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Bleomycin-Induced Pulmonary Fibrosis in C57Bl/6 mice

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We use this protocol and it's working

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Abstract

This protocol describes the induction of pulmonary fibrosis in C57Bl/6 mice using bleomycin (BLM) by oropharyngeal (OA) administration. It also outlines the key methods for disease severity assessment, including lung function analysis using FlexiVent, bronchoalveolar lavage fluid (BALF) analysis, blood biochemical analysis, hematology analysis, flow cytometry of BALF cells, histological staining, RNA isolation-qPCR, acellular extracellular matrix preparation, fibroblast isolation and culture/functional assays.

Attachments



Bleomycin-induced pu...

49KB

Materials

Mice and housing conditions

- C57Bl/6 mice, 8-12 weeks old.
- Specific Pathogen-Free (SPF) facility.
- Housing conditions: 21–24°C, 50–60% humidity, 12h day/night cycle conditions.
- Food & water ad libitum.

Chemicals and Reagents

- Bleomycin hydrochloride (BLM) (Baxter, 15000 I.U./vial)
- Xylazine/ketamine/atropine mixture (6.6mg/100mg/0.033mg/kg, respectively)
- Atipamezole (1 mg/kg)
- Corneregel
- Sodium chloride (0.9% NaCl)
- Red blood cell lysis buffer
- 0.4% Trypan Blue solution
- Formalin (Formaldehyde solution 10% neutralized, 143091.1214, Panreac-Applichem)
- Direct Red (120 µg/mL for soluble collagen quantification or powder for Fast Green-Sirius Red staining) (Direct Red 80, Sigma-Aldrich)
- collagen from rat tail (C7661, Sigma Aldrich)
- Bradford reagent (Bio-Rad, Hercules, CA, USA)
- Bovine serum albumin (A9647, Sigma Aldrich)
- Surgical sutures without needle
- 50, 70, 96 and 100% EtOH
- Eosin G (7089.2, ROTH)
- Scott's solution
- Papanicolaou's solution 1a Harris' Hematoxylin solution (1.09253.0500, Sigma Aldrich)
- Formaldehyde solution 37% (A0877, 0250, Panreac-Applichem)
- Acetic acid (33209, Sigma Aldrich)
- Fast Green (GT3407, Glentham Life Sciences)
- DPX (06522, Sigma Aldrich)
- Tri Reagent (TR-118, MRC)
- M-MLV Reverse Transcriptase (28025-013, Invitrogen)
- Sybr Select master mix (4472913, Applied Biosystems)
- Pepsin (Sigma-Aldrich, P6887)
- Hydrochloric acid
- Alcian Blue 8GX (A5268, Sigma Aldrich)
- DAB peroxidase substrate kit (SK-4100, Vector Laboratories)
- Hydrogen peroxide
- sodium citrate

- Fluoroshield with DAPI (F6057-20ML, Sigma Aldrich)
- Collagenase type IV C5138 Sigma-Aldrich
- 10X Phosphate-buffered saline (PBS) (70011-044, Gibco)
- 1X DMEM high glucose (41966-029, Gibco)
- Fetal Bovine Serum (FBS) (A5256701, Gibco)
- Penicillin – Streptomycin (10,000U/mL) (15140-122, Gibco)
- Amphotericin B (15290-018, Gibco)
- Trypsin-EDTA 0.5% (10X) (15400054, Gibco)
- Optimem (11058021, Gibco)
- MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (475989, Sigma Aldrich)
- Alexa Fluor 633 phalloidin (A22284, Molecular probes)
- RIPA Lysis Buffer
- Paraformaldehyde (PFA) (158127, Sigma Aldrich)
- Crystal Violet (C3886, Sigma Aldrich)
- Reagent for Alanine Transaminase (ALT, OSR6007, Beckman Coulter, Brea, CA, USA)
- Reagent for Aspartate Aminotransferase (AST, OSR6009, Beckman Coulter, Brea, CA, USA)
- Reagent for Albumin (OSR6102, Beckman Coulter, Brea, CA, USA)
- Reagent for Cholesterol (OSR6116, Beckman Coulter, Brea, CA, USA)
- Reagent for Creatine kinase (CK, OSR6179, Beckman Coulter, Brea, CA, USA)
- Reagent for Direct bilirubin (OSR6111, Beckman Coulter, Brea, CA, USA)
- Reagent for Iron (OSR6186, Beckman Coulter, Brea, CA, USA)
- Reagent for Total protein (OSR6132, Beckman Coulter, Brea, CA, USA)
- Reagent for Triglycerides (OSR60118, Beckman Coulter, Brea, CA, USA)
- Reagent for Urea (OSR6134, Beckman Coulter, Brea, CA, USA)
- Reagent for Uric acid (OSR6098, Beckman Coulter, Brea, CA, USA)
- V-52D Diluent (105-005962-00, Mindray)
- V-52DIFF Lyse (105-005964-00, Mindray)
- V-52LH Lyse (105-005965-00, Mindray)
- Probe cleanser (105-002225-00, Mindray)
- CBC-5DMR (05M444TA3.1508, Mindray)
- anti-CD45 BD cat. no. 566095; Biosciences
- anti-CD11b cat. no. 561690; BD Biosciences
- anti-CD11c cat.no. 117317; Biolegend
- anti-MHCII cat.no. 107627; Biolegend
- anti-Ly-6G cat. no. 127621; Biolegend
- anti-Ly-6C cat. no. 553104; BD Biosciences
- anti-CD64 cat. no. 139320; Biolegend
- anti-Siglec-F cat. no. 740956; BD Biosciences
- anti-CD24 cat. no. 101823; Biolegend
- anti-CD206 cat.no. 141705; Biolegend
- DAPI cat.no. 10236276001; Roche
- Water for injection

Equipment

- Fiberoptic light source
- Vertical plastic support
- Laryngoscope
- Electrical Heating blanket
- FlexiVent system (SCIREQ, Montreal, Canada)
- CO₂ chamber with gradual filling
- hemocytometer
- Spectrophotometer (Sunrise, TECAN, Austria)
- Olympus Slideview VS200 slide scanner
- Microtome
- T25 digital Ultraturax (IKA)
- BioRad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories)
- Lyophilizer or Speedvac (Savant SPD1010 Speedvac Concentrator, Thermo Scientific)
- mortar and pestle
- Laminar Flow hood for cell culture
- Beckman Coulter AU480 Clinical Chemistry Analyzer (Beckman Coulter, Brea, CA, USA)
- BC-5000Vet from Mindray
- Cytex Aurora Spectral Flow Cytometer

Consumables

- Luer Syringes 1mL (303172, BD)
- I.V. Cannulas (391452, BD)
- Histology cassettes (S.074.03.001.500, Isolab)
- Microscopic slides (631-0108, VWR)
- Coverslips (631-1574, VWR)
- 6-well plates (140685, Thermo Scientific)
- T75 flasks (83.3911.002, Sarstedt)
- Boyden Chambers: Corning® 6.5 mm Transwell® with 8.0 µm Pore Polycarbonate Membrane Insert (3422, Corning)

Software

- FlowJo™ v10.10 Software (BD Life Sciences)
- Fiji 2.9.0

Procedures

6w 5d 21h

1 Oropharyngeal (OA) Bleomycin (BLM) administration

2w

- 1.1 Weigh the mouse and record the body weight.
- 1.2 Anesthetize mouse by intraperitoneal injection of anesthesia mix (5 μ L/g body weight) so that the mouse receives Xylazine/ketamine/atropine mixture at 6.6 mg/ 100 mg/ 0.033 mg/kg, respectively.
- 1.3 Monitor anesthetic depth of the mouse by observing the loss of pedal reflexes (pinch at both foot pads).
- 1.4 Stabilize the mouse on a vertical plastic support by hanging it with a rubber band from the two upper front teeth.
- 1.5 Place the flexible fiberoptic of the light source on the neck of the mouse just below the vocal cords to provide the best view of the trachea.
- 1.6 Open the mouth of the mouse by using a small metal laryngoscope. Simultaneously, with blunt forceps, gently pull out the tongue to get a clear view of the trachea. (The trachea can be easily visualized in the mouth cavity, and it appears as a small white light spot, while the mouth cavity has an orange shade.)
- 1.7 While holding the mouth open with the laryngoscope, block the nares with a tong and immediately administer 0.8U/kg BLM or SAL to the oropharyngeal cavity with a pipette.

Note

Blocking the nares with a tong prevents obligatory nasal breathing and permits the complete inhalation of the deliverable.

- 1.8 Remove the tong from the nose as soon as the solution is completely inhaled.
- 1.9 Release the mouse from the plastic support and add moisturizing gel to its eyes.

- 1.10 Place the mouse on an electrical heating blanket to help the quick recovery from anesthesia.
- 1.11 Administer atipamezole (1 mg/kg). This must be at least 15 min after anaesthesia.
- 1.12 Return the mouse to its cage.
- 1.13 Record the mouse weight on day 7.
- 1.14 The disease phenotype will be observed 14 days after the BLM administration.

Note

Dose and route were selected upon prior extensive local testing to induce a solid fibrotic profile, while minimizing lethality. However, it is advised to try two different doses of BLM in your setup.

Note

The health status of the mice must be monitored at least once per day and all measures must be taken to minimize animal suffering and distress.

2 **Measurement of respiratory functions with flexiVent (14 days after BLM administration)**

1h

- 2.1 Weigh the mouse and record the body weight.
- 2.2 Anesthetize the mouse using xylazine/ketamine mixture 10mg/ 100mg/kg via intraperitoneal injection (5µL/g body weight). Do not use atropine in order to avoid tachycardia.
- 2.3 Calibrate the Flexivent system following the instructions of the software (two calibrations must be made: one at the beginning of the session and one prior to each mouse).
- 2.4 Enter the name and the initial weight of the mouse (prior to BLM or SAL administration).

- 2.5 Verify anesthetic depth of the mouse by checking the loss of pedal reflexes.
- 2.6 Perform a tracheostomy by making a small midline incision in the neck.
- 2.7 Expose trachea and carefully insert a cannula in the trachea.
- 2.8 Secure the cannula in place using sutures.
- 2.9 Connect the tracheostomized mouse to the FlexiVent ventilator system.
- 2.10 Wait until the mouse acquires the breathing rhythm of the machine.
- 2.11 Take the measurements of the lung mechanics according to manufacturer instructions.
- 2.12 Euthanize the mouse using a CO₂ chamber with gradual filling, followed by exsanguination. Blood collection is from the inferior vena cava. See step 11 for serum preparation and storage for biochemical analysis and step 12 for hematology analysis.

3 **Bronchoalveolar Lavage Fluid (BALF) Collection & Analysis**

1h

- 3.1 Insert a cannula into the trachea and fix it by making a loose knot with the sewing thread.
- 3.2 Lavage lungs with 3×1mL of sterile 0.9% NaCl. Keep the first lavage (BALF 1) separately from the other two lavages (BALFs 2 & 3).
- 3.3 Centrifuge all BALFs at 1200 rpm, 10 min, 4°C
- 3.4 Store the supernatant (-80°C) from BALF 1 for protein & collagen assays.



- 3.5 Combine cell pellets from all 3 BALFs in a single tube by resuspending them in 1 mL red blood cell lysis buffer.
- 3.6 Incubate on ice for 2-3 minutes.
- 3.7 Centrifuge 1200 rpm, 10min, 4°C.
- 3.8 Resuspend cell pellet in 1mL saline for cell count.
- 3.9 Count cells using 0.4% Trypan Blue solution and a hemocytometer to estimate pulmonary inflammation.
- 3.10 Perform total protein quantification (Bradford Assay, 595nm) as an indication of pulmonary edema and vascular leak
- 3.11 i) Prepare a bovine serum albumin standard curve in saline (BSA 0–2 mg/mL).
- 3.12 ii) Dilute 5x Bradford reagent to 1x Bradford reagent or as the manufacturer indicates.
- 3.13 iii) Place 5 μ L of BALF 1 supernatant in a 96-well plate in duplicate wells
- 3.14 iv) Add 245 μ L of 1xBradford reagent to all wells.
- 3.15 v) Take measurement of absorbance in a spectrophotometer at 595 nm.
- 3.16 vi) Convert absorbance values to protein concentration values (mg/ml) using the BSA standard curve.
- 3.17 vii) If the protein concentration values fall outside the BSA standard curve, dilute samples and repeat protein quantification.
- 3.18 Perform soluble collagen quantification (Sirius Red Assay, 540nm)

30m

- 3.19 i) Prepare a rat tail collagen I standard curve in saline (0–1000 µg/mL).
- 3.20 ii) Place 10 µL of the BALF 1 supernatants from mice that had received BLM in 1,5 mL tubes and add 40 µL of saline in order to dilute the samples.
- 3.21 iii) Place 50 µL of the BALF 1 supernatants from mice that had received SAL in 1,5 mL tubes.
- 3.22 iv) Place 50 µL of the standards in 1,5 mL tubes.
- 3.23 v) Add 350 µL of 0.5M acetic acid and 400 µL of Direct Red (120 µg/mL) to all tubes.
- 3.24 vi) Incubate tubes for 30 min at RT, in the dark.
- 3.25 vii) Centrifuge the tubes at 12000 rpm for 10 min and distribute 200 µL of each supernatant to the wells of a 96-well plate in duplicates.
- 3.26 viii) Measure the absorbance at 540nm. Convert absorbance values to collagen concentration values using the collagen standard curve taking into account the dilution of the BLM samples.
- 3.27 ix) If the collagen concentration values fall out of the standard curve, prepare new dilutions of the samples and repeat collagen quantification.

4 Lung lobes isolation

- 4.1 After BALF isolation, perform perfusion of the lung lobes through the heart using PBS and until the lobes turn white.
- 4.2 Ligate the left lung lobe with a surgical suture doing a double knot, isolate it and quickly snap-freeze in liquid nitrogen and store longterm at -80° C.
- 4.3 Insert a cannula to the trachea.



- 4.4 Make a loose knot surrounding the trachea and the cannula.
- 4.5 Remove the needle and administer 1 mL formalin to the right lung lobes through the trachea.
- 4.6 Remove the cannula and tighten the knot just before the final part of the cannula leaves the trachea.
- 4.7 Isolate the right lobes together with the trachea and the heart and fix them in 10% formalin overnight.
- 4.8 The next day remove the formalin and replenish with 1x PBS. Store the tissues at 4° C.

5 **Biochemical analysis**

4h

For biochemical analysis serum from blood is needed.

- 5.1 After blood collection store blood at RT for at least 30 min to clot.
- 5.2 Centrifuge at 2200g, 10min, 8°C.
- 5.3 Immediately centrifuge again at 6000g, 10min, 8°C.
- 5.4 Isolate supernatant (serum) and centrifuge again at 16200g, 10min, 8°C.
- 5.5 Isolate supernatant.
- 5.6 Store at -20°C until further usage.
- 5.7 Dilute serum 1:1 with water for injection.



- 5.8 Perform Biochemical analysis with a Clinical Chemistry Analyzer. Measure Alanine Transaminase, Aspartate Aminotransferase, Albumin, Cholesterol, Creatine kinase, Direct bilirubin, Iron, Total protein, Triglycerides, Urea, and Uric acid levels.

6 Hematology analysis

1h

For hematology analysis fresh whole blood is needed.

- 6.1 Collect blood and add 1 μ L of EDTA 50 mM per 50 μ L of whole blood

- 6.2 Perform hematology analysis in 15 μ L of EDTA-containing whole blood with a Vet hematology analyzer according to the manufacturer protocol.

Note

It is critical to avoid placing the samples on ice.

7 Flow cytometry of BALF cells

3h

If flow cytometry of BALF cells is desired, proceed from step 3.8 substituting saline with FACS buffer (1xPBS/ 2% FBS). Continue as following:

- 7.1 Determine cell number using an improved Neubauer hemocytometer under an inverted light microscope.
- 7.2 Centrifuge cells at 1200 rpm, 10min, 4°C.
- 7.3 Resuspend cell pellets in 50 μ L blocking buffer (1xPBS/ 2% FBS/ 1:400 anti-mouse CD16/32) and incubate for 10 min at 4°C.
- 7.4 Stain the cells with the addition of extra 50 μ L blocking buffer containing appropriate dilutions of the following antibodies anti-CD45 (1:300), anti-CD11b (1:250), anti-CD11c (1:250), anti-MHCII (1:250), anti-Ly-6G (1:200), anti-Ly-6C (1:200), anti-CD64 (1:100), anti-SiglecF (1:200), anti-CD24 (1:200) and anti-CD206 (1:100) for 30 min, 4°C.
- 7.5 Centrifuge cells at 1200 rpm, 10min, 4°C.

- 7.6 Wash with blocking buffer (1xPBS /2% FBS/ 1:400 anti-mouse CD16/32).
- 7.7 Centrifuge cells at 1200 rpm, 10min, 4°C.
- 7.8 Resuspend cells in 250 µL of filtered 1xPBS containing DAPI (1:15000).
- 7.9 Acquire data on a flow cytometer and analyze it with a specified software.
- 7.10 The used gating strategy for the identification and quantification of the different immune cell populations is as follows. After unwanted debris and aggregates elimination by forward scatter (FSC) and side scatter (SSC) gating, dead cell elimination by DAPI and taking into consideration only single cells by using the FSC-A and FSC-H gating, immune cells were identified based on the expression of CD45. Neutrophils were distinguished from all other immune cells based on the expression of the neutrophil-specific marker Ly6G. Subsequently, positive staining for either CD11b or CD11c on the remaining cells used to discern the remaining immune cells from CD11b- / CD11c- B, T lymphoid cells. The lymphoid cells were further discerned in B cells (MHC II+ / CD45+) and T cells (MHC II-/CD45+). The remaining CD11b+/CD11c+ immune cells were further categorized based on their pattern of SSC and MHC II. The cell pool of low SSC-A and MHCII (low and high) was used to discern Monocytes (CD64+/CD11b+). The pool of high SSC-A was divided using CD64 and CD24 markers. The pool of low CD64 was used to discern Dendritic cells (MHCII+/CD11b-) and Eosinophils (MHCII-/CD11b+). The pool of high CD64 was further divided with CD11c and CD11b, and the pool of CD11c+/CD11b+/- was used for discrimination of alveolar macrophages (SiglecF+/CD11b-) and monocyte-derived alveolar macrophages (SiglecF-/CD11b+). Monocyte-derived alveolar macrophages were further subdivided as CD206 positive or negative monocyte-derived alveolar macrophages.

8 Histology (H&E, Fast Green-Sirius Red and Alcian Blue staining)

3d

- 8.1 Insert the 2 largest right lung lobes into tissue embedding cassettes.
- 8.2 Subject cassettes to standard tissue processing (dehydrate sequentially in graded ethanol, clear with xylene and infiltrate tissue with molten paraffin wax under vacuum).



- 8.3 Transfer lungs to metal embedding molds filled with molten paraffin.
- 8.4 Generate 5µm slice sections in microtome
- 8.5 Let them dry overnight at 37°C.
- 8.6 Subject some sections to H&E staining
- 8.7 i) Deparaffinize the sections by incubating them in xylene 3 times for 5 min each.
- 8.8 ii. Rehydrate the sections in EtOH 100% for 3 min.
- 8.9 iii. Rehydrate the sections in EtOH 96% 2 times for 2 min each.
- 8.10 iv. Rehydrate the sections in EtOH 70% for 2 min.
- 8.11 v. Rehydrate the sections in EtOH 50% for 2 min.
- 8.12 vi. Rinse in dH₂O for 2 min.
- 8.13 vii. Incubate the sections in hematoxylin solution for 1 min.
- 8.14 viii. Wash in tap water until no color is observed.
- 8.15 ix. Incubate the sections in acid alcohol for 3 sec.
- 8.16 x. Wash in dH₂O for 2 sec.

1h



- 8.17 xi. Incubate in Scott's solution for 2 min and 15 sec.
- 8.18 xii. Wash in dH₂O for 5 sec.
- 8.19 xiii. Incubate in Eosin solution for 30 sec.
- 8.20 xiv. Wash in dH₂O for 5 sec.
- 8.21 xv. Dehydrate in 50, 70, 96 and 100% EtOH for 20 sec. each.
- 8.22 xvi. Clear in two changes of xylene, 2min. per change.
- 8.23 xvii. Mount with DPX and seal with a coverslip.
- 8.24 xviii. Scan slides on a slide scanner.
- 8.25 xix. Observe the slides and quantify fibrosis development in a blinded manner (by two independent reviewers) based on the Ashcroft score (0, normal lung; 1, isolated alveolar septa with gentle fibrotic changes; 2, fibrotic changes of alveolar septa with knot-like formation; 3, contiguous fibrotic walls of alveolar septa; 4, single fibrotic masses; 5, confluent fibrotic masses; 6, large contiguous fibrotic masses; 7, air bubbles among the contiguous fibrotic mass; 8, fibrous obliteration).
- 8.26 Subject some sections to staining with Fast Green-Sirius Red

Note

Sirius Red is the same as Direct Red

4h

Note*Use superfrost slides*

- 8.27 i. Before deparaffinization, incubate the sections for 1h at 56°C.
- 8.28 ii. Deparaffinize and rehydrate the sections as in H&E staining.
- 8.29 iii. Incubate the sections in Bouin's solution (75% picric acid, 25% formaldehyde and 1% acetic acid) for 1h at 56°C.
- 8.30 iv. Incubate the sections in 0,04% Fast Green dissolved in picric acid for 15 min.
- 8.31 v. Rinse in dH₂O for 10 sec.
- 8.32 vi. Incubate the sections in Sirius Red 0,1% / Fast Green 0,04% dissolved in picric acid for 40 min.
- 8.33 vii. Wash for 5 sec. in picric acid.
- 8.34 viii. Wash for 5 sec. in 1% acetic acid.
- 8.35 ix. Dehydrate in 50, 70, 96 and 100% EtOH for 20 sec. each.
- 8.36 x. Clear in two changes of xylene, 2 min. per change.
- 8.37 xi. Mount with DPX and put on a coverslip.
- 8.38 xii. Scan slides with a slide scanner or observe under microscope.



1h 30m

- 8.39 Subject some sections to Alcian Blue staining
- 8.40 i. Deparaffinize and rehydrate sections as in H&E.
- 8.41 ii. Place sections in 3% acetic acid for 3 min (7,5 mL glacial acetic acid plus 242,5 mL ddH₂O). This step protects the subsequent Alcian Blue solution from pH changes.
- 8.42 iii. Incubate sections in 1% Alcian Blue 8GX solution in 3% acetic acid, pH 2.5 for 30 min at RT or 15 min at 37 °C.
- 8.43 iv. Rinse briefly in 3% acetic acid.
- 8.44 v. Wash in running tap water for 10 min.
- 8.45 vi. Rinse well with dH₂O.
- 8.46 vii. Incubate in Scott's solution for 2 min and 15 sec.
- 8.47 viii. Wash in dH₂O for 5 sec.
- 8.48 ix. Dip sections briefly in eosin (1-2 dips).
- 8.49 x. Rinse briefly with dH₂O.
- 8.50 xi. Dehydrate as in H&E.
- 8.51 xii. Clear in two changes of xylene, 2min per change.



- 8.52 xiii. Mount with DPX and seal with a coverslip.

Note

Alcian Blue is expected to stain weakly acidic sulfated mucins, hyaluronic acid and sialomucins dark blue, background pink to red and nuclei red.

9 Immunohistochemistry

2d

- 9.1 Generate 5µm slice sections in microtome and put them on superfrost slides.
- 9.2 Let them dry overnight at 37°C.
- 9.3 Incubate the sections for 1h at 60°C.
- 9.4 Deparaffinize the sections by incubating them in xylene 3 times for 5 min.
- 9.5 Rehydrate the sections in a gradient of EtOH as in H&E staining
- 9.6 Rinse in dH₂O (leave the slides there until next step)
- 9.7 Perform antigen retrieval with sodium citrate buffer (2.94 gr trisodium citrate dihydrate in 1L H₂O, pH 6.0) by heating in microwave for 20 min.
- 9.8 Let the antigen retrieval solution (with the sections) come to RT
- 9.9 Wash with dH₂O.
- 9.10 In the case of DAB staining, perform peroxidase blocking in 3% H₂O₂ in dH₂O, in the dark, for 10 min.

- 9.11 Prepare PBS-T (phosphate buffer saline with Tween-20 0.05%) which will serve as a washing buffer and as a basis for the blocking and antibody solutions
- 9.12 Wash sections 2x with dH₂O and 2x with PBS-T, shaking. Each wash lasts 5 min.
- 9.13 Block sections (10% normal goat serum/2% BSA in PBS-T) in a humid chamber, at room temperature for 1h.

Note

Use normal goat serum when the secondary antibody that will be used derives from goat. If the secondary antibody derives from another animal, use the respective serum.

- 9.14 Incubate the sections with primary antibody in 2% BSA in PBS-T in a humid chamber, overnight at 4°C. Include sections that will be incubated with the isotype antibody as a control, at the same concentration as the primary antibody.
- 9.15 Next day wash the sections 3x with PBS-T, shaking. Each wash lasts 5min.
- 9.16 Incubate the sections with secondary antibody in 2% BSA in PBS-T in a humid chamber, in the dark for 1 hr.
- 9.17 Wash the sections 3x with PBS-T, shaking in the dark. Each wash lasts 5min.
- 9.18 In the case of DAB staining, visualize the staining with DAB peroxidase substrate kit according to the kit's instructions and then stain sections with hematoxylin (1 dip, no more than 10 seconds). Wash with tap water and dehydrate as in H&E. Mount with DPX and seal with coverslip.

In the case of fluorescence staining, mount slides with Fluoroshield with DAPI and seal with coverslip.

Note

Dehydration does not take place in fluorescence staining.



10 RNA isolation and real-time PCR

- 10.1 Homogenize 50–100mg of the left lung lobe using the Tri Reagent and a T25 homogenizer (IKA) at 13000 rpm.
- 10.2 Isolate the RNA following the manufacturer's protocol.
- 10.3 Perform cDNA synthesis using 2 µg of total RNA per sample in 20-µl reaction using M-MLV RT.
- 10.4 Perform Real-time PCR for a housekeeping gene (*B2m*), collagen 1a1 and fibronectin with SYBR Select Master mix on a BioRad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). The annealing temperature for all primers is 60°C. Primers sequences for RT PCRs are depicted below.

A	B
<i>B2m</i> forward	5'-TTCTGGTGCTTGTCTCACTGA-3'
<i>B2m</i> reverse	5'-CAGTATGTTCCGGCTTCCCATTTC-3'
<i>Col1a1</i> forward	5'-CTACTACCGGGCCGATGATG-3'
<i>Col1a1</i> reverse	5'-CGATCCAGTACTCTCCGCTC-3'
<i>Fn1</i> forward	5'-GGCCACCATTACTGGTCTGG-3'
<i>Fn1</i> reverse	5'-GGAAGGGTAACCAGTTGGGG-3'

- 10.5 Normalize Ct values of *col1a1* and *Fn1* with the Ct values of *B2m*. Calculate relative gene expression with the $2^{-\Delta\Delta C_t}$ method.

11 Acellular ECM (aECM) preparation from whole lungs

In the case of aECM preparation one needs the whole lung, which means that histology and RNA isolation will not take place.

- 11.1 Isolate the lung
- 11.2 Treat the lung with increasing concentrations of SDS (0.01, 0.1, 1%) in a PBS solution, with 24 h incubation for each SDS concentration.
- 11.3 Wash the decellularized lung tissue with PBS for at least 3 days
- 11.4 Cut the lung into small pieces and store at -80°C
- 11.5 Lyophilize the frozen lung with a lyophilizer (or a Speedvac)
- 11.6 Weigh the lyophilized lung and mill it in liquid nitrogen with a mortar and pestle.
- 11.7 Solubilize the milled tissue (powder) with enzymatic digestion in a pepsin solution (pepsin is dissolved in 0.1 M HCl at a final concentration of 1 mg/ml) for 48 hrs at 37°C , shaking. Approximately 10 mg of the ECM powder should be digested in 1 mL of pepsin solution, in order to solubilize the ECM components.
- 11.8 Go to cell culture laminar flow hood.
- 11.9 Dilute the matrix in 0.1 M acetic acid to make a 5 mg/ml concentration of lung ECM solution, which will be used as a coating substrate for cells.
- 11.10 Coat a well of a 6-well plate with 700 μL of aECM or a well of a 24-well plate with 150 μL of aECM. Incubate o/n at RT.
- 11.11 Wash the coated plates with sterile 1xPBS three times. The wells are then ready to be seeded with cells.

12 Isolation and culture of primary murine lung fibroblasts

2h

For the isolation of primary lung fibroblasts, the use of 8-10-week- old C57Bl6/J mice and/or BLM-challenged mice is required. The isolation procedure requires the whole lung. Use 2-3 mice for a T75 flask.



- 12.1 Sterilize all instruments (forceps, scissors and lancets) before use.
- 12.2 Humanely euthanize the mouse using a CO₂ chamber with gradual filling.
- 12.3 Sterilize the animal with 70% EtOH.
- 12.4 Perfuse the lungs with 10mL sterile PBS through the heart using a 26G-needle.
- 12.5 Dissect the lungs using sterilized scissors and place them in ice-cold 1X DMEM (w/o FBS).
- 12.6 Mince the lungs in a sterile-petri dish using two lancets.
- 12.7 Digest the minced lung tissue in 0.6 mg/mL collagenase type IV (prepared in DMEM w/o FBS) at 37°C for 1h shaking.
- 12.8 Filter the digested cell suspension through a 70 µm cell strainer. Wash the cell strainer with 1x DMEM/10% FBS to inactivate collagenase.
- 12.9 Centrifuge at 1200 rpm for 5 min.
- 12.10 Resuspend the cell pellet in 1X DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin and 0.1% Amphotericin B.
- 12.11 Incubate the cells at 37°C in a humidified chamber with 5% CO₂ in a T75 flask.
- 12.12 After 2 days wash the cell layer twice with 1X PBS to remove non-adherent cells.
- 12.13 Trypsinize the adherent cells using 5X Trypsin for 5min at 37°C.
- 12.14 Transfer the detached cells to a T125 flask for further expansion.

**Note**

Primary murine lung fibroblasts can be used for up to three passages.

12.15 Quantify proliferation of LF culture with the MTT assay.

3d

12.16 i. Seed cells in a 96-well plate at a density of 4000 cells per well.

12.17 ii. Leave o/n in the CO₂ incubator for cells to attach.

12.18 iii. Remove medium and replace it with starvation medium (FBS-free medium, supplemented with 0,2% BSA) and incubate o/n.

12.19 iv. Treat LFs with an appropriate stimulus.

12.20 v. Wash cells with phenol red-free Optimem.

12.21 vi. Remove the Optimem.

12.22 vii. Add 100 µl phenol red-free Optimem and 15 µl of stock MTT solution (5 mg/ml in phenol red-free Optimem) in each well.

12.23 viii. Incubate for 3 hrs at 37°C in the CO₂ incubator

12.24 ix. Check purple crystal formation under microscope

12.25 x. Remove medium, add 200 µl acidic isopropanol (0.04 N HCl in isopropanol) and leave for 30 min, RT, with FOIL under rotation.

12.26 xi. Make sure the crystals are solubilized



- 12.27 xii. Measure Abs at 570 nm in a plate reader and subtract background at 660 nm
- 12.28 Estimate migration and invasion with Boyden chambers according to manufacturer's instructions
- 12.29 i. Coat the upper chamber of a Boyden chamber (attached to a well of a 12-well plate) with aECM (5 mg/ml) if you want to estimate invasion (for migration skip this step). Incubate o/n, RT.
- 12.30 ii. Add 40000 cells to the upper chamber of the Boyden chamber (diluted in FBS-free DMEM supplemented with 0,02%BSA plus stimulus)
- 12.31 iii. Allow the cells for 6 h to invade/migrate through the transwell membrane to the lower side which is in touch with FBS-free DMEM supplemented with 0,02%BSA
- 12.32 iv. Remove the cells which remain in the upper chamber with a cotton swab
- 12.33 v. Wash invasive or migratory cells with PBS by adding PBS in the lower chamber.
- 12.34 vi. In a similar way fix invasive or migratory cells with PFA 4% in PBS by leaving it for 15 min.
- 12.35 vii. Stain invasive or migratory cells with Crystal Violet (5mg/ml in 2% EtOH) for 20 min.
- 12.36 viii. Wash with dH₂O until residual Crystal Violet is removed.

Note

It is critical to remove remaining stain on the surrounding plastic of the Boyden chamber with a cotton swab.

- 12.37 ix. Lyse cells with RIPA Lysis Buffer for 20 minutes.
- 12.38 x. Measure absorbance at 550 nm.

1d



12.39 Perform LF immunofluorescence staining

12.40 i. Insert a coverslip in a well of a 24-well plate. Sterilize with UV. Seed 20000 cells on each coverslip. Incubate o/n to allow cells to attach.

12.41 ii. Replace medium with FBS-free DMEM supplemented with 0,02% BSA. Incubate o/n.

12.42 iii. Add stimulus (eg. TGF β 10ng/mL for 24 h).

12.43 iv. Wash cells with 1xPBS. Fix cells with 4% PFA for 15 min.

12.44 v. Wash cells with 1xPBS three times.

12.45 vi. Permeabilize cells with 0.1% Triton X in PBS for 10 min.

12.46 vii. Wash cells with 1xPBS.

12.47 viii. Perform blocking with 2% BSA in PBS for 1h at RT.

12.48 ix. Incubate o/n at 4°C with primary antibodies in 2% BSA in PBS.

12.49 x. Wash cells with 1xPBS three times.

12.50 xi. Incubate with a secondary antibody and conjugated phalloidin in 2%BSA in PBS for 60min at RT.

12.51 xii. Wash cells with 1xPBS three times.



- 12.52 xiii. Mount coverslips with a drop of mounting Fluoroshield medium (containing DAPI for nucleus labelling).

13 **Acellular ECM (aECM) preparation from lung fibroblasts (LFs)**

1w 3d

- 13.1 Culture LFs in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.1% amphotericin.
- 13.2 Seed LFs at a density of 3×10^5 /ml into 6-well cell culture dishes and culture them for 7–10 days until confluence. Supplement the culture medium with 50 µg/mL ascorbic acid and 10 ng/ml TGFβ to enhance cell-derived ECM deposition.
- 13.3 Replenish the culture medium (supplemented with ascorbic acid and TGFβ) every 2 days.
- 13.4 Perform decellularization by treating the confluent cells with 0.2 M NH₄OH for 5–15 minutes until cells are killed. Cells are constantly observed with a light microscope at 10x to confirm proper removal of all fibroblast debris.
- 13.5 After decellularization, wash the matrices 3 times with PBS. Use the fibroblast-derived matrices immediately or store at 4 °C for up to 10 days.