time and handled just as the primary antibody.

First, the specimen were incubated at room temperature for one hour and fiftyone minutes at room temperature before they were left in the cooling room at 4°C over night.

6.8.1.7. Secondary Antibody

The secondary antibody from the kit was used. 9µl of the secondary antibody solution were added to 1791 µl 2% BSA/PBS. Before putting the secondary antibody on the slides, they were washed three times with PBS.

The solution of the secondary antibody was then applied on all of the organs the slides using a pipette again, but not on the buffer controls. The buffer control organs were incubated with BSA/PBS only in the meantime. The incubation time was 30 minutes at room temperature.

6.8.1.8. ABC Reagent

The Vectastain ABC-reagent is used to produce to create high sensitivity and low background staining in Biotin-conjugated antibody staining procedures. The principle is the interaction of the avidin-biotin complex. (Source: Vectastain-ABC HRP Kit, last accessed on the 24th of September 2017 on: <https://vectorlabs.com/vectastain-abc-kit-standard.html>)

The ABC kit has to be prepared 30 minutes prior to use. One drop of reagent A in the Vectastain Kit was added to 5 ml 2% BSA/PBS first, the one drop of reagent B was added to the solution.

Before applying the ABC-reagent on the slides, they were washed three times with PBS. The incubation with ABC reagent was performed at room temperature for 30 minutes. Following after the incubation, the slides were washed three times with PBS before being left in a solution of Triton-X/PBS for 6 minutes that was prepared during the incubation time.

0.5% Triton-X/PBS

1.25 ml Triton-X

248.75 ml PBS

Table 23 - Reagents used for the preparation of a 0.5% Triton-X/PBS solution that is used for washing after the ABC-reagent incubation

6.8.1.9. Chromogenic Detection

The kit that that was used works with a chromogenic detection system with horse raddish peroxidase (HRP). In presence of an electron donor, the formation of a complex between enzyme and substrate takes place. After this, the electron donor is being oxidated. The continuing catalysis of Hydrogenperoxide is driven by the force of the electron donor. Without the presence of H_2O_2 the reaction is stopped.

In this case, the electron donor used was 3,3'-Diaminobenzidinetetrahydrochloride (DAB). Upon oxidation, this substrate produces a brown staining, that is not soluble in organic solvents or alcohol. The oxidation also leads to polymerization of DAB. (Dako, 2013)

DAB

10 tabs DAB

250 ml PBS

1.25 ml Triton X

Table 24 - Reagents needed to make a DAB solution for the chromogenic detection during IHC experiments

The DAB was prepared in a bottle and a magnetic stirrer was used to ensure complete dissolution of the tablets. The solution was poured into a staining cuvette. Before putting the slides into the DAB, 25 µl of Hydrogenperoxide were added. The incubation time was 9 minutes at room temperature. Afterwards, the slides were washed with distilled water.

6.8.1.10. Counterstaining and Mounting the Slides with Entellan

Hematoxyllin was used as the counterstaining agent to stain the nuclei. The procedure of the counterstaining is equal to the HE-staining described in the chapter of topic one (Evaluation of alveoli diameters), but the staining is only performed until the point, where the slides are left in Ammonium-water for 20 seconds and washed with tap water. Afterwards, the same techniques for the dehydration in the ascending alcohol row and mounting the slides with Entellan were used as already explained in a previous chapter.

The slides were dried overnight. Afterwards the staining results were evaluated using the microscope and software on the computer.

6.8.2. IHC-Staining with the monoclonal Antibody

For the monoclonal antibody, there was no established protocol, which is why different retrieval methods in various experiments were performed. Regarding the incubation times, the protocol of the manufacturer was followed, using a 1:100 and a 1:1000 dilution and an Isotype control.

In contrast to the polyclonal antibody, the monoclonal antibody is murine, thus the kit used was a MOM-Kit (Mouse on Mouse). The first experiments were made on the lungs of mouse 67 as a Luciferase-positive specimen with mouse 53 as a negative control.

6.8.2.1. Buffers for Antigen Retrieval

During the experiments, the antigen retrieval was performed with with a pH 6 citrate buffer, a pH 8 and a pH 9 EDTA buffer.

Sodium-Citrate-Buffer pH 6	10mM Sodium-Citrate, 0,05% Tween 20
Tri-sodium-citrate	2.94 g
Distilled water	1 L
Tween-20	0.5 ml

Table 25 - Reagents used for the preparation of a Sodium Citrate buffer with pH 6 that is used for the demasking of antigens

The pH adjustment and storage methods were the same as described for the pH 8 EDTA buffer, which was used for this retrieval.

Tris-EDTA-Buffer pH 9	10mM Tris Base, 1mM EDTA solution, 0.05% Tween 20
Tris: Tris(hydroxymethyl)aminomethane)	1.21 g
EDTA (Ethylenediaminetetra- aceticacid), Disodium salt dihydrate	0.37 g
Distilled water	1 L
Tween-20	0.5 ml

Table 26 - Reagents used for the preparation of a Tris-EDTA buffer with pH 9 that is used for the demasking of antigens

The reagents used for this buffer were the same, but the pH was set to 9 instead of 8. All buffers were stored at 4°C.

6.8.2.2. Antigen Demasking for IHC-Staining with the monoclonal Antibody

The first experiments were performed with the waterbath method for antigen retrieval. The same technique was used as described in 6.7.1.2., but opposed to that not at pH 8 but with 2 different buffers at pH 6 and 9.

To be able to compare the two different retrieval methods, the same organs at different pH were used for the stainings.

Later on, the same specimen, slide scheme, dilution and staining technique for another antigen retrieval were used for another experiment. In this case, the microwave antigen retrieval method was performed after the same deparaffinization and rehydration methods as described above. The slides were put into the same cuvettes that were used for the waterbath method.

In this case, the demasking of antigens was done at 3 different pHs using the pH 6 citrate buffer and two Tris-EDTA buffers (pH 8 and 9). The microwave was set on 650 W. Then the retrieval solutions were heated up until they were close to their boiling points. When the boiling started, the retrieval was continued by microwave heating for ten minutes. Afterwards the slides were washed three times with PBS and the IHC performed like in the waterbath retrieval experiments.

Slide-Scheme:

Luc.-pos. pH ... Mouse 67 Lu2 Date	Luc.-pos. pH ... Mouse 67 Lu2 Date	Luc.-neg. pH... Mouse 53 Lu2 Date	Luc.-neg. pH... Mouse 53 Lu2 Date
AB 1:100	2nd. Control	AB 1:100	2nd. Control
AB 1:1000	Isotype-Control	AB 1:1000	Isotype-Control

Table 27 - Slide Scheme for IHC stainings with the monoclonal antibody. Two different dilutions (1:100 and 1:1000), a second control and an Isotype control were used. For each pH, there was a Luciferase positive and a Luciferase negative organ.

6.8.2.3. Endoperoxidase Blocking

As already described in the introduction and methods for the polyclonal antibody stainings, the principle and reagent for the endoperoxidase blocking were the same. But in this case, due to the manufacturers recommendation, this blocking step was performed for ten minutes instead of six minutes.

After the blocking, the slides were washed with PBS. This washing step also differs from the methods used on the polyclonal antibody. In this case, the slides get washed with PBS two times for two minutes respectively. Afterwards, the specimen were processed as described above using a humidity chamber and incubated for 5 minutes with the "MOM-Diluent":

MOM-Diluent	
	600 µl protein-concentrate
	7.5 ml PBS

Table 28 - Reagents used for preparation of MOM-diluent used as a diluent during the IHC stainings with the monoclonal antibody

6.8.2.4. Blocking Procedure

Before blocking, the diluent was dabbed off carefully with a paper towel. The reagent was applied on the lungs with a pipette. Then the lungs were incubated with the blocking solution for an hour:

Blocking Solution	2 drops stock solution
	2.5ml PBS

Table 29 - Reagents used for preparation of the blocking solution used during the IHC stainings with the monoclonal antibody to avoid unspecific background staining

After an hour, the blocking solution was dabbed off carefully.

6.8.2.5. Primary monoclonal Antibody

Since the plan was to evaluate the ideal protocol for Luci-21, two different dilutions were applied on the organs: 1:100 and 1:1000, as shown in the slide-scheme above.

1:100	6µl MAB in 594µl MOM-Diluent
1:1000	2µl MAB in 1498µl MOM-Diluent
Isotype-control (1:500)	2µl Isotype in 998µl diluent

Table 30 - Dilution scheme for the primary antibody and the Isotype control used during IHC staining with the monoclonal antibody

The antibody solutions and Isotype were dripped on the specimen with a pipette, the 2nd control organs were treated with the MOM-diluent during the incubation time of 30 minutes at room temperature. Afterwards, the slides were washed two times with PBS for 2 minutes respectively. Then the slides were put back on the beams in the humidity chamber.

6.8.2.6. Secondary Antibody

2nd AB
10µl in 2500µl MOM-Diluent

Table 31 - Reagents used for preparation of the secondary antibody during IHC with monoclonal antibody

Since the secondary antibody was used on all organs (1:100, 1:1000, Isotype-control and 2nd control), a bigger volume of this reagent was needed. A volume of about 40-70µl was applied on each organ and incubated for ten minutes. Following the incubation, the slides were washed with PBS two times for two minutes again.

6.8.2.7. ABC Reagent

ABC reagent	
	2 drops of reagent A
	2.5ml PBS
	2 drops of reagent B

Table 32 - Reagents used for the preparation of ABC-reagent used during IHC stainings with the monoclonal antibody to ensure better detection of the biotinylated secondary antibody

The principle and method of the ABC-reagent treatment is the same as already described, but the incubation time in this case was 5 minutes only. Afterwards the slides were washed with PBS for 2 times two minutes respectively.

6.8.2.8. Counterstaining and Mounting the slides

Procedure as described in 6.7.1.9.

7. Results

The results will be described in different chapters since there were 3 different topics. As for the IHC chapters, there will be a comparison of the differences between results obtained with monoclonal and polyclonal antibodies.

7.1. Evaluation of the ideal Fixation Technique

Six different animals were used, there were 3 different fixation methods, whereas the same fixation method was performed on two mice respectively:

Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6
Mouse 53	Mouse 143	Mouse 29	Mouse 127	Mouse 121	Mouse 249
No intra-tracheal fixation	No intra-tracheal fixation	Inflation fixation, 5 minutes Formalin with a syringe pump at a rate of 200ml/hour	Inflation fixation, Syringe pump at a rate of 200ml/hour, 50ml in 14 minutes, 24 minutes formalin	Intra-tracheal Instillation of 4% Formalin/ 10% buffered Formalin for ten minutes	Intra-tracheal instillation of 4% Formalin/ 10% buffered Formalin for ten minutes

Table 33 - Overview of the fixation methods performed on the lungs of different animals, performed to evaluate the diameter of the alveoli

7.1.1. Microscopic Analysis and Results of the Experiments for the evaluation of the ideal fixation technique

7.1.1.1. Lungs with no performed intratracheal Fixation

The most significant results were observable in the organs, that had no intratracheal fixation at all (Mouse 53 and mouse 143):

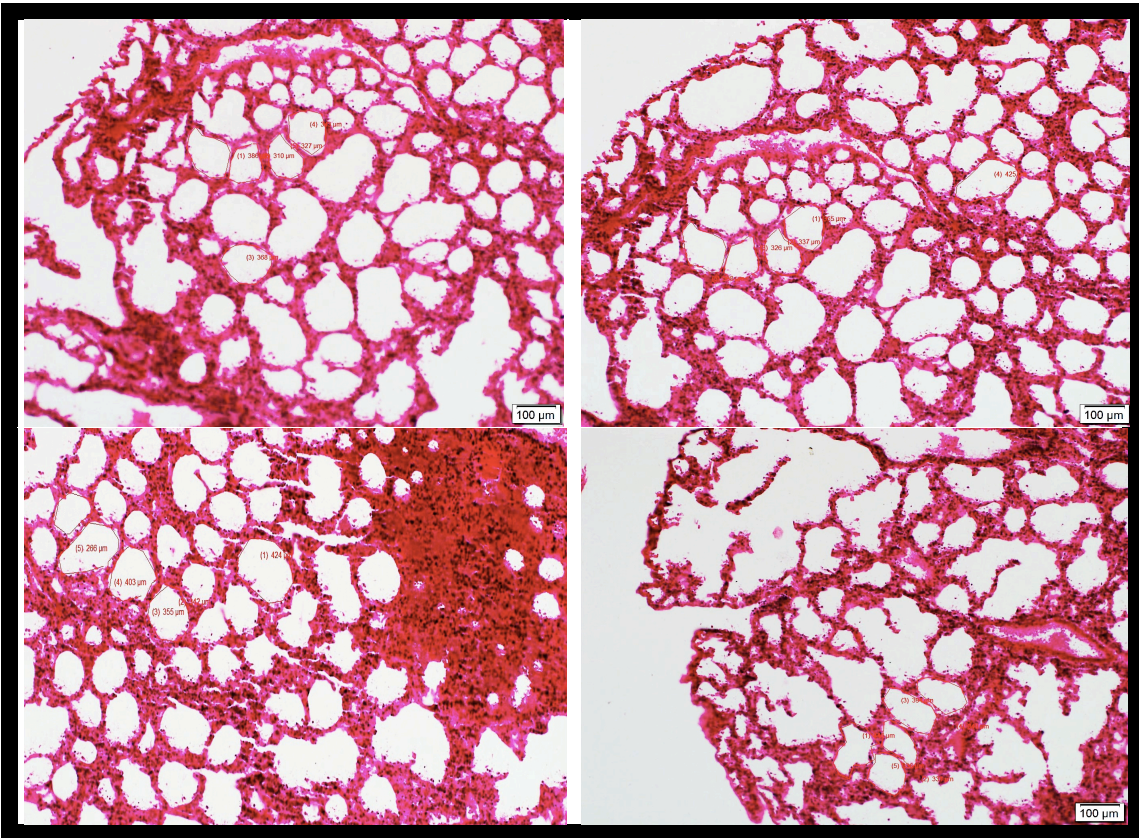


Figure 9 - Microscopic images of the lung of mouse 53 (10x magnification) HE Stain of a lung with no intratracheal fixation

Measured diametres	
Mouse lung 53 in μm (Animal 1)	Mean diameter in μm
368, 386, 337, 373, 335	359.8

Table 34 - Diameters and mean diameter of the alveoli in the lung of mouse 53, where no intratracheal fixation method was performed

In the lung of mouse 53, the outcome of the fixation was probably the worst. There were no thin-walled alveoli at all. The walls were thickened and alveoli were enlarged. Also, many alveolar structures looked disrupted and it was impossible to morphologically evaluate the lungs, since there was barely anything left of the natural lung morphology.

Looking at the images of lung of mouse 143, where also no intratracheal fixation was made, there were smaller alveoli on the one hand but on the other hand also a lot of fixation artefacts, thickened alveolar walls, plenty of disrupted areas and only few remnants of the natural lung architecture:

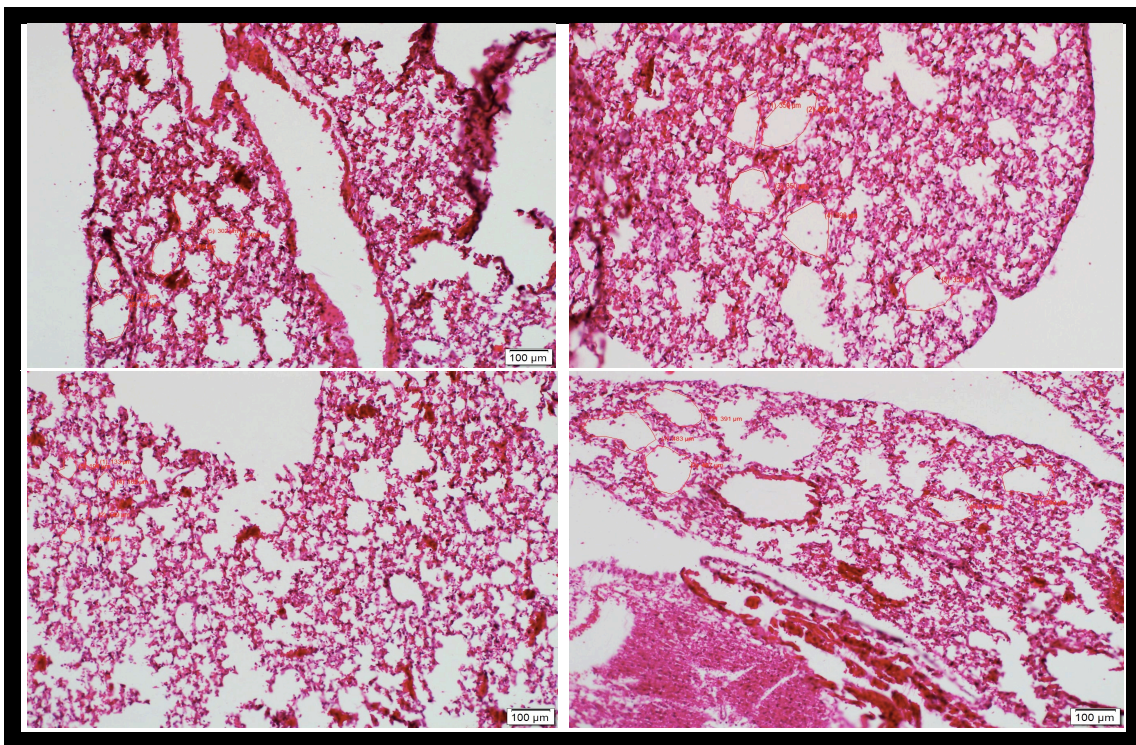


Figure 10 - Microscopic images of the lung of mouse 143 after HE staining (10x magnification)

Measured diametres

Mouse lung 143 in μm

Mean diameter in μm

(Animal 2)

Enlargened alveoli: : 349, 342, 368, 356, 358	354.6
Smaller alveoli: 133, 163, 191, 169, 149	161

Table 35 - Diameters and mean diameter of the alveoli in the lung of mouse 143, where no intratracheal fixation method was performed

Also in the case of mouse lung 143, it is obvious that this fixation was not ideal for optimal preservation of the lung morphology and that this method is not suitable for proper investigation of specimen.

7.1.1.2. Inflation-fixation

Now following are the results of the lungs that were fixed with inflation-fixation. The lungs of mouse 29 and 127 were fixed this way.

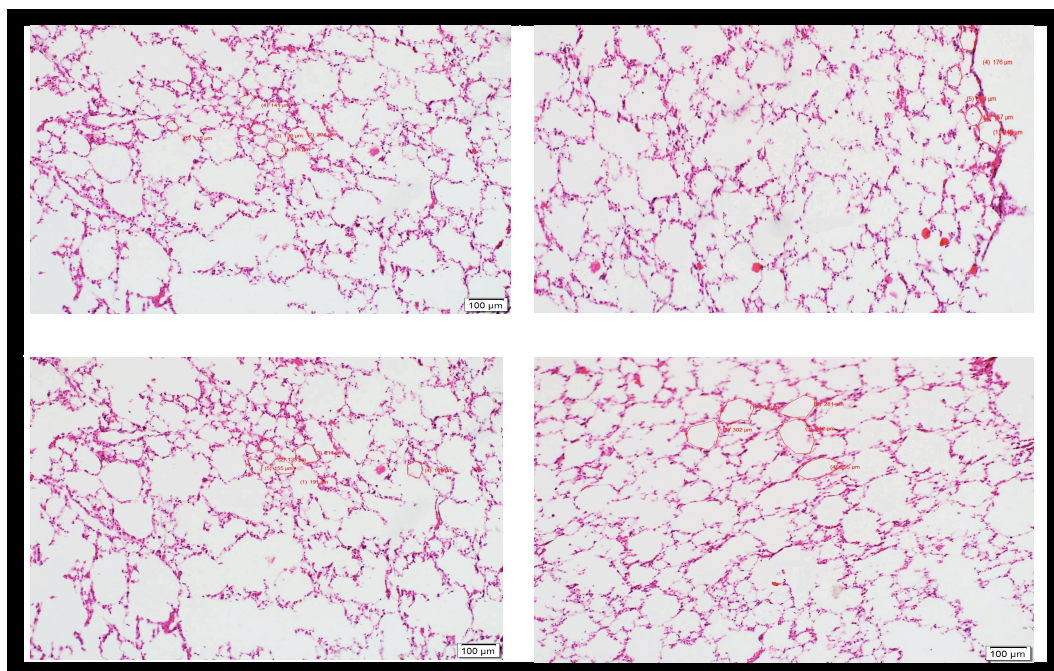


Figure 10 - Microscopic images of the lung of mouse 29 after inflation fixation and HE staining, 10x augmentation

Measured diametres

Mouse lung 29 in μm

Mean diameter in μm

(Animal 3)

Enlargened alveoli: : 302, 304, 281, 295, 303	297.0
Smaller alveoli: 141, 136, 164, 157, 155	150.6

Table 36 - Diameters and mean diameter of the alveoli in the lung of mouse 29, where inflation fixation method was performed

With the inflation fixation method that was performed on the lungs of mouse 29, it was possible to preserve parts of the fine, thin walls of the alveoli. Unfortunately, also alveoli enlargement, alveoli wall disrapture and some artefacts occured also in this case.

The lungs of mouse 127 were also fixed with the same inflation fixation technique, but there were way more artefacts, torn and thickened alveolar walls and a less well preserved lung architecture:

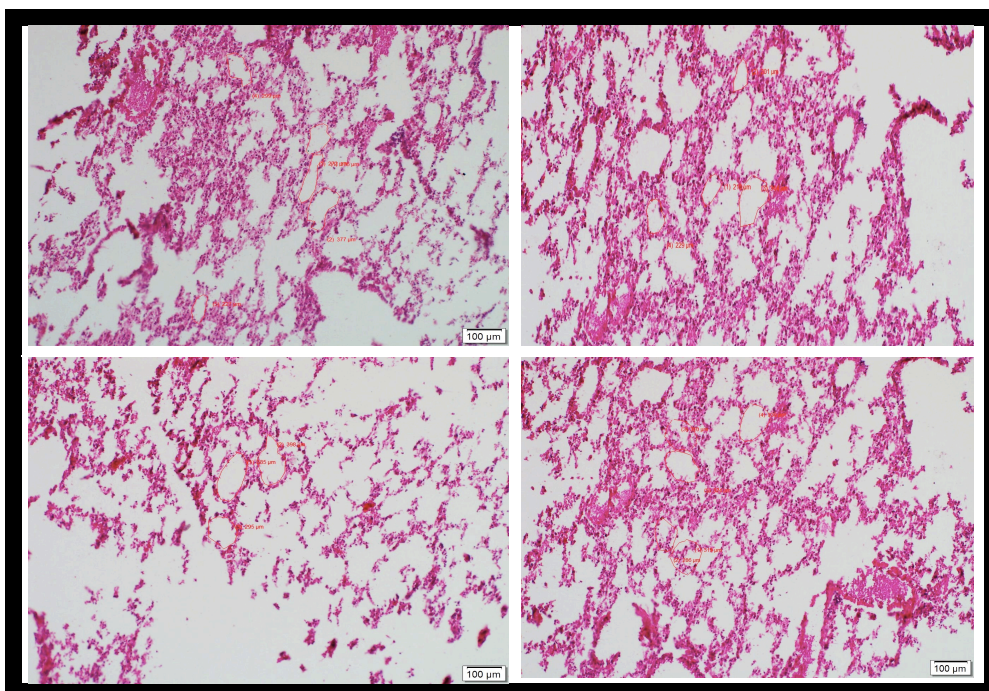


Figure 11-Microscopic images of the lung of mouse 127 after inflation fixation and HE staining, 10x augmentation

Measured diametres	
Mouse lung 127 in μm	Mean diameter in μm
(Animal 4)	
Enlargened alveoli: : 398, 408, 361, 371, 375	382.6
Smaller alveoli: : 229, 205, 283, 335, 327	275.8

Table 37 - Diameters and mean diameter of the alveoli in the lung of mouse 29, where inflation fixation method was performed

In genereal, the lung of mouse 127 had only very few well preserved alveoli that were suitable for a proper morphological analysis.

7.1.1.3. Intratracheal Instillation

Now the effect of intratracheal instillation will be shown. This fixation technique was performed on the lungs of mouse 121 and 249. The alveoli remained thin walled and there were the least fixation artefacts in comparison to all other fixation methods:

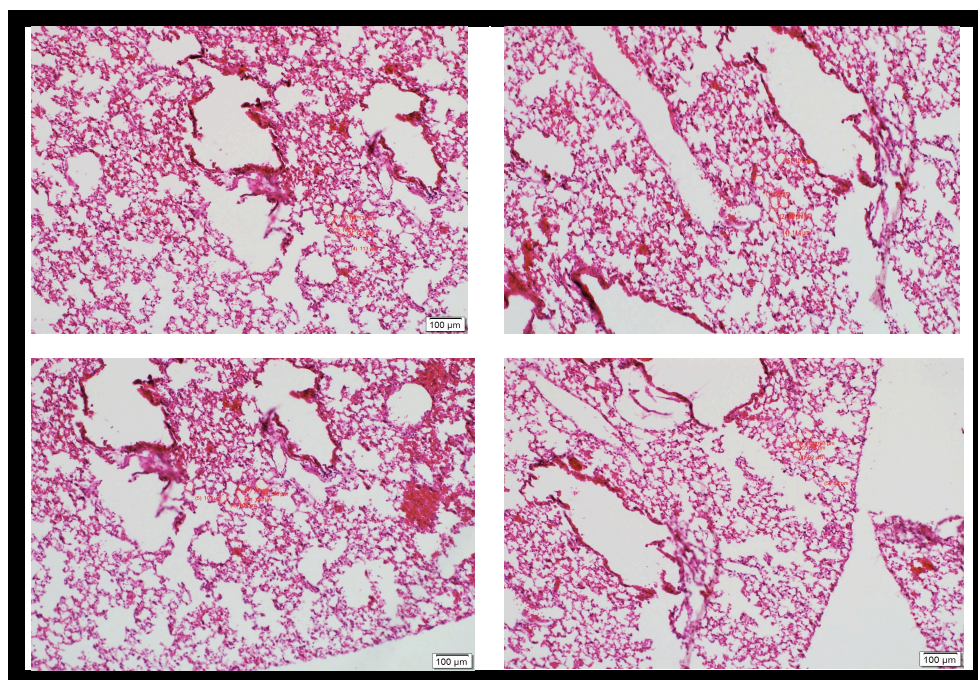


Figure 12 - Microscopic images of the lung of mouse 121 after intratracheal instillation and HE staining, 10x magnification

Unfortunately, there were also some disrupted areas, but compared to the outcomes of the other fixation methods this was obviously the best technique regarding preservation of lung architecture and alveoli diametres.

Measured diametres

Mouse lung 121 in μm

Mean diameter in μm

(Animal 5)

114,93, 101, 95, 113

103.2

Table 38 - Diameters and mean diameter of the alveoli in the lung of mouse 121, where the intratracheal instillation fixation method was performed

In case of the lung of mouse 249, the measured alveoli diameters were also mainly small. Unfortunately, there were some disrupted alveoli walls also:

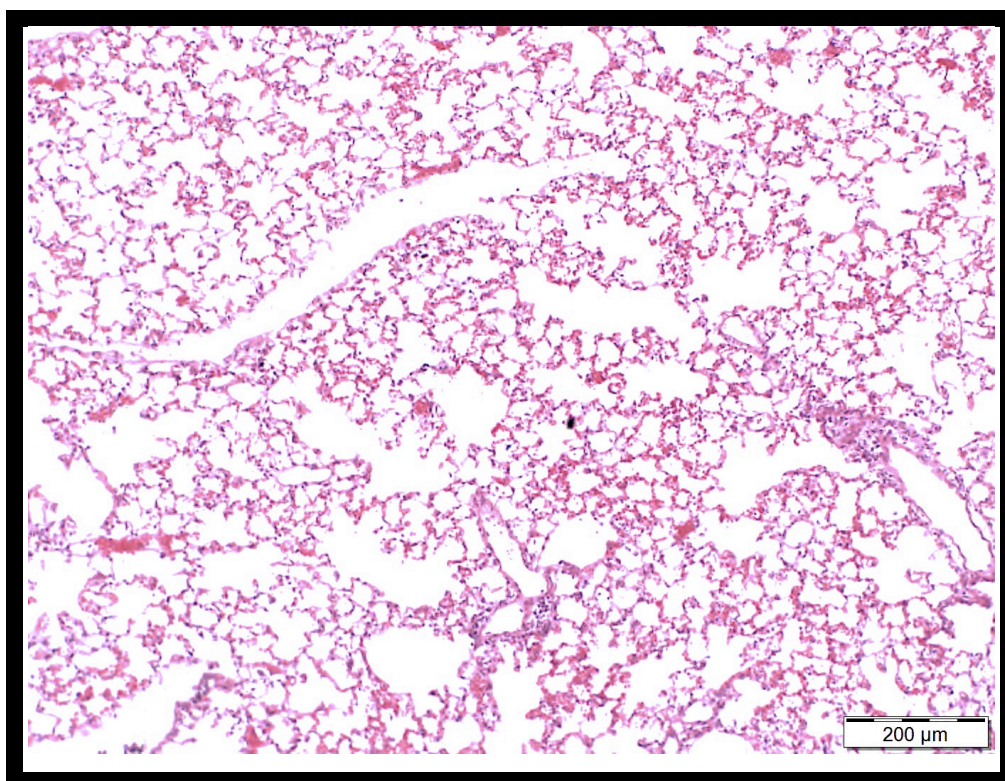


Figure 13 - Microscopic image of the lung of mouse 249 after intratracheal instillation and HE staining, 20x magnification

The lung of mouse 249 had a very well preserved lung structure and only very little fixation artefacts. The alveoli were thin walled and hardly any rupture occurred.

Measured diametres	
Mouse lung 249 in μm (Animal 6)	Mean diameter in μm
Enlargened alveoli: : 160, 156, 149, 141, 132	147.6
Smaller alveoli: : 80, 81, 92, 95, 94	88.4

Table 39-Diameters and mean diameter of the alveoli in the lung of mouse 249 after intratracheal fixation was performed

Mouse number	Fixation technique	Mean diameter in μm
53 (Animal 1)	No intratracheal fixation	359.8 (enlargened alveoli)
143 (Animal 2)	No intratracheal fixation	354.6 (enlargened alveoli) 161 (smaller alveoli)
29 (Animal 3)	Inflation fixation	297.0 (enlargened alveoli) 150.6 (smaller alveoli)
127 (Animal 4)	Inflation fixation	382.6 (enlargened alveoli) 275.8 (smaller alveoli)
121 (Animal 5)	Intratracheal instillation	103.2 (smaller alveoli)
249 (Animal 6)	Intratracheal instillation	147.6 (enlargened alveoli) 88.4 (smaller alveoli)

Table 40 - Overview of the mean diameters of the alveoli after performing different fixation methods

Shown in this table is an overview over the influence of the fixation technique on the diameters of the alveoli. It is not only obvious in the microscopic images of the lungs but also in the measurement of the mean diametres, that the intratracheal fixation method is the most suitable for ideal preservation of the lung architecture during fixation.

	no fix. a	no fix. a	infla. a	infla. a	intratr.a.	intratr.a.
Mouse	1	2	3	4	5	6
m.point 1		133	141	229	114	80
m.point2		163	136	205	93	81
m.point 3		191	164	283	101	92
m.point 4		169	157	335	113	95
m.point 5		149	155	327	95	94

Table 41 - Overview of measured diametres of smaller alveoli after different fixation methods in μm

m.point: Measurement point

no fix.: No intratracheal fixation method performed

infla.: Inflation fixation

a: Small alveoli

Intratr.: Intratracheal instillation

	no fix. b	no fix. b	infla. b	infla. b	intratr.b	intratr. b
Mouse	1	2	3	4	5	6
m.point 1	368	349	302	398		160
m.point 2	386	342	304	408		156
m.point 3	373	368	295	361		149
m.point 4	335	358	303	371		141
m.point 5	337	356	281	375		132

Table 42 - Overview of measured diametres of bigger alveoli after different fixation methods in μm

m.point: Measurement point

no fix.: No intratracheal fixation method performed

infla.: Inflation fixation

Intratr.: Intratracheal fixation

b: Big alveoli

	small alveoli no fix	small alveoli no fix	small alveoli inflation	small alveoli inflation	small alveoli intratr.	small alveoli intratr.
Mouse	1	2	3	4	5	6
mean		161.00	150.60	275.80	103.20	88.40
stdev		19.47	10.44	51.73	8.82	6.53
min		133	136	205	93	80
max		191	164	335	114	95
range		58	28	130	21	15
variation		379.2	109.04	2676.16	77.76	42.64

Table 43-Overview over mean diametres, standard deviations, minima, maxima, range and variation of small alveoli after performing different fixation methods

	big alveoli no fix	big alveoli no fix	big alveoli inflation	big alveoli inflation	big alveoli intratr.	big alveoli intratr.
Mouse	1	2	3	4	5	6
mean	359.80	354.60	297	382.60		147.60
stdev	20.31	8.75	8.60	17.56		10.13
min	335	342	281	361		132
max	386	368	304	408		160
range	51	26	23	47		28
variation	412.56	76.64	74	308.24		308.24

Table 44-Overview of mean diametres, standard deviations, minima, maxima, range and variation of big alveoli after performing different fixation methods

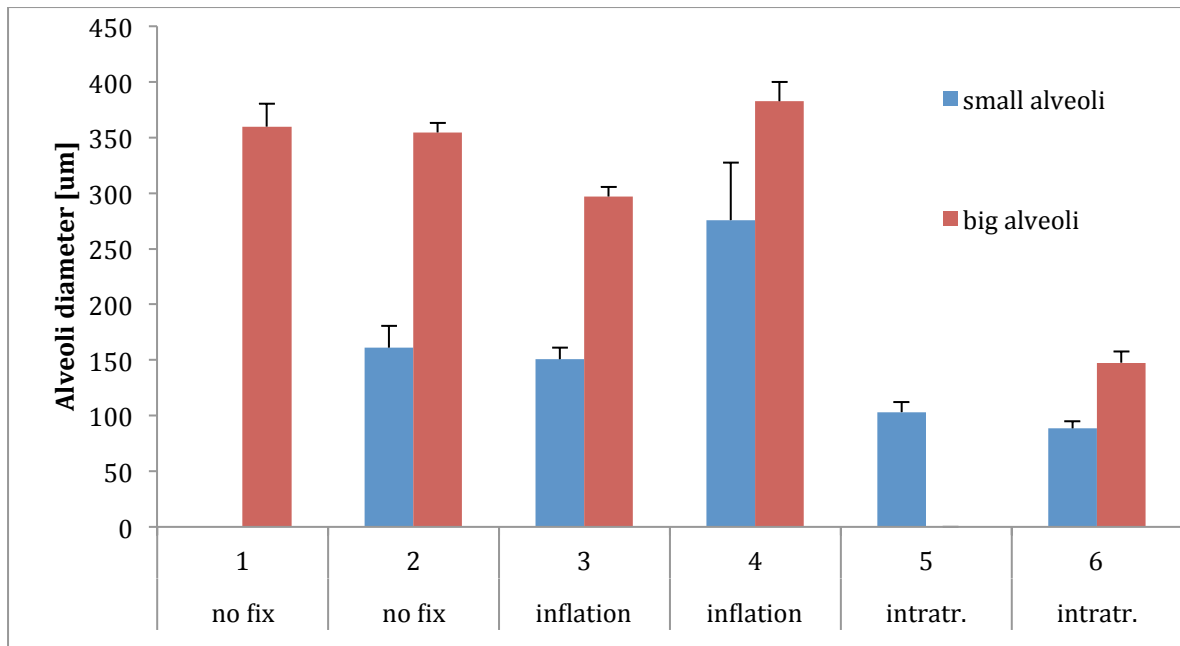


Figure 13 - Graphic illustration of the impact of different fixation methods on the diametres of alveoli on six different animals. Shown here is a comparison between no performed intratracheal fixation (mouse 1 and 2), inflation fixation (mouse 3 and 4) and intratracheal instillation (mouse 5 and 6)

7.2. Results after IHC-Staining with the polyclonal Antibody

7.2.1. IHC-Staining without Isotype Control

As already mentioned in materials and methods, the first experiments with the polyclonal antibody were performed without an isotype control on the lung of mouse 67 as a Luciferase-positive sample and lung of mouse 211 as a negative control.

Looking at the staining results from mouse 67's tissue, the Luciferase-expression was detectable through specific staining especially in the fine capillaries of the alveolar walls, as it is expected after a PAMAM transfection.

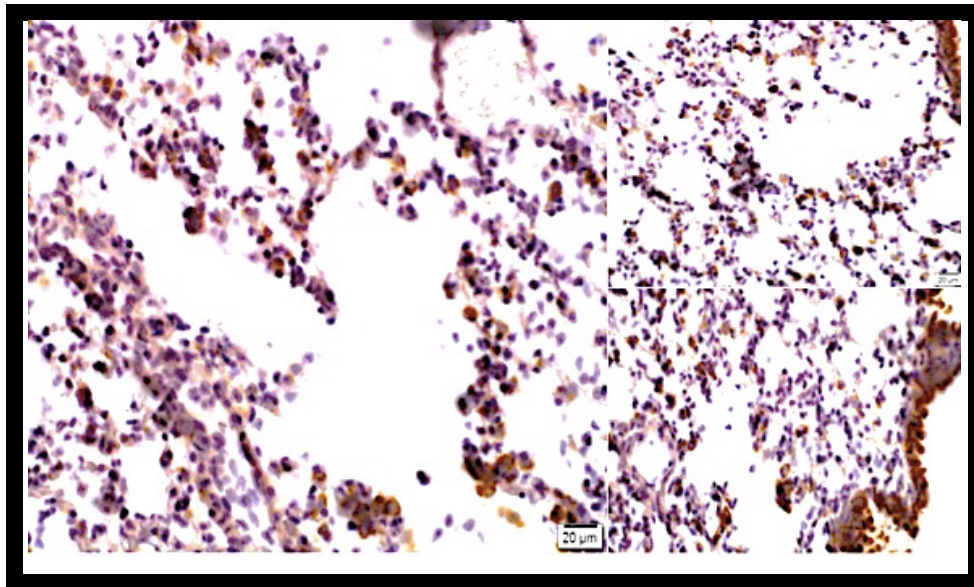


Figure 14 - Microscopic images of the lung of mouse 67 stained with polyclonal antibody and counterstained with Hematoxylin, 40x magnification

Besides the specific staining pattern there were also unspecific stainings on the margins of the lung. When compared to the 2nd control, the specific stainings did not occur, which suggests that the enzyme was really expressed in the areas only stained with Goat vs. Firefly antibody. Unfortunately the slide with the buffer control broke.

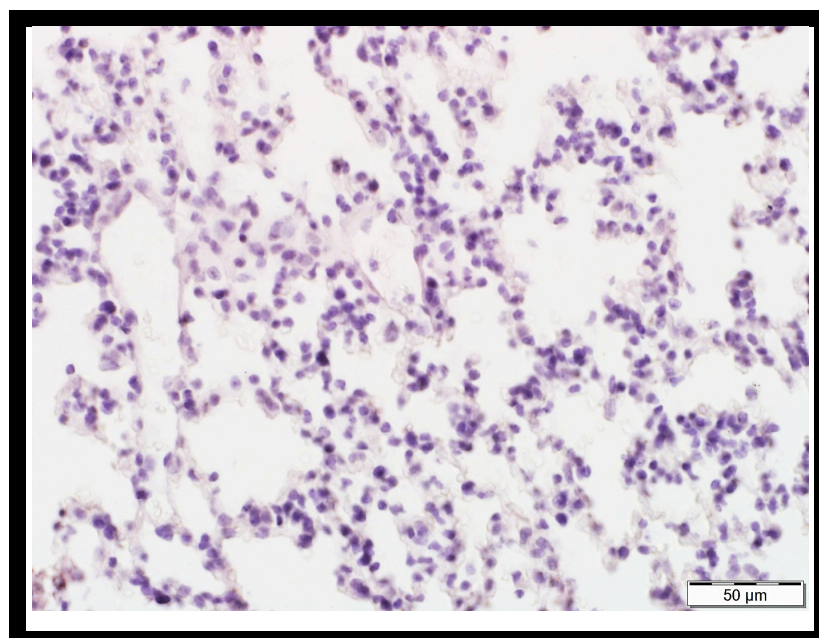


Figure 15 - Microscopic images of the lung of mouse 67 treated with secondary antibody control only and counterstained with Hematoxylin, 40x magnification

Compared to the Luciferase-expressing tissue of mouse 67's lungs, the negative control of mouse 211 did not show this staining pattern.

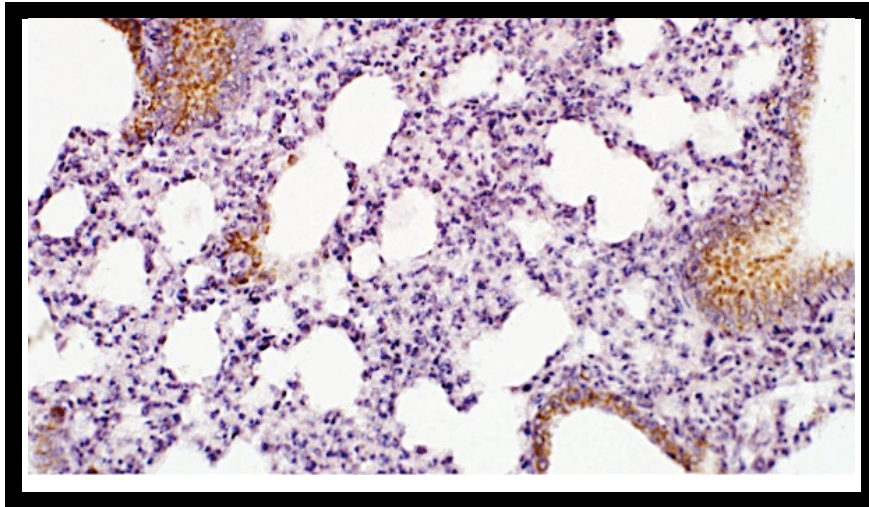


Figure 16 - Microscopic images of the lung of mouse 211 treated with polyclonal antibody and counterstained with Hematoxylin, 40x magnification. In this image, the brown staining is obviously unspecific

There were plenty of unspecific stainings, but they were easy to identify, since there was mainly no Hematoxylin counterstaining visible in the brown-stained areas. The unspecific staining was again mainly present at epithelial cells.

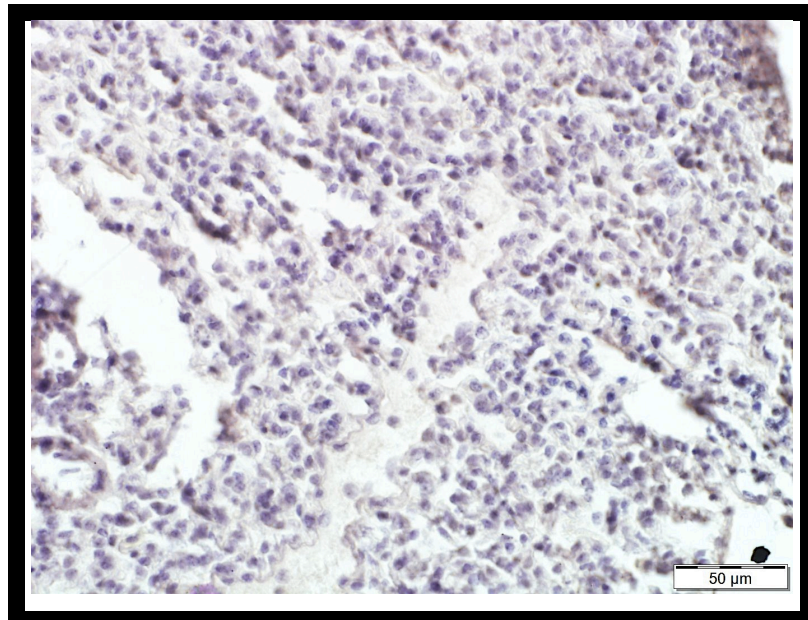


Figure 17 - Microscopic images of the lung of mouse 211 treated with buffer control only and counterstained with Hematoxylin, 40x magnification

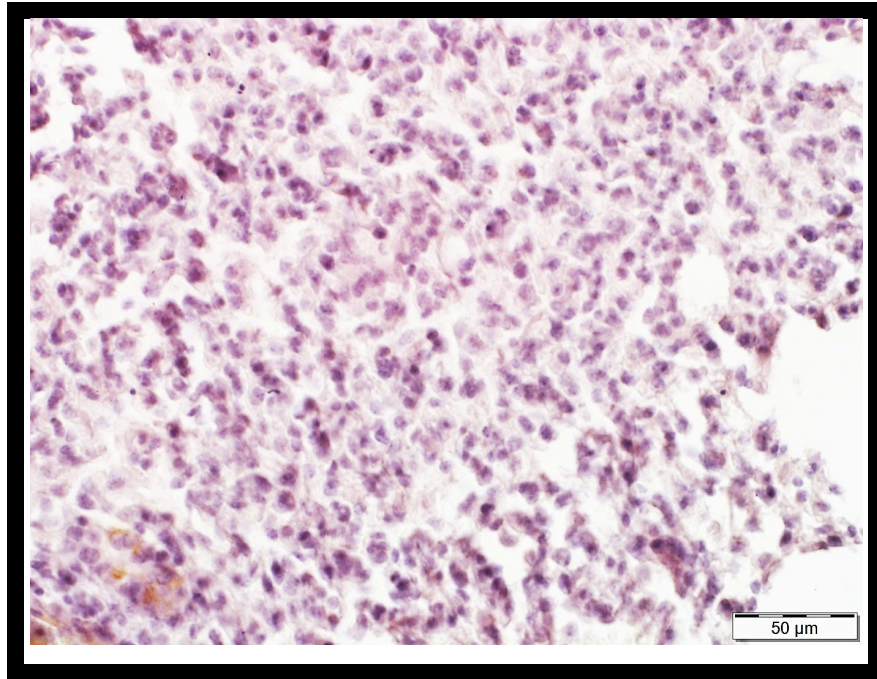


Figure 18 - Microscopic images the Luciferase-negative specimen of the lung of mouse 211 treated with secondary antibody control only and counterstained with Hematoxylin, 40x magnification

7.2.2. IHC-Staining with Isotype Control

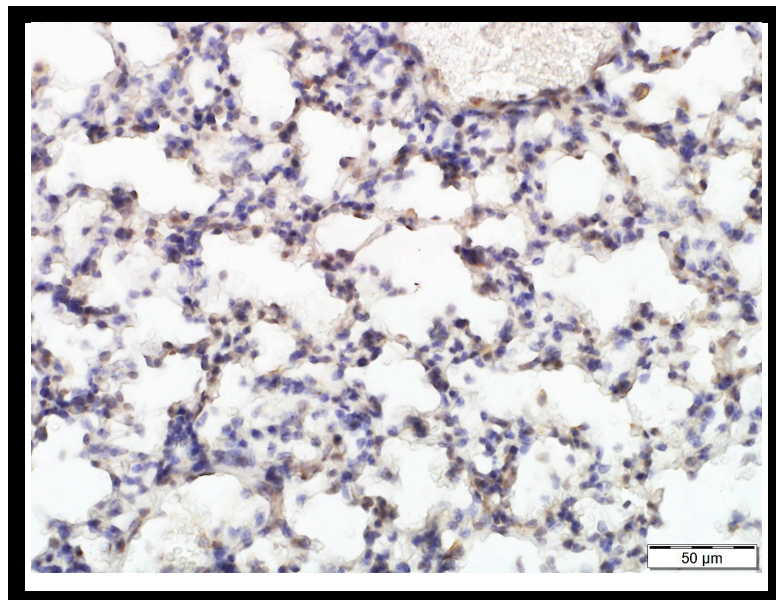


Figure 19 - Microscopic images of the lung of mouse 67 treated with polyclonal antibody and counterstained with Hematoxylin, 40x magnification. There are obvious specific stainings

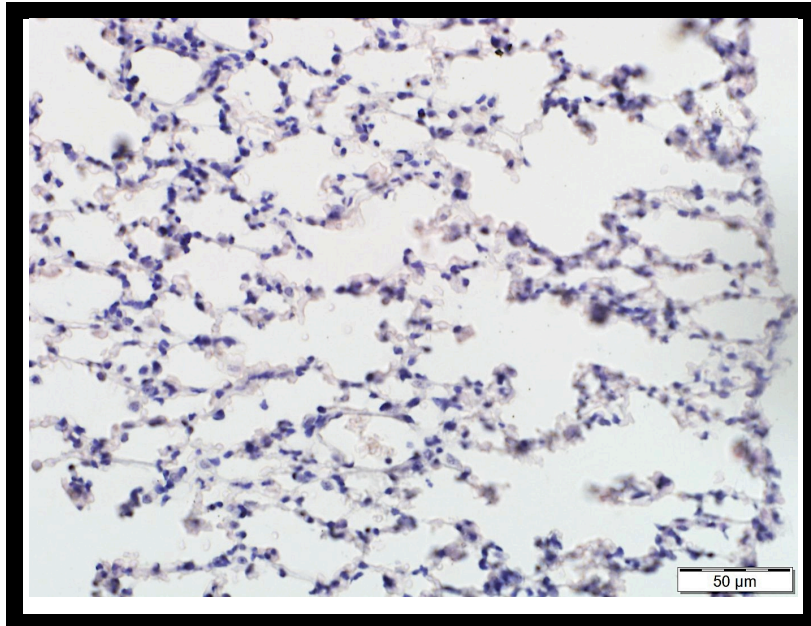


Figure 20 - Microscopic images of the lung of mouse 67 treated with secondary antibody control only and counterstained with Hematoxylin, 40x magnification. No staining visible here

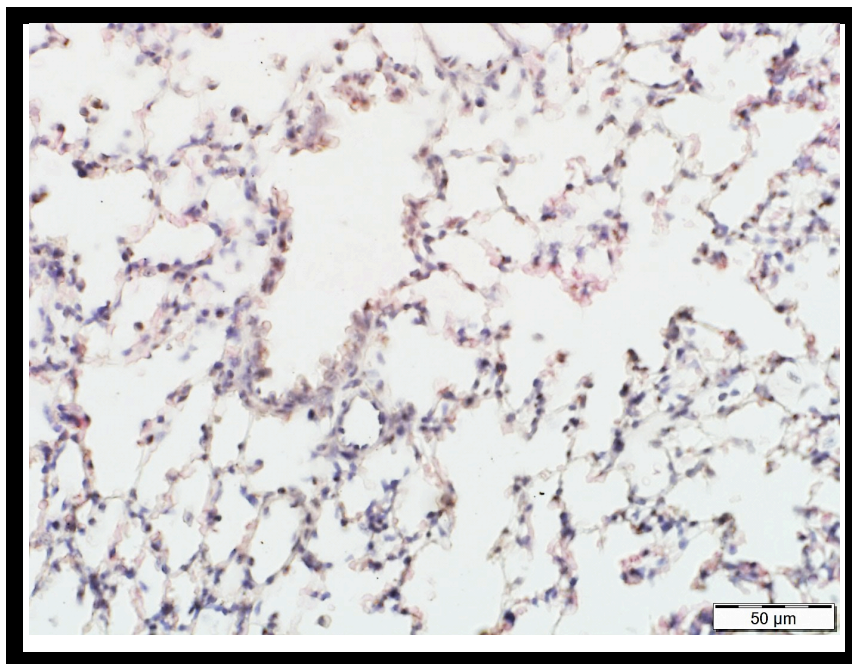


Figure 21 - Microscopic images of the lung of mouse 67 treated with Isotype antibody and counterstained with Hematoxylin, 40x magnification. Mainly background staining occurred after IHC with the Isotype-antibody

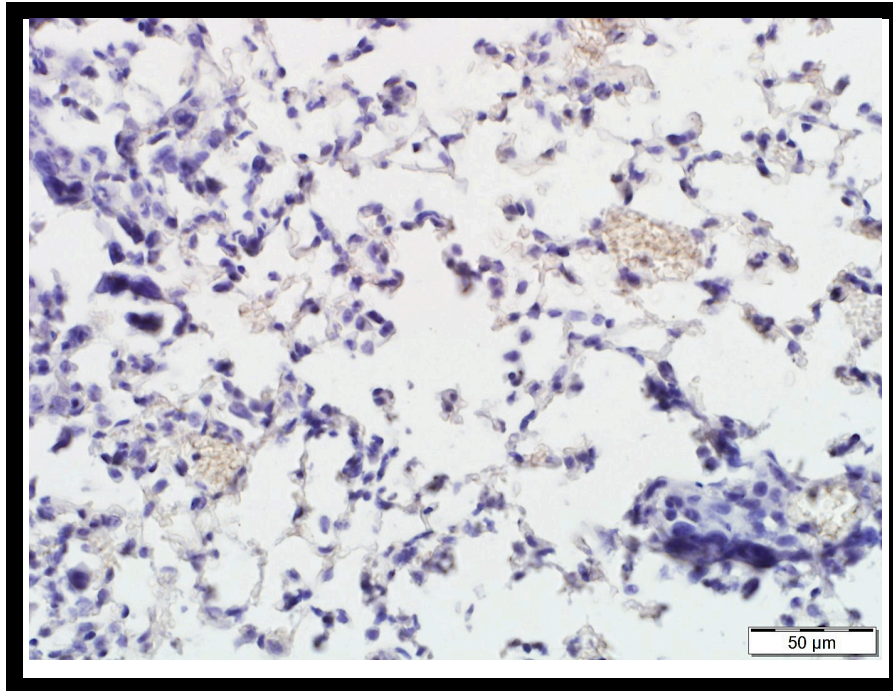


Figure 22 - Microscopic images of the lung of mouse 240 treated with polyclonal antibody and counterstained with Hematoxylin, 40x magnification. The staining pattern of the Luciferase-positive lung did not occur in the control organ here

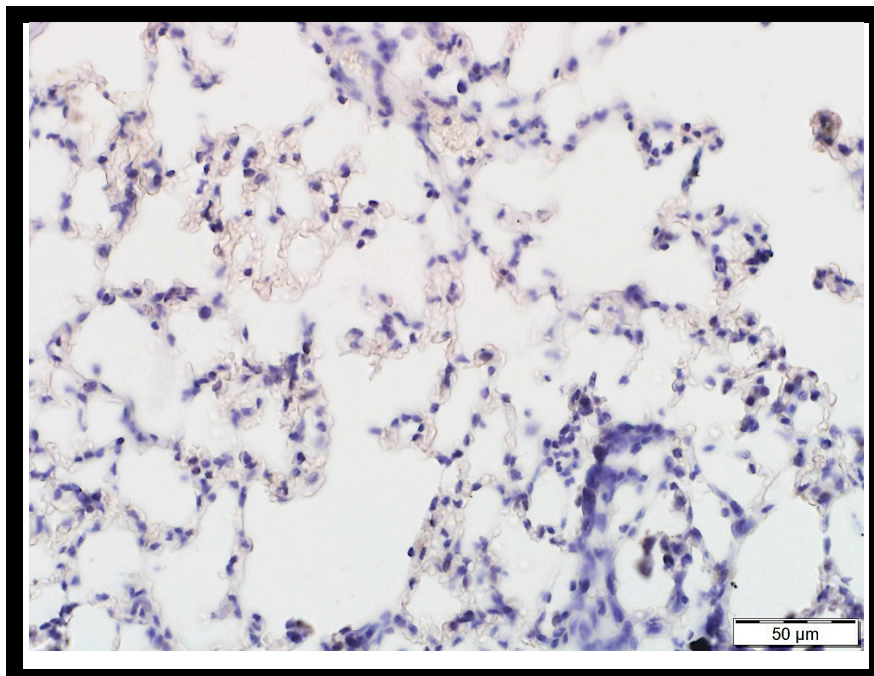


Figure 23-Microscopic images of lung of mouse 240 treated with secondary antibody control only and counterstained with Hematoxylin, 40x magnification. Also only little background staining visible here

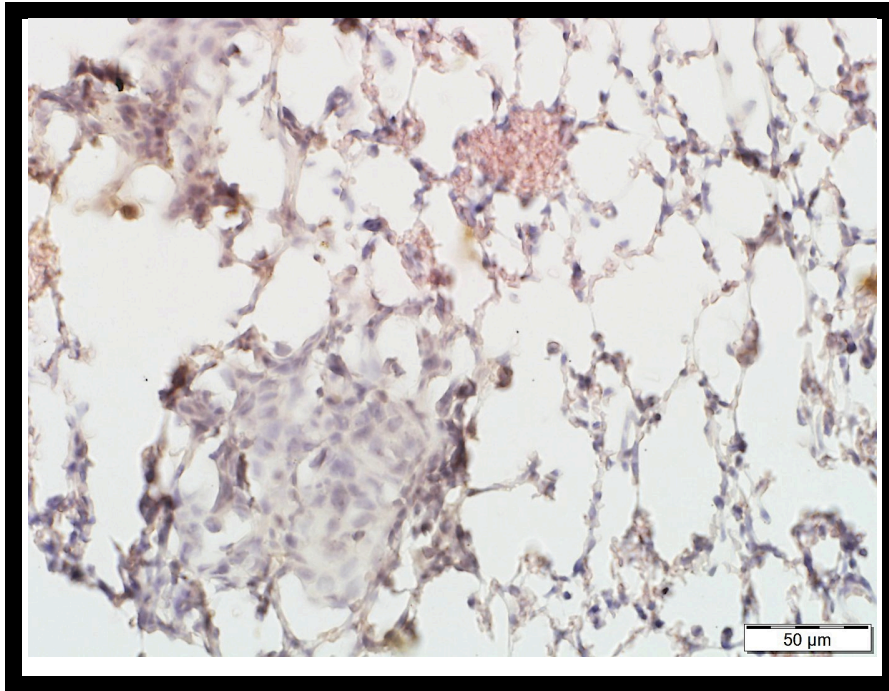


Figure 24 - Microscopic images of the lung of mouse 240 treated with Isotype antibody control and counterstained with Hematoxylin, 40x magnification. In this case, quite a lot of background staining occurred, but no specific staining either.

7.3. IHC with the monoclonal Antibody

7.3.1. Waterbath Antigen Retrieval

In general, the results in both cases were not ideal (pH 6 and pH9 waterbath antigen retrieval), but there were more unspecific stainings at pH 9, which will be demonstrated with the following microscopic images.

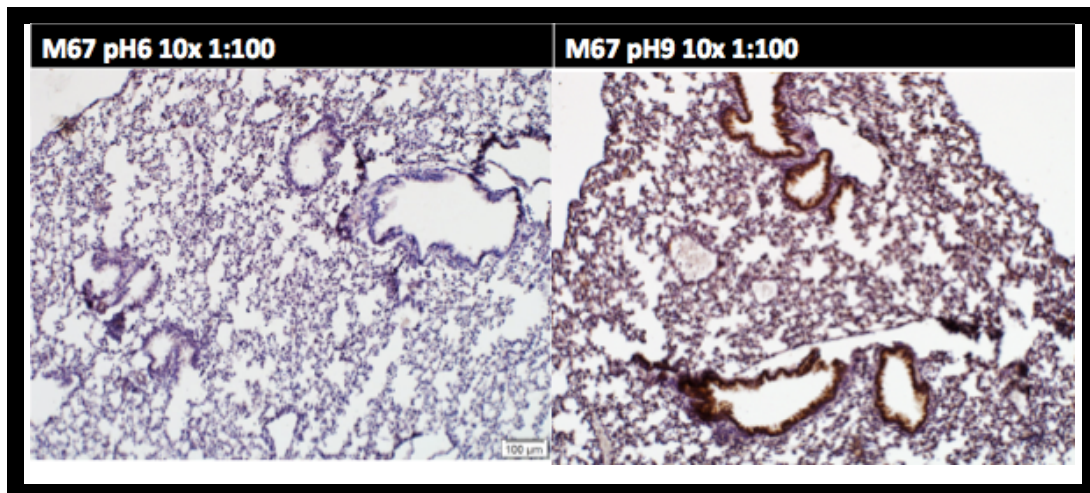


Figure 25 - Microscopic images of the lung of mouse 67 treated with monoclonal primary antibody in 1:100 dilution after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 10x magnification

In this direct comparison of the staining patterns after retrieval with different buffers, it is obvious, that the unspecific staining occurs in more areas and also in a lot stronger manner at a dilution of the primary antibody of 1:100 at pH 9.

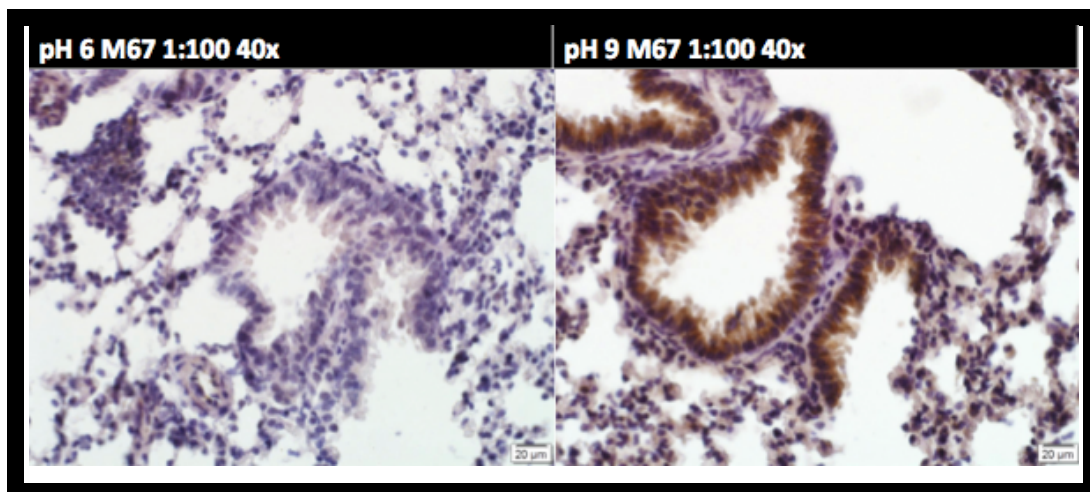


Figure 26 - Microscopic images of the lung of mouse 67 treated with monoclonal primary antibody in 1:100 dilution after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 40x magnification

As shown in these images, especially the epithelial cells were unspecifically stained. The biggest problem here was to differentiate between unspecific and specific stainings, because the staining looked exactly how it is supposed to look like when the primary antibody recognizes its antigen, which leads to the assumption, that there must be some type of cross-reactivity or unsatisfying specificity of the antibody. The unspecific staining also occurred in the 1:1000 dilution.

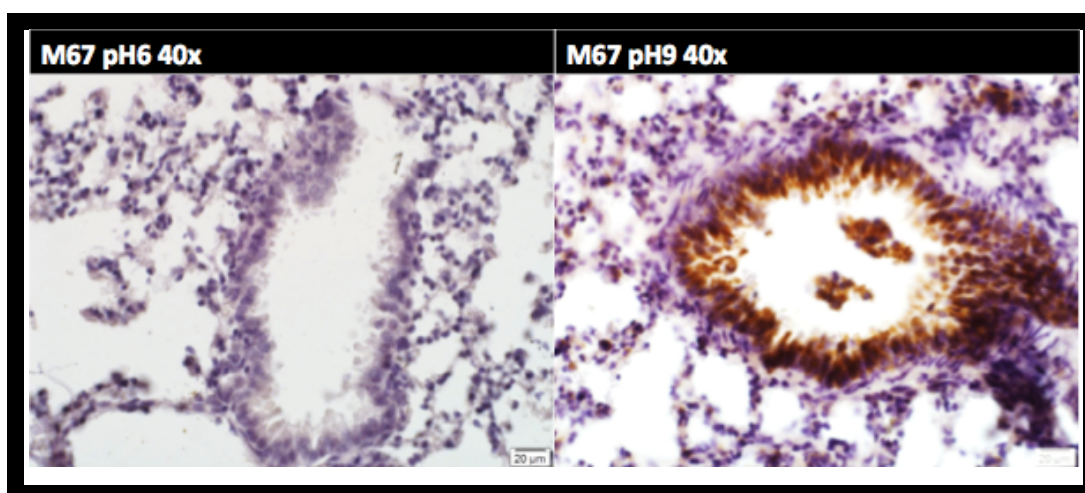


Figure 27 - Microscopic images of the lung of mouse 67 treated with monoclonal primary antibody in 1:1000 dilution after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9. Counterstained with Hematoxylin, 40x magnification

Looking at the negative control organ of mouse 53, there was also unspecific staining, but not as strong as it was in the lung of mouse 67. Some of the unspecific stainings were easier to identify in these 1:100 dilution of the primary antibody staining results:

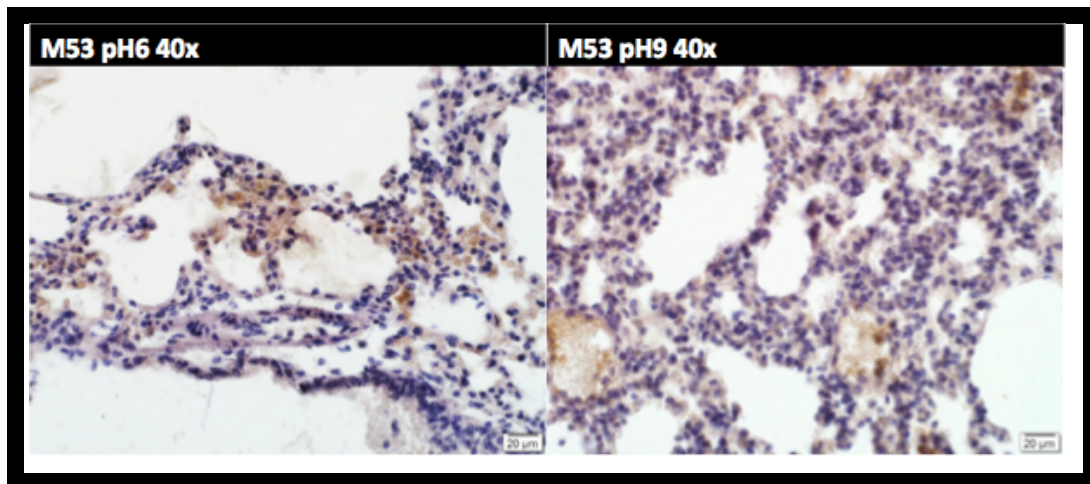


Figure 28 - Microscopic images of the lung of mouse 53 treated with monoclonal primary antibody in 1:100 dilution after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 40x magnification

Unfortunately the anatomic structure of this lung was not well preserved which is what makes the comparison to the Luciferase-positive lung a challenge. Again, also in the 1:1000 dilution of the primary antibody the staining pattern remained similar also in the negative control organ:

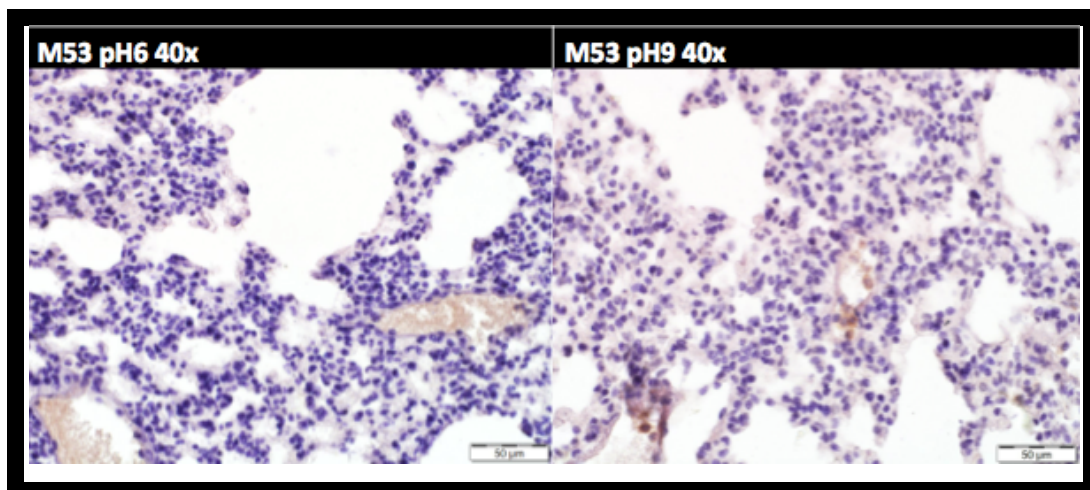


Figure 29 - Microscopic images of the lung of mouse 53 treated with monoclonal primary antibody in 1:1000 dilution after waterbath antigen retrieval with Sodium-Citrate buffer at pH 6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 40x magnification

The Isotype control also showed a lot of stained areas in the microscopic analysis.

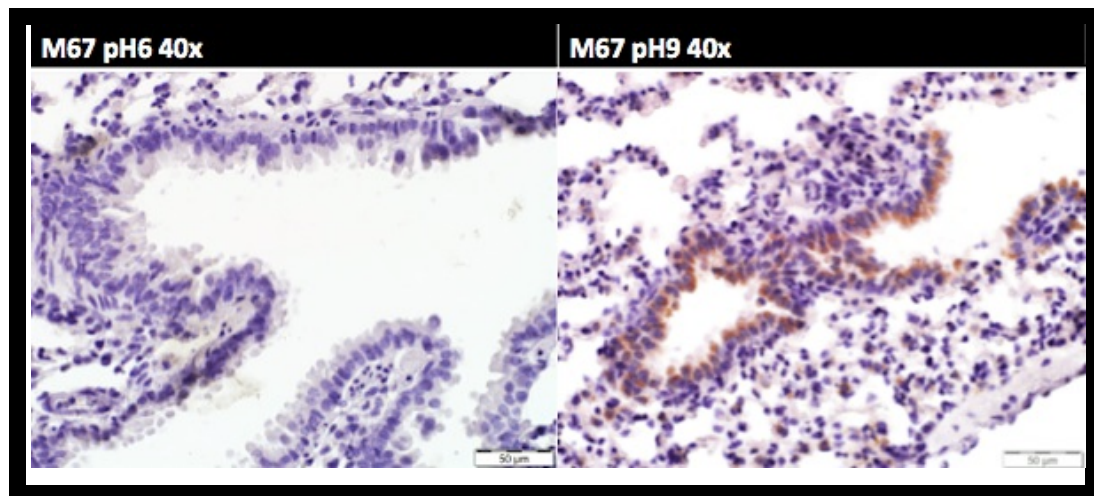


Figure 30 - Microscopic images of the lung of mouse 67 treated with Isotype primary antibody in 1:500 dilution after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 40x magnification

This image shows a very similar outcome to the primary antibody staining, but with the Isotype of the primary antibody. This outcome is not desirable in IHC. The staining was not as intense as it was in the different dilutions of the primary antibody, but actually there should be no staining at all with a good antibody specificity.

Unfortunately the results in the 2nd controls were also not satisfying.

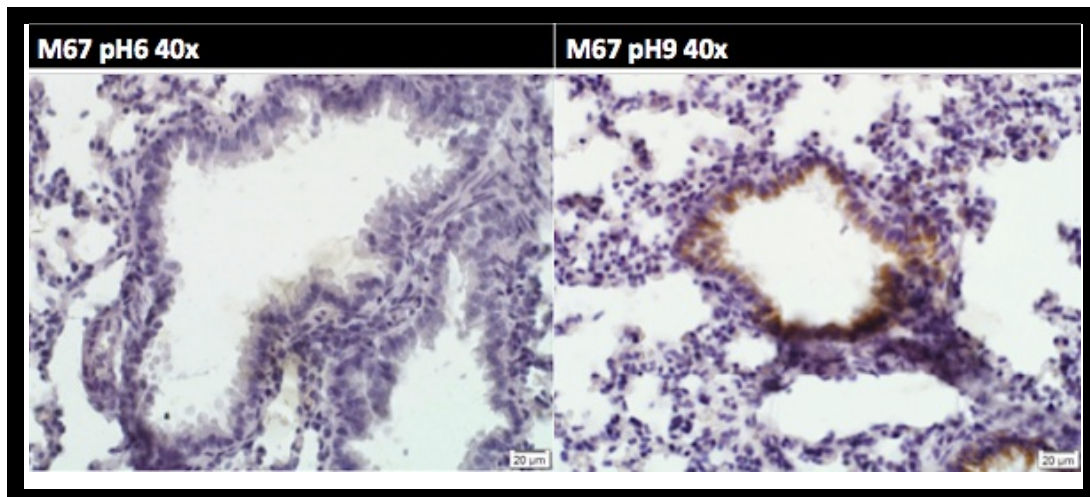


Figure 31 - Microscopic images of the lung of mouse 67 treated with secondary antibody only after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 40x magnification

Also in the 2nd control, there were specific looking stained areas in the epithelial cells and also in alveoli walls. Even though the manufacturer's protocol was followed the different reagents were handled with care, the outcome of the antibody-stained specimen and control organs are very similar, also to be seen in the negative control organs on the 2nd control specimen.

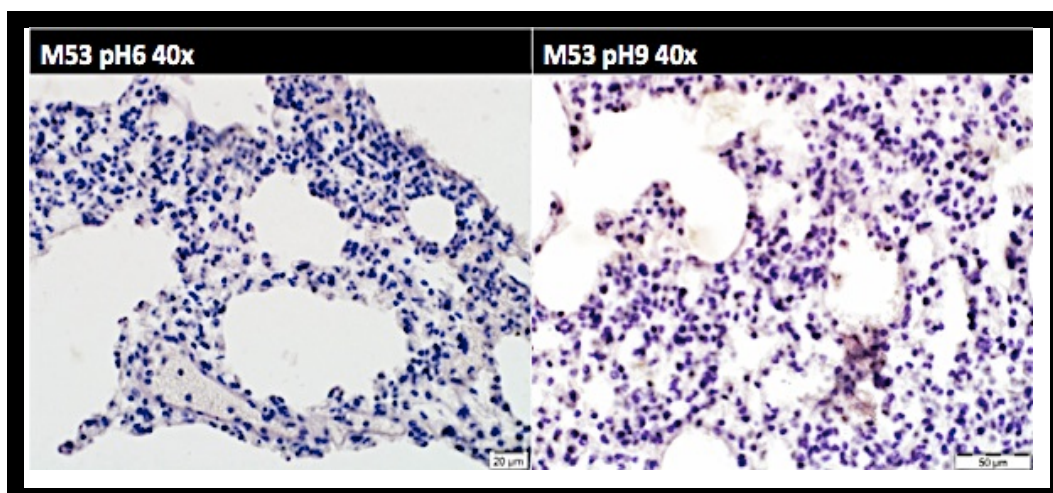


Figure 32 - Microscopic images of the lung of mouse 53 treated with secondary antibody only after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 40x magnification

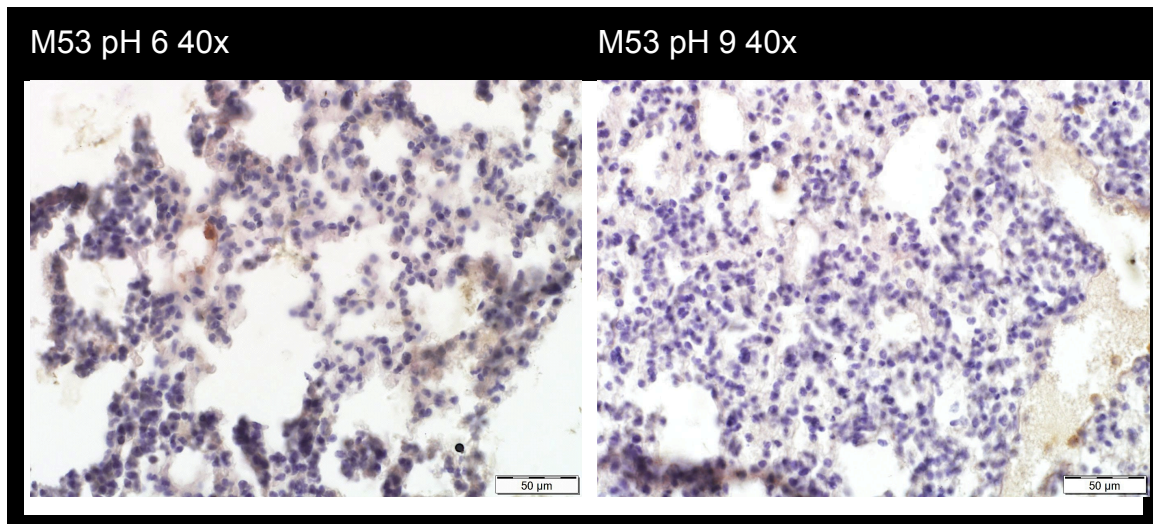


Figure 33 - Microscopic images of the lung of mouse 53 treated with Isotype antibody after waterbath antigen retrieval with Sodium-Citrate buffer at pH6 and Tris-EDTA buffer at pH 9, counterstained with Hematoxylin, 40x magnification

7.3.2. Microwave Antigen Retrieval

In general, the results of the microwave retrieval were also not very satisfying, if not even worse. Some of the specimen had a brown staining all over the tissue, whereas the others did not show this staining pattern.

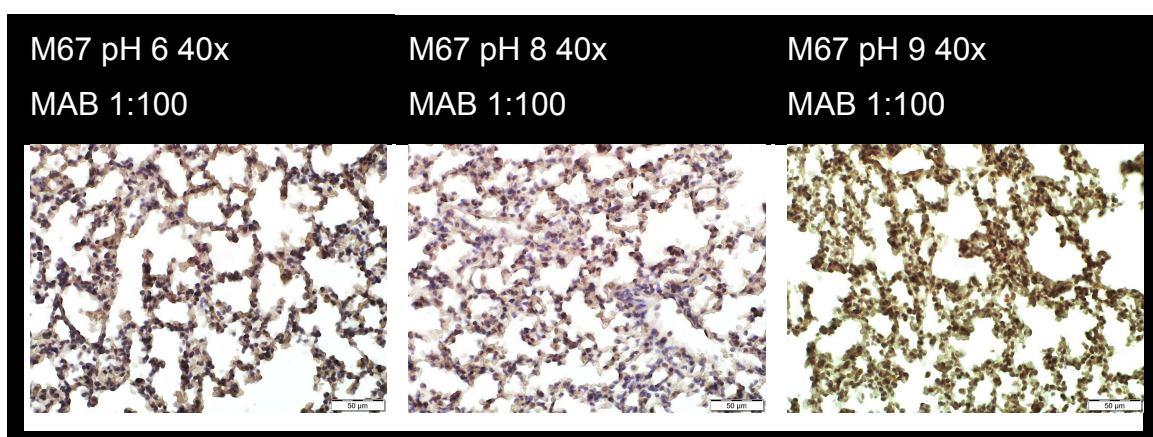


Figure 34 - Microscopic images of the lung of mouse 67 after IHC with the monoclonal antibody in a 1:100 dilution after performing microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxylin, 40x magnification

The outcome is not optimal. Especially at pH 9 the whole tissue had strong background staining in the 1:100 dilution, which did not occur during the IHC in the 1:1000 dilution. There was also specific staining in the microwave experiment, but due to a stronger background signal it was harder to identify.

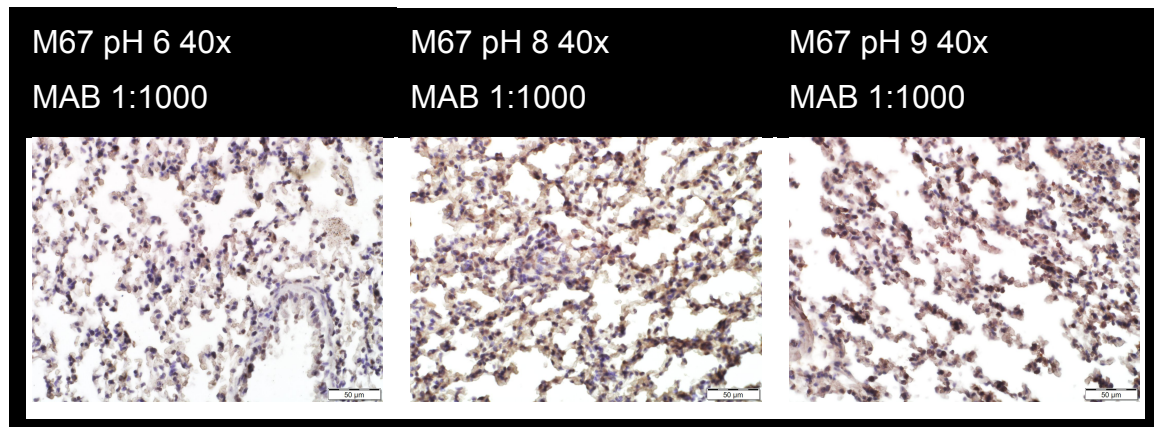


Figure 35 - Microscopic images of the lung of mouse 67 after IHC with the monoclonal antibody in a 1:1000 dilution after performing microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxylin, 40x magnification

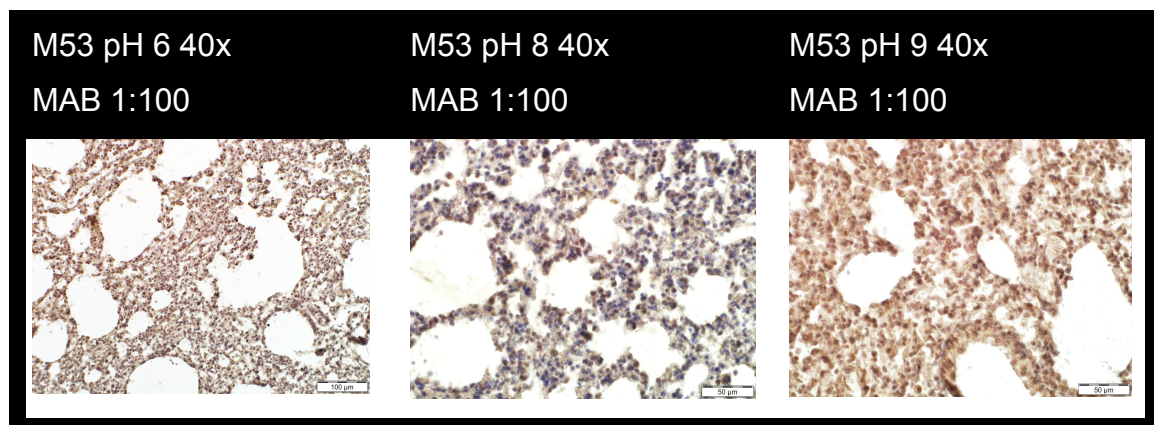


Figure 36 - Microscopic images of the lung of mouse 53 after IHC with the monoclonal antibody in a 1:100 dilution after performing microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxylin, 40x magnification.

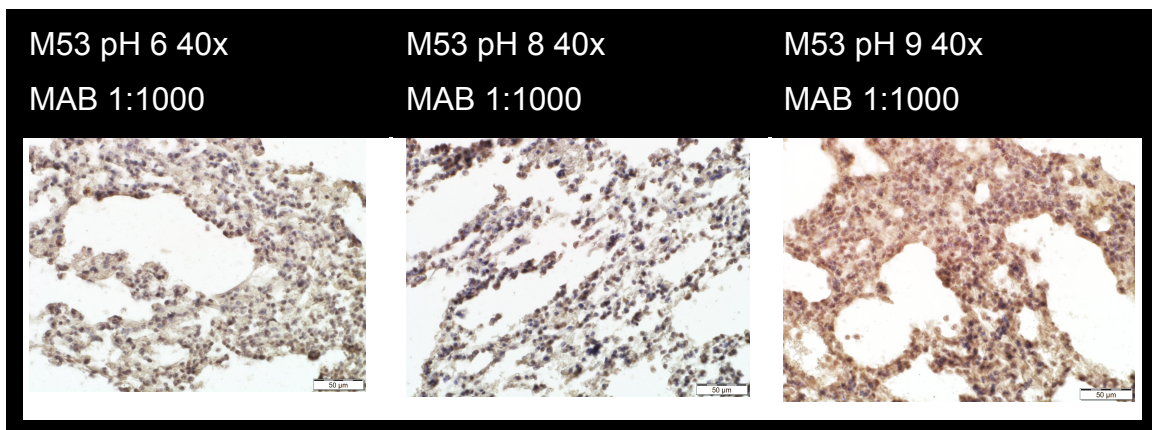


Figure 37 - Microscopic images of the lung of mouse 53 after IHC with the monoclonal antibody in a 1:1000 dilution after performing microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxyllin, 40x magnification

In the Luciferase negative control tissue of the lung of mouse 53, the background staining was stronger than in the Luciferase positive tissue of mouse 67. Especially at pH 6 and 9, the background staining was significant and the strongest after the retrieval with a pH 9 buffer solution.

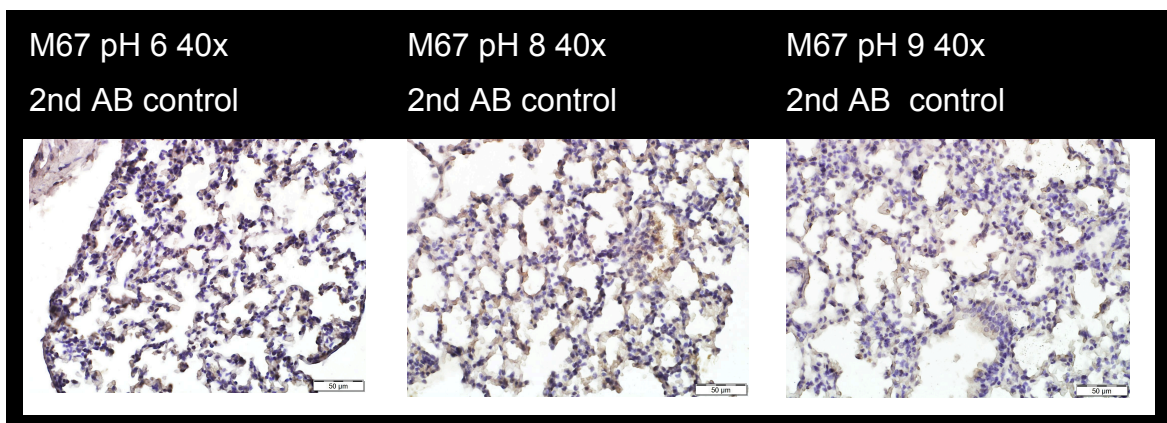
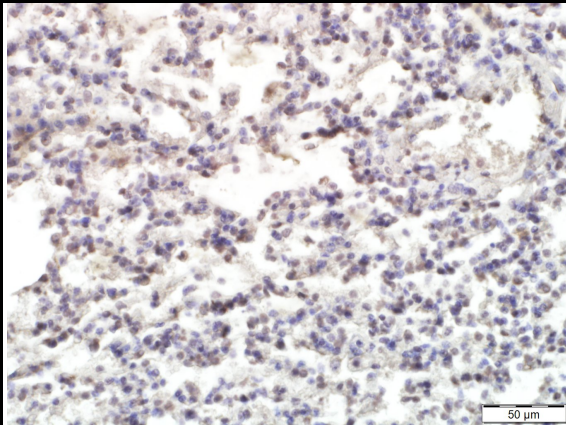


Figure 38 -Microscopic images of the lung of mouse 67. IHC performing the secondary antibody control after microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxyllin, 40x magnification

M53 pH 8 40x
2nd AB control



M53 pH 9 40x
2nd AB control

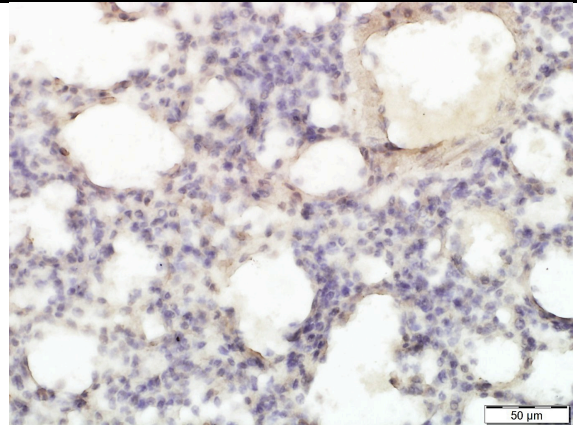
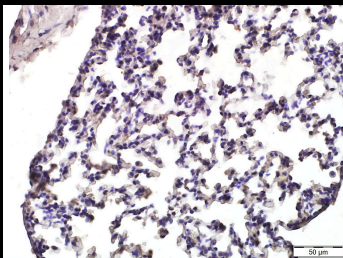
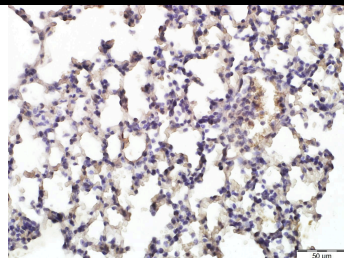


Figure 39 - Microscopic images of the lung of mouse 53. IHC performing the secondary antibody control after microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxyllin, 40x magnification. Due to unfortunate circumstances the 2nd antibody and Isotype control at pH 6 for mouse 53's lung got discarded.

M67 pH 6 40x
Isotype control



M67 pH 8 40x
Isotype control



M67 pH 9 40x
Isotype control

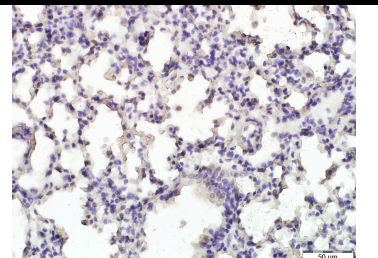
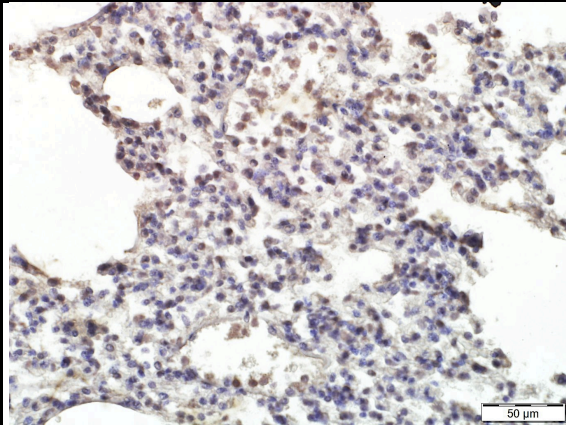


Figure 40 - Microscopic images of the lung of mouse 67. IHC with the monoclonal antibody after microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxyllin, 40x magnification

M53 pH 8 40x
Isotype control



M53 pH 9 40x
Isotype control

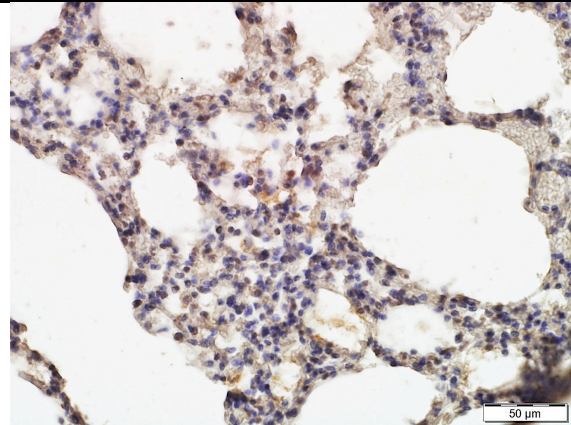


Figure 41 - Microscopic images of the lung of mouse 53. IHC performing the Isotype control after microwave antigen-demasking with 3 different retrieval buffers. Counterstaining with Hematoxylin, 40x magnification. Due to unfortunate circumstances the 2nd antibody and Isotype control at pH 6 for mouse 53's lung got discarded.

8. Discussion

8.1. Comparison of Fixation Methods

The microscopical images as well as the measurement of the mean diametres showed, that the best fixation technique during the experiments was intratracheal instillation.

In a previous study by S. Braber in 2010, there was a comparison between different fixatives, fixation methods and embedding materials. The results of the paraffine-embedded tissue were similar to the outcome during our experiments. In their study, it also showed that via intratracheal instillation, the lung architecture remained normal with curled alveoli and little to no fixation artifacts.

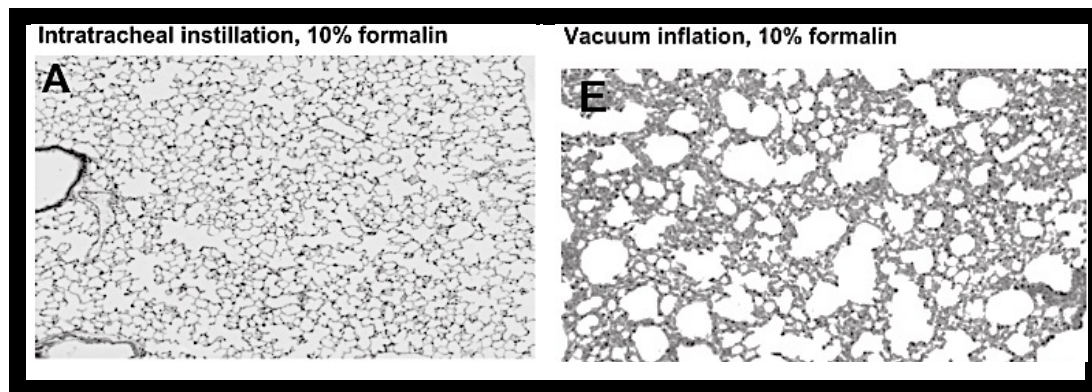


Figure 42 - Microscopic black and white images of the outcome of the comparison of different fixation methods in a work done by S.Braber after HE-staining in 100x magnification et.al. Source: (Braber et al., 2010)

To assess the degree of emphysema, it is essential to conserve the natural lung architecture as well as the size, architecture and the connectivity of all air spaces. The preservation of the native lung structure is also important for the evaluation of HE, IHC and several other staining methods, which was therefore relevant for the other two topics of the diploma thesis.

No fixation was ideal to reflect the condition of the lung in the living mouse, but the 25 centimeter lung pressure during intratracheal instillation helped preserve the in vivo appearance of the lungs in the best way compared to other fixation methods.

The results of the study by S. Braber and the experiments during the work at MMCT both showed, that intratracheal instillation is the ideal method, if the aim of an experiment is a quantitative study, that involves the morphometry of the alveoli. (Braber et al., 2010)

8.2. Immunohistochemistry

During the experiments, there were significant differences especially depending on the type of antigen retrieval with IHC staining using monoclonal and polyclonal antibody.

Working with the polyclonal primary antibody, the effect of different pH was not evaluated, since this was part of a previous work at MMCT, but there was a remarkable difference between the two heat induced antigen retrieval methods. In this case, the waterbath method seems to be more reliable and less background staining occurred compared to microwave antigen retrieval.

Working with the monoclonal antibody to detect Luciferase expression in murine lung tissue, different retrieval buffers and methods were used. Reliable results of IHC staining are indispensable in several fields of science and medicine.

Our experiments showed that microwave heating does not lead to reliable results compared to other heat induced antigen retrieval methods such as waterbath heating.

In a comparative study, that compared different heat induced antigen retrieval methods to evaluate Estrogen receptor expression expression in patient tissue, it was also found that microwave heating showed less specificity and sensitivity, but in this case especially compared to pressure cooker heating. (Qadir, Mushtaq, Sharif, Mamoon, & Khadim, 2010)

Regarding the establishment of the ideal protocol for the monoclonal antibody, it would have been necessary to do more experiments. We did not try the pressure cooking method for antigen retrieval and also no positive cell control, to see how exactly a positive, specific staining result is supposed to look like.

9. Appendix

Polyclonal Antibody Goat vs. Firefly

Product name: Anti-Firefly Luciferase Antibody

Quantity: 100µg

Product: ab181640

Description: Goat polyclonal to Firefly Luciferase

Lot number: GR226575-1

Concentration: 1mg/ml

Suggested dilution: 1:1000

Storage: Shipped 4°C, storage -20°C avoiding thawing/freezing circles

Table 45 - Polyclonal antibody used during IHC experiments

Source: Datasheet Abcam

Mouse IgG κ Isotype Control

Clone: P3.6.2.8.1

Quantity: 100 µg

REF: 14-4714-82

Lot number: 4305295

Concentration: 0,5mg/ml

Suggested dilution: same as primary antibody used in experiment

Storage: +4°C

Manufacturer: Thermo Fisher

Table 46 - Isotype used for IHC with polyclonal antibody, Source: Datasheet Thermo Fisher

Monoclonal Antibody	Firefly Luciferase Monoclonal Antibody (Luci 21 1-107)
Host/Isotype	Mouse/IgG1, kappa
Clone	Luci 21 1-107
Storage buffer	Ascites
Storage	-20°C
Manufacturer	Thermo Fisher
Lot	RH2257531
Size	100 µl
Concentration	Can only be estimated, about 10mg/ml

Table 47 - Monoclonal antibody used for IHC experiments

Source: Datasheet ThermoFisher

Isotype	Purified mouse IgG1 κ-Isotype
Material	#557273
Size	0,5mg
Concentration	0,5mg/ml
Clone	MOPC-31C
Isotype	Mouse (BALB/c) IgG κ
Storage buffer	Aqueous buffered solution ≤0,09% sodium azide
Storage	4°C
Manufacturer	BD Biosciences

Table 48 - Isotype used for IHC with monoclonal antibody, Source: Datasheet BD Biosciences

References

- Al-Dosari, M. S., & Gao, X. (2009). Nonviral Gene Delivery: Principle, Limitations, and Recent Progress. *The AAPS Journal*, 11(4), 671–681. <https://doi.org/10.1208/s12248-009-9143-y>
- Braber, S., Verheijden, K. a T., Henricks, P. a J., Kraneveld, a D., & Folkerts, G. (2010). A comparison of fixation methods on lung morphology in a murine model of emphysema. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 299(6), L843–L851. <https://doi.org/10.1152/ajplung.00192.2010>
- Bussolati, G., & Radulescu, R. T. (2011). Blocking Endogenous Peroxidases in try : A Mandatory , Yet Also Subtle Measure. *Applied Immunohistochemistry & Molecular Morphology*, 19(5), 2011. <https://doi.org/10.1097/PAI.0b013e318219a6e6>
- Contag, C. H., & Bachmann, M. H. (2002). Advances in in vivo bioluminescence imaging of gene expression. *Annu. Rev. Biomed. Eng.*, 4, 235–60. <https://doi.org/10.1146/annurev.bioeng.4.111901.093336>
- Crisp, S. J., Kullmann, D. M., & Vincent, A. (2016). Autoimmune synaptopathies. *Nature Reviews. Neuroscience*, 17(2), 103–17. <https://doi.org/10.1038/nrn.2015.27>
- Dako. (2013). EDUCAT IO NA L Immunohistochemical Staining Methods.
- Fehrenbach, H. (2001). Alveolar epithelial type II cell: defender of the alveolus revisited. *Respiratory Research*, 2(1), 33–46. <https://doi.org/10.1186/rr36>
- Fischer, A. H., Jacobson, K. A., Rose, J., & Zeller, R. (2008). Hematoxylin and eosin staining of tissueand cell sections. *Cold Spring Harbor Protocols*, 3(5), 3–5. <https://doi.org/10.1101/pdb.prot4986>
- Geyer, A., Taschauer, A., Alioglu, F., Anton, M., Maier, J., Drothler, E., ... Ogris, M. (2017). Multimodal fluorescence- and bioluminescence imaging reveals transfection potential of intratracheally administered polyplexes for breast cancer lung metastases. *Human Gene Therapy*, hum.2017.137. <https://doi.org/10.1089/hum.2017.137>
- Jacques, S. L. (2013). Corrigendum: Optical properties of biological tissues: a review. *Physics in Medicine and Biology*, 58(14), 5007–5008. <https://doi.org/10.1088/0031-9155/58/14/5007>

- Lyons, S. K., Patrick, P. S., & Brindle, K. M. (2013). Imaging mouse cancer models in vivo using reporter transgenes. *Cold Spring Harbor Protocols*, 2013(8), 685–699. <https://doi.org/10.1101/pdb.top069864>
- Marques, S. M., & Esteves Da Silva, J. C. G. (2009). Firefly bioluminescence: A mechanistic approach of luciferase catalyzed reactions. *IUBMB Life*, 61(1), 6–17. <https://doi.org/10.1002/iub.134>
- Measurements, S. V. (1966). MEASUREMENT OF PULMONARY EMPHYSEMA. 2., 752–764.
- Michalet, X., Pinaud, I., Michalet, X. et al. Quantum Dots for Live Cells, in Vivo Imaging, and Diagnostics. *Science* (80-.). 307, 538–544 (2005). F F, Bentolila, L. A., Tsay, J. M., Doose, S., Li, J. J., ... Weiss, S. (2005). Quantum Dots for Live Cells, in Vivo Imaging, and Diagnostics. *Science*. <https://doi.org/10.1126/science.1104274>
- Qadir, A., Mushtaq, S., Sharif, M. A., Mamoon, N., & Khadim, M. T. (2010). Comparison of heat induced antigen retrieval techniques for immunohistochemical estrogen receptor expression in ductal carcinoma of breast in females. *Journal of the College of Physicians and Surgeons Pakistan*, 20(8), 519–523. <https://doi.org/10.2010/JCPSP.519523>
- Taschauer, A., Geyer, A., Gehrig, S., Maier, J., Sami, H., & Ogris, M. (2016). Up-Scaled Synthesis and Characterization of Nonviral Gene Delivery Particles for Transient *In Vitro* and *In Vivo* Transgene Expression. *Human Gene Therapy Methods*, 27(3), 87–97. <https://doi.org/10.1089/hgtb.2016.027>
- Thavarajah, R., Mudimbaimannar, V., Rao, U., Ranganathan, K., & Elizabeth, J. (2012). Chemical and physical basics of routine formaldehyde fixation. *Journal of Oral and Maxillofacial Pathology*, 16(3), 400. <https://doi.org/10.4103/0973-029X.102496>
- Thurlbeck, W. M. (1967). Internal surface area and other measurements in emphysema. *Thorax*, 22, 483–496. <https://doi.org/10.1136/thx.22.6.483>
- Van Kuppevelt, T. H., Robbesom, A. A., Versteeg, E. M. M., Veerkamp, J. H., Van Herwaarden, C. L. A., & Dekhuijzen, P. N. R. (2000). Restoration by vacuum inflation of original alveolar dimensions in small human lung specimens. *European Respiratory Journal*, 15(4), 771–777. <https://doi.org/10.1034/j.1399-3003.2000.15d23.x>

