Generation and characterization of a murine model of pulmonary fibrosis

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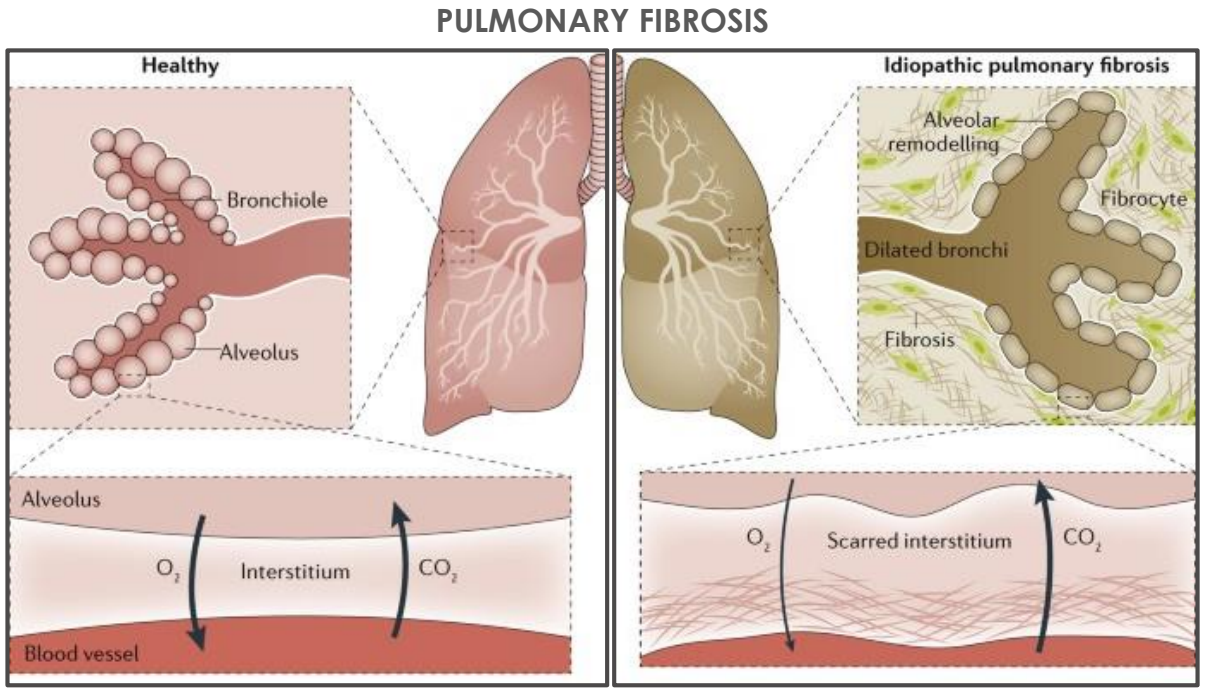
UNIVERSITÀ DEGLI STUDI DI MILANO BICOCCA

TeCSBi PhD TECNOLOGIE CONVERGENTI PER I SISTEMI BIOMOLECOLARI

Background

Fibrosis is a reparative or reactive process that is majorly characterized by the formation and deposition of excess fibrous connective tissue resulting in progressive architectural remodeling in nearly all tissues and organs



REPETITIVE EPITHELIAL INJURY - TISSUE DAMAGE

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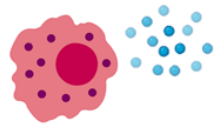
INFLAMMATORY RESPONSE – CYTOKINES PRODUCTION
(TISSUE MACROPHAGES)



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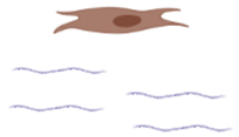
LOCAL ACTIVATION OF MESENCHYMAL CELLS
(ECM PRODUCTION)



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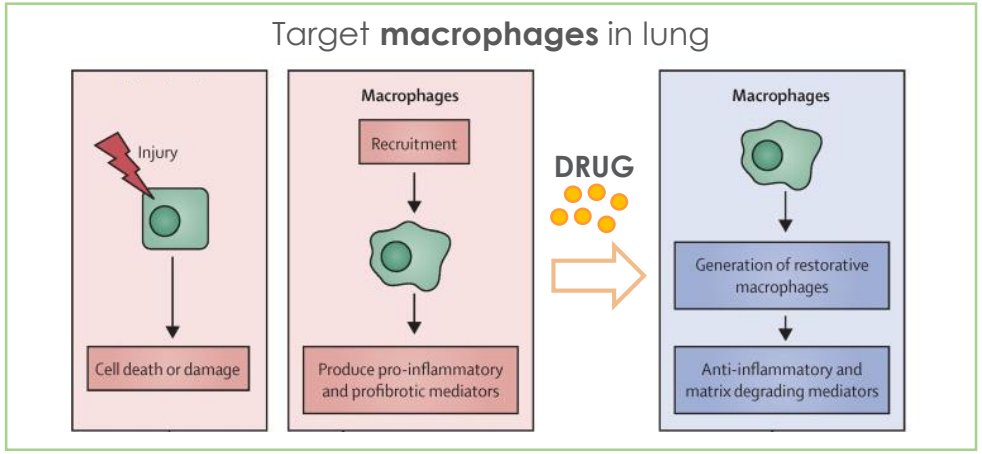
Uncontrolled and progressive accumulation of fibrotic tissue in affected organs causing their dysfunction and ultimate failure

- Incomplete knowledge of the fibrotic process pathogenesis
- The marked heterogeneity in their etiology and clinical manifestations
- The absence of appropriate and fully validated biomarkers
- Limited treatment options available

Background

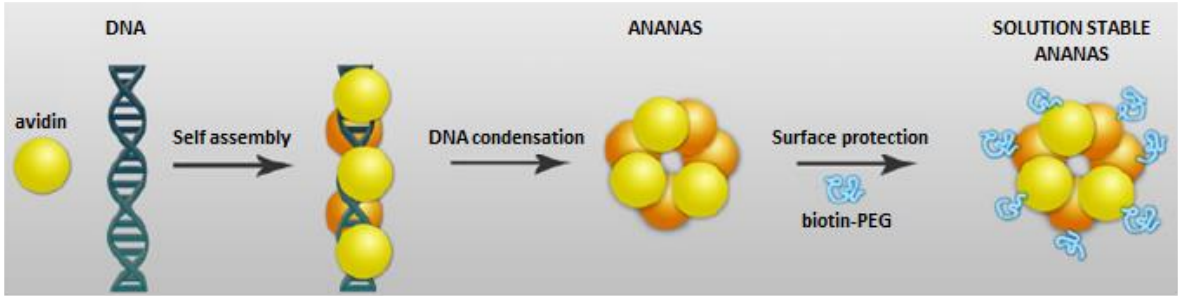


CORTICOSTEROIDS

- High efficacy
- High bioavailability in target organ

- Circulating corticosteroids easily pass from the bloodstream to vital organs
- Serious side effects upon chronic treatment

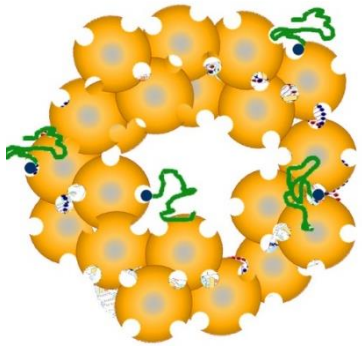
Use of nanotechnology for medical therapeutics by developing **nanoscale agents** for the treatment of various kinds of diseases



ADVANTAGES

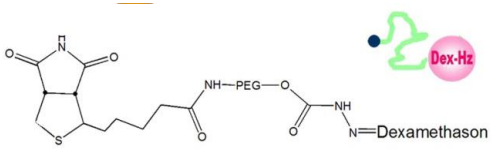
Improved bioavailability
Enhancing aqueous solubility
Increasing resistance time in the body
Targeting drug to specific location in the body

REDUCE STEROID SIDE EFFECT ON OFF TARGET ORGANS AND SPECIFICALLY ACT ON INFLAMMATORY CELLS



ANANAS core

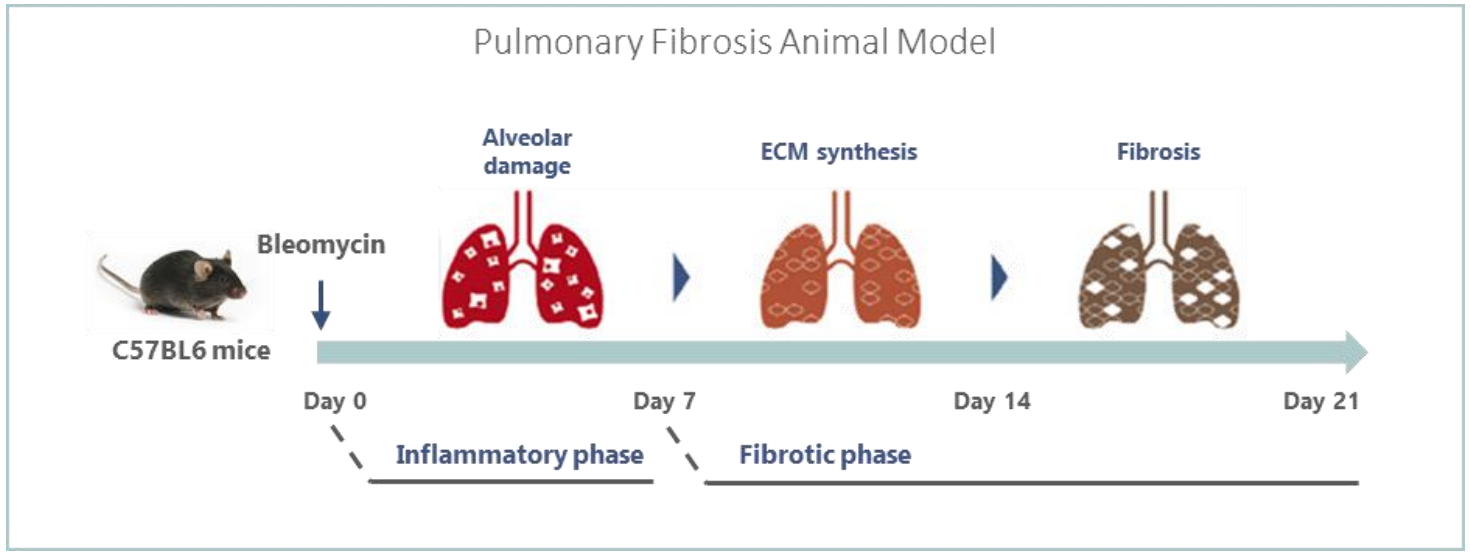
+



biotin-PEG-Hz-dexamethasone

Aim of the study and Methods

1. Characterization of the animal model of pulmonary fibrosis induced by bleomycin



Murine model generation and Histological processing

2. Intranasal administration of the free drug (dex) or linked to biodegradable avidin-nucleic acid nanoassemblies (ANANAS-dex) in healthy animals and biodistribution study

IN VIVO/EX VIVO Imaging

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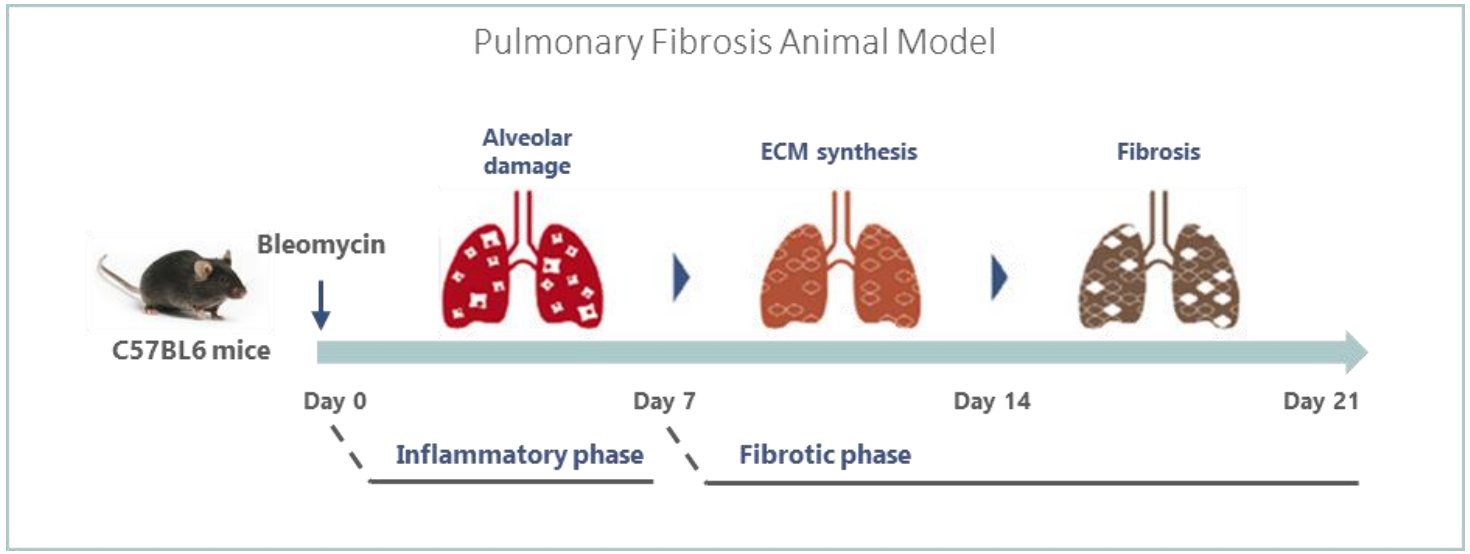

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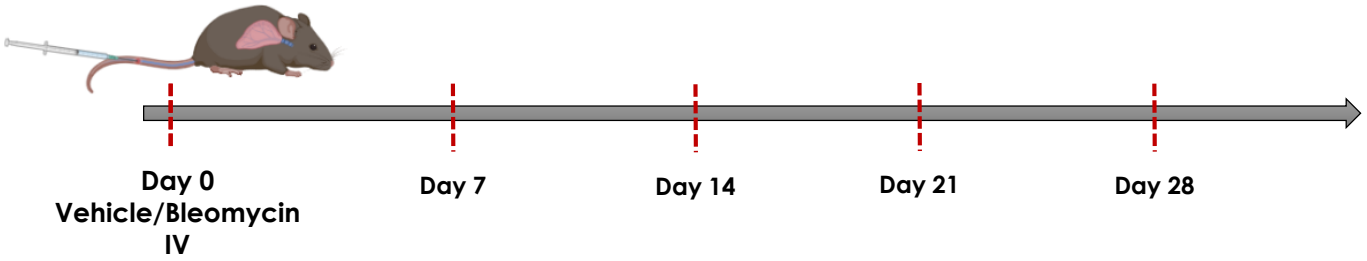
IN VIVO/EX VIVO Imaging

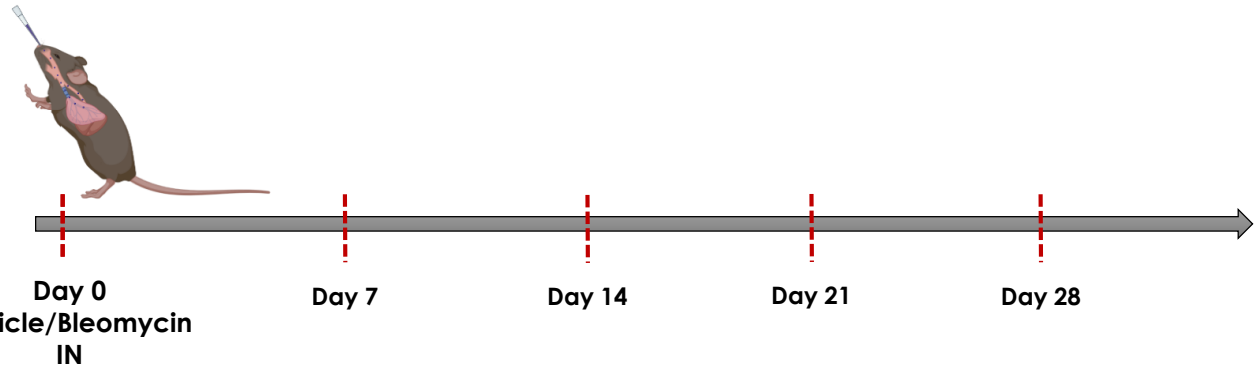
1. Generation of the model

C57BL/6, 10 weeks old mice, male



Dose: **150 mg/kg** (200 µl)

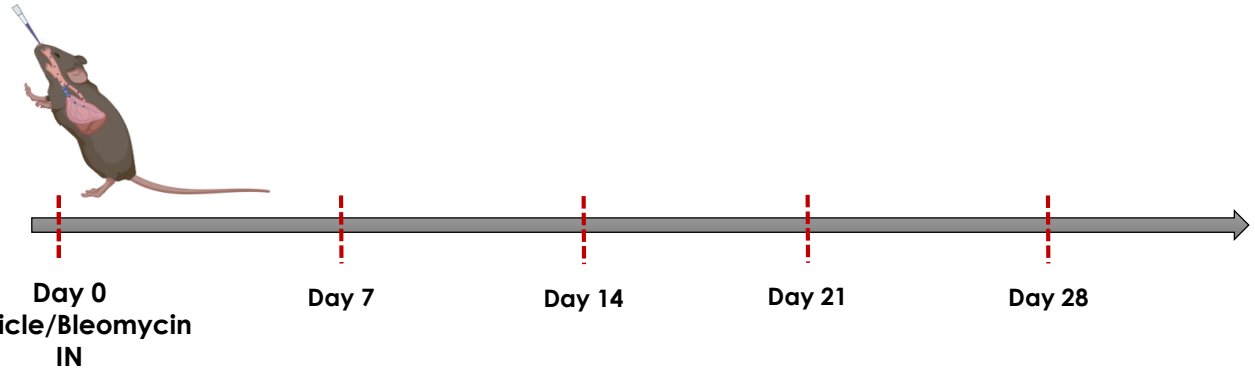
25 µg/mouse



Dose: **1 mg/kg** (25 µl)

25 µg/mouse

Anesthetized animals with **Isoflurane**



Dose: **5 mg/kg** (50 µl)

125 µg/mouse

Anesthetized animals with **Ketamine/Medetomidine**

1. Histological processing

Microscopic analysis of cells and tissues requires the preparation of very thin, high-quality sections (slices) mounted on glass slides and appropriately stained to highlight normal and abnormal structures

- Freeze the tissue (liquid nitrogen) and cryostat section → "**FROZEN SECTIONS**"

- Infiltrate tissue with a liquid agent (Paraffin wax) → "**PARAFFIN SECTIONS**"

NECROSCOPY

Procedures carried out to evaluate gross anatomic changes in the body of a dead animal

SAMPLING

Biological sample collection

FIXATION and TRIMMING

Conservation of the biological sample

PROCESSING

Processing of the biological sample and paraffin embedding

SECTIONING

Sample are cut into slices to be placed on a slide

STAINING

Highlight important features of the tissue with proper dyes

MICROSCOPY

Digital image acquisition, image analysis, histopathological evaluation and study report

Necroscopy and Sampling

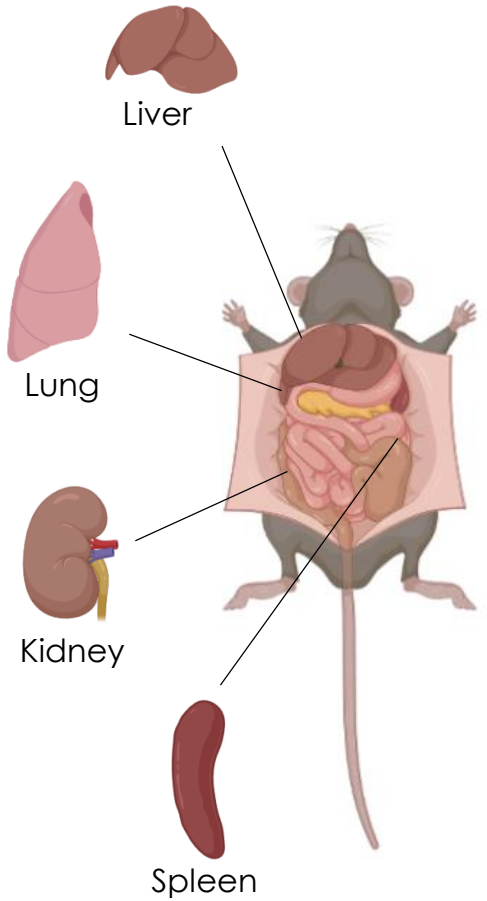
NECROSCOPY

Procedures carried out to **evaluate gross anatomic changes** in the body of a dead animal and to sample tissues/fluids for (microscopic) examination.

It must be performed **immediately** after sacrifice

Observe macroscopic details

Post mortem changes: autolysis and putrefaction (gastrointestinal tract, pancreas, bone marrow, CNS) happen very fast in small animals.



SAMPLING

Collection of a biological sample consisting of organs (or parts of organs) and/or tissues during necroscopy (including liquids: blood, urine, CSF)

Planning of sampling protocol

Standardization of procedures, that are fundamental to ensure histopathological analysis which is:

- Reproducible
- Comparable
- Reliable

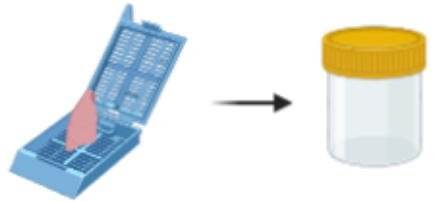
Fixation and Trimming

Chemical process by which organs are **preserved and strenghten** avoiding alterations, autolysis and damage caused by manipulation.

Preferred fixative: Neutral Formalin Buffered 10% (or PFA 4%)

It must take place as soon as possible after sacrifice
Rapid penetration of the tissue
Preservation of morphological detail and tissue molecular characteristics (immuno-histochemical staining, PCR, WB)

Toxicity: Observe the safety regulations expected
Overfixation: avoid fixing times that exceed 24h, then transfer in ETOH 70%/PBS



TIPS

- Specimens dimensions allow rapid penetration of the fixative
- Containers of an appropriate size with large Ø opening
- Adequate volume of tissue/fixative - ratio 1/10
- Adequate type of biocassettes
- Specimens should be properly identified and all details recorded

Processing

"**Tissue processing**" describes the steps required to take an animal or human tissue from fixation to the state where it is completely infiltrated with an histological wax and can be embedded to create a permanent block ready for section cutting on the **microtome**. Processing means extracting all the water from the tissue to allow the penetration of paraffin wax, in which the tissue is finally embedded.

Solvent	Time	Temperature
70% ethanol	1h	37°C
80% ethanol	1h	37°C
96% ethanol	1h	37°C
100% ethanol	1h	37°C
Xylene	45'	37°C
Xylene	45'	37°C
Paraffin	30'	58°C
Paraffin	30'	58°C
Paraffin	1h	58°C
Paraffin	1h	58°C

Because melted paraffin wax is hydrophobic, most of the water in a specimen must be removed before it can be infiltrated with wax. This process is commonly carried out by immersing specimens in a **series of ethanol** solutions of increasing concentration until pure, water-free alcohol is reached

The **EMBEDDING CENTER** where a mold is filled with molten wax and the specimen placed into it to form a block.

TIPS

- Specimens are carefully orientated
- Choose adequate mold and labelled biocassettes
- Handle the specimens with care to avoid damage and artifacts

Sectioning and Staining

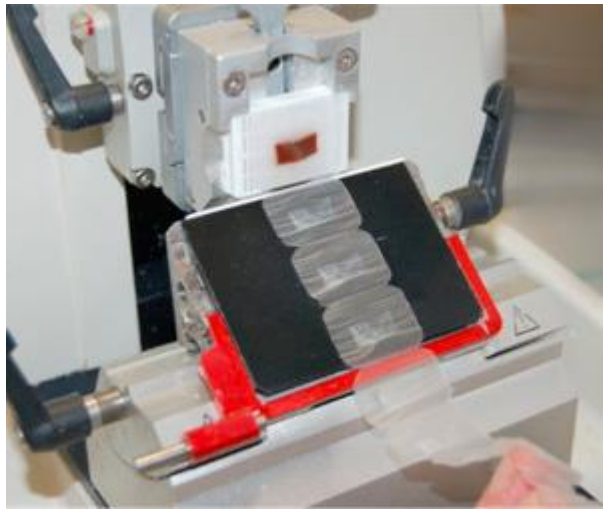
SECTIONING

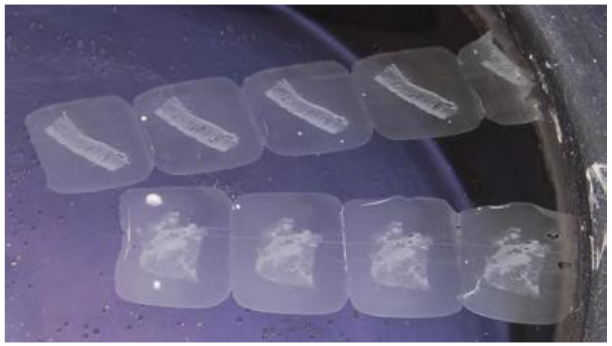
Process of cutting tissue into thin slices. Embedded tissue is sectioned with the **microtome**.

TIPS

- High quality, sharp blades are always used for cutting
- Blocks are chilled on a cold wet surface
- Flotation bath temperature is carefully checked (40 °C).

  The wax should not melt





Sections of 4-7 µm are left on the flotation bath for just long enough to flatten then promptly picked up on a slide

STAINING

Highlight important features of the tissue as well as to enhance the tissue contrast.

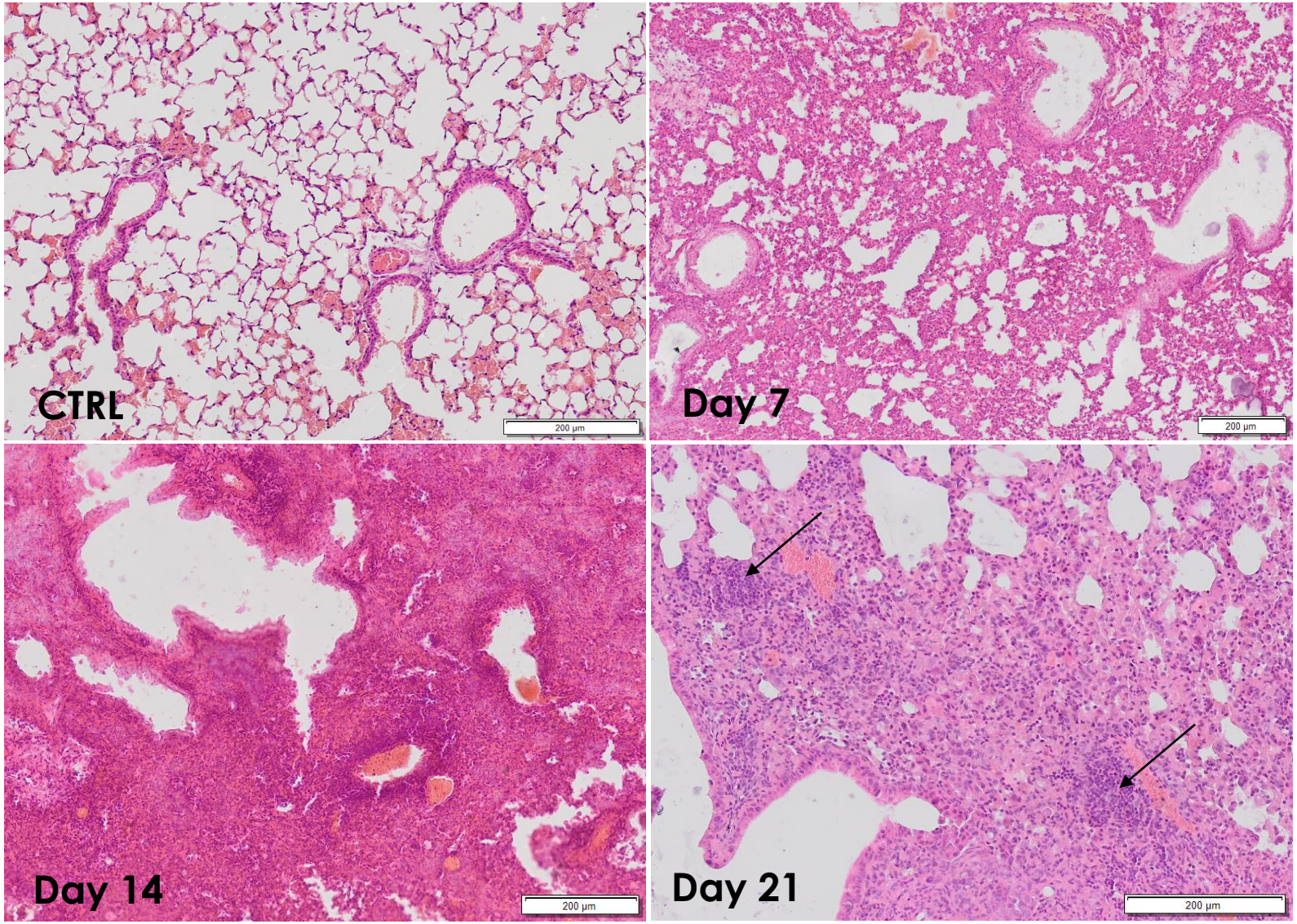
The first staining step is de-waxing which uses a solvent to remove the wax and then re-hydrate in a series of ethanol solutions of increasing concentration of water prior to staining.
When the staining is complete, the section is covered with a coverglass that makes the preparation permanent.

1. Results

Hematoxylin is a basic dye that stains nucleic acids by a complex, incompletely understood reaction giving it a bluish color while **Eosin** is an acidic dye that stains cell cytoplasm giving it a pinkish stain.

Assess tissue morphology and histopathological lesions.

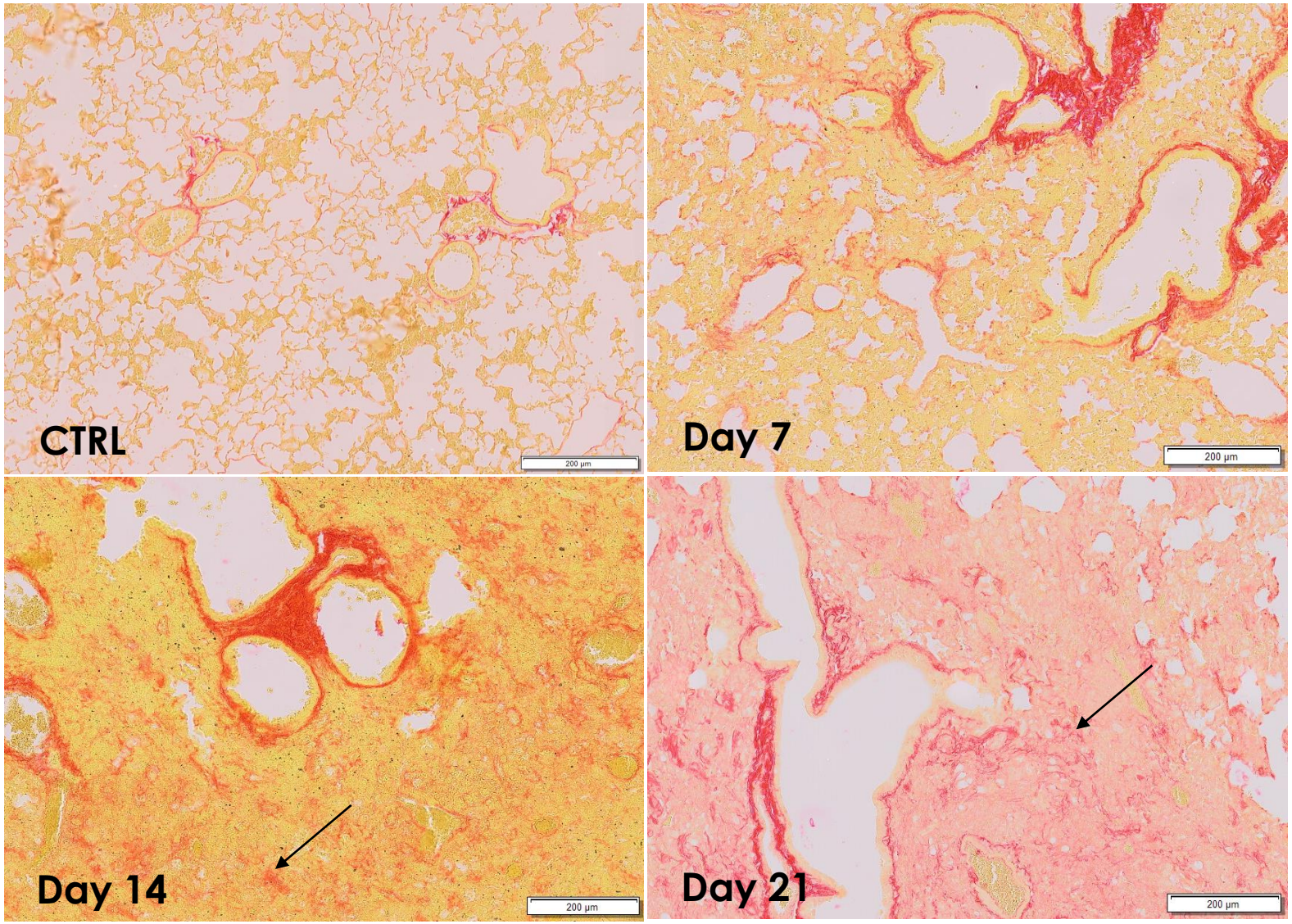
5 mg/kg Bleomycin
Inflammation peak at 1-2 weeks after administration, 80% macrophages - granulocytes



1. Results

Sirius Red, a strong anionic dye, stains collagen by reacting, via its sulphonic acid groups, with basic groups present in the collagen molecule. The elongated dye molecules are attached to the collagen fiber in such a way that their long axes are parallel. This parallel relationship between dye and collagen results in an enhanced birefringency.

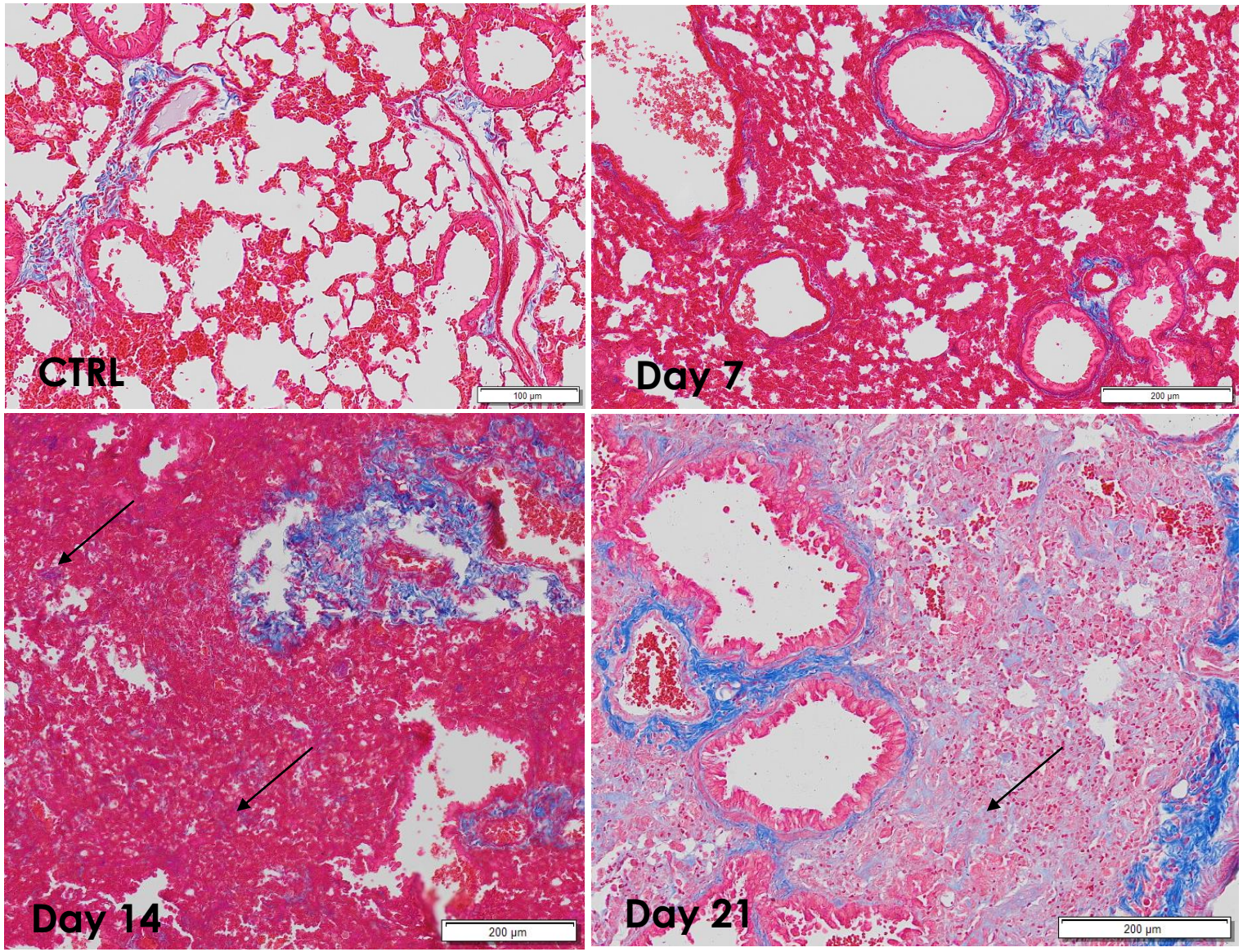
5 mg/kg Bleomycin
Diffuse fibrotic collagen in lung parenchima after 14 days post administration



1. Results

Trichrome staining is used to visualize connective tissues, particularly collagen, in tissue sections. In a standard Masson's Trichrome procedure, **collagen** is stained blue, muscle tissue is stained red, and cytoplasm is stained pink/red.

5mg/kg Bleomycin
Newly synthesized collagen in lung parenchima at 14-21 days post treatment



1. Results

Immunohistochemistry (IHC) is the application of monoclonal as well as polyclonal antibodies to determine the tissue distribution of an **antigen of interest** in health and disease samples.

5mg/kg Bleomycin
Iba1 staining signal is higher at 21 – 28 days post treatment