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Title: Refined Murine Model of Idiopathic Pulmonary Fibrosis

Authors and Affiliations:

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**

3. **Filming location:** Will the filming need to take place in multiple locations? **No.**

Current Protocol Length
Number of Steps: 20
Number of Shots: 51

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Author's NOTE: Interview statements are only slightly modified as indicated by red fonts.

1.1. **Chuwen Lin:** Pulmonary fibrosis is a devastating and irreversible disease characterized by fibrotic scars and bronchiolization of alveoli. This research addresses the limitations in existing animal models by introducing an improved method in mice that more closely mimics the pathology of pulmonary fibrosis **[1].**

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 1B*.

What are the current experimental challenges?

1.2. **Chuwen Lin:** The standard model of pulmonary fibrosis uses intratracheal injection of a single dose of bleomycin, but it fails to mimic the key features. A method using 4 to 8 repetitive injections is developed, but it is time-consuming and results in high mortality **[1]**.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What advantage does your protocol offer compared to other techniques?

1.3. **Chuwen Lin:** We use a twice-repeated oropharyngeal injection to induce pulmonary fibrosis. It recapitulates the core traits of the disease while saving time and reducing mortality of mice compared to the 4 to 8 repetitive injection models **[1]**.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.2.*

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions:

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.4. **Chuwen Lin:** Publishing with JoVE will allow more researchers to watch the video protocol and visually understand what our lab has been studying. Hopefully, this will encourage them to read more of our publications and cite them **[1]**.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Laboratory Animal Research Center of Sun Yat-sen University.

Protocol

2. Oropharyngeal Administration of Bleomycin in Mice

Demonstrator: Fengqing Zhu

2.1. To begin, weigh each mouse prior to oropharyngeal administration to calculate the required volume of bleomycin solution based on its body weight **[1]**

2.1.1. WIDE: Talent placing a mouse on the scale and noting the weight.

2.2. Place the mouse on the tracheal injection stand **[1-TXT]** and secure it by hooking its teeth **[2]**. Using forceps, gently extend the tongue and hold it with the thumb and index finger **[3]**. Cover the mouse's nose with the middle finger to ensure the solution is delivered properly **[4]**.

2.2.1. Talent placing the mouse on the tracheal stand. **TXT: Anesthesia:; Induction: 3% Isoflurane**

2.2.2. Talent hooking the mouse's teeth to secure it on the tracheal stand.

2.2.3. Talent pulling the tongue forward with forceps and holding it with the thumb and index finger.

2.2.4. Talent covering the mouse's nose using the middle finger.

2.3. Using a 200-microliter pipette tip, aspirate the required volume of diluted bleomycin solution **[1]** and slowly administer the solution into the oropharynx, avoiding deep insertion of the pipette tip **[2-TXT]**.

2.3.1. Talent drawing up bleomycin solution using a 200-microliter pipette.

2.3.2. Talent administering the bleomycin solution into the oropharynx. **TXT: Listen for a gurgling sound to confirm lung delivery of the solution**

2.4. Remove the mouse from the stand **[1]** and place it on a heating pad for recovery **[2-TXT]**.

2.4.1. Talent gently removing the mouse from the stand.

2.4.2. Talent placing the mouse on a heating pad. **TXT: Repeat oropharyngeal administration 7 days after the initial dose**

3. Tissue Collection Procedure

Videographer: Please make sure the labels on the reagents are visible in the frame to avoid having shots that look identical on camera.

3.1. After euthanizing the mice, perform a midline sternotomy to open the thoracic cavity **[1-TXT]**. Perfuse the lungs via the right ventricle using 10 milliliters of ice-cold PBS to flush out the blood cells **[2]**.

3.1.1. Talent performing the midline incision and exposing the thoracic cavity. **TXT: Euthanasia: Cervical dislocation**

3.1.2. Talent perfusing the lungs using a syringe filled with ice-cold PBS through the right ventricle.

3.2. Insert a cannula into the trachea **[1]** and inflate the lungs using 1 percent paraformaldehyde to ensure complete tissue fixation **[2]**.

3.2.1. Talent inserting the tracheal cannula.

3.2.2. Talent inflating the lungs with paraformaldehyde.

3.3. Excise the lungs **[1]** and immerse them in 1 percent paraformaldehyde at room temperature for 1 hour to allow fixation **[2]**.

3.3.1. Talent removing the lungs.

3.3.2. Talent placing the lungs in a container filled with paraformaldehyde.

3.4. For tissue processing, rinse the fixed tissues three times in PBS for 5 minutes each **[1]**. Dehydrate the samples using 70 percent ethanol, ensuring complete dehydration **[2-TXT]**.

3.4.1. Talent rinsing the tissues in PBS.

3.4.2. Talent transferring tissues in a 70% ethanol-labeled container. **TXT: Repeat the dehydration with 95% and 100% ethanol**

3.5. After dehydration, clear the tissues in xylene for 1 hour at room temperature **[1]**. Then, embed the tissues in molten paraffin wax at 60 degrees Celsius for at least 2 hours or until fully infiltrated **[2]**. Using a rotary microtome, section the embedded tissues **[3]** into 7-micrometer slices **[4]**.

3.5.1. Talent placing tissues into a beaker containing xylene.

3.5.2. Talent placing tissues into a mold filled with molten paraffin wax held at 60 degrees Celsius.

3.5.3. Talent using a rotary microtome to slice paraffin-embedded tissues.

3.5.4. A shot of the thin slices.

4. Sample Assessment Procedure

Demonstrator: Yonghui He

Videographer: Please make sure the labels on the reagents are visible in the frame to avoid having shots that look identical on camera.

4.1. For hematoxylin and eosin staining, perform sequential xylene deparaffinization twice for 5 minutes **[1]**. Then, rehydrate the slides in 100 percent ethanol for 5 minutes **[2-TXT]**. Rehydrate again in 95 percent ethanol for 1 minute **[3]**, and 80 percent ethanol for 1 minute **[4]**. Rinse with distilled water **[5]**.

4.1.1. Talent immersing the slides in xylene bath.

4.1.2. Talent transferring the slides in 100% ethanol. **TXT: Repeat rehydration in 100% ethanol for 1 min** *Videographer: Please make sure the ethanol labels are properly visible in the frame.*

4.1.3. Talent transferring the slices in 95% ethanol.

4.1.4. Talent transferring the slices in 80% ethanol

4.1.5. Talent rinsing slices in distilled water.

4.2. Stain the tissue with hematoxylin for 5 minutes **[1]**, rinse three times with tap water **[2]**, and soak in water for 5 minutes **[3]**. Then, dip in 80 percent ethanol for 1 minute **[4]** and counterstain with eosin for 45 seconds **[5-TXT]**.

4.2.1. Talent pipetting hematoxylin onto the slide for staining.

4.2.2. Talent rinsing slides in tap water.

4.2.3. Talent soaking slides in water.

4.2.4. Talent dipping slides in 80 percent ethanol bath.

4.2.5. Talent pipetting eosin onto slides for counterstaining. **TXT: Perform dehydration with 95% and 100% ethanol**

4.3. After performing dehydration with graded ethanol, clear the slides in xylene by dipping for 5 minutes twice **[1]**, then mount the samples using neutral balsam **[2]**.

4.3.1. Talent dipping slides in xylene bath for clearing.

4.3.2. Talent applying neutral balsam and sealing coverslip on the slide.

4.4. For Masson's staining, after dewaxing, rinse the slides in double-distilled water **[1]** and stain with Ponceau S-fuchsin for 5 seconds **[2]**. Then, rinse with 0.2 percent weak acid solution for 1 minute **[3]**.

4.4.1. Talent rinsing the slides in double-distilled water.

4.4.2. Talent applying Ponceau S-fuchsin stain with a pipette.

4.4.3. Talent rinsing slides with a weak acid solution.

4.5. Differentiate in phosphomolybdic acid solution for 5 minutes **[1]**, then rinse again with weak acid for 1 minute **[2]**. Counterstain using aniline blue for 1 minute before rinsing again with weak acid **[3]**.

4.5.1. Talent immersing slides in phosphomolybdic acid.

4.5.2. Talent rinsing slides with a weak acid solution.

4.5.3. Talent applying aniline blue stain to the slide.

4.6. After dehydrating the sections again through graded ethanol, clear the slides in xylene **[1]** and mount them with resin **[2]**.

4.6.1. Talent clearing the slides in xylene.

4.6.2. Talent mounting stained sections using resin and applying a coverslip.

4.7. For immunohistochemical staining, after dewaxing and rehydrating the paraffin-embedded slides, perform antigen retrieval by placing the slides in boiling 0.1 molar sodium citrate buffer with 0.5 percent Tween-20 for 90 seconds **[1]**.

4.7.1. Talent placing slides in a boiling sodium citrate buffer bath.

4.8. Quench endogenous peroxidase activity with 3 percent hydrogen peroxide **[1]**. Permeabilize membranes using 0.5 percent Triton X-100 in PBS to block nonspecific binding **[2]**.

4.8.1. Talent applying hydrogen peroxide solution to slides.

4.8.2. Talent pipetting Triton X-100 solution to slides to permeabilize membranes and to block non-specific binding.

4.9. Add primary antibody to the slides **[1]** and incubate at 4 degrees Celsius overnight **[2]**. Add species-matched secondary antibody and incubate again for 1 hour at room temperature **[3]**. Now, add streptavidin-HRP and incubate for 30 minutes **[4]**.

4.9.1. Talent adding primary antibody to the slides.

4.9.2. Talent placing slides in an incubator.

4.9.3. Talent adding secondary antibody solution to the slides.

4.9.4. Talent pipetting streptavidin-HRP and covering slides.

4.10. For immunofluorescence staining, after repeating the immunohistochemistry steps, label biotinylated targets with Streptavidin-Alexa Fluor 594 or 647 *(Streptavidin-Alexa-Fluor-five-ninety-four or six-forty-seven)* at a 1:500 *(one to five hundred)* dilution for 30 minutes at room temperature **[1]**.

4.10.1. Talent adding fluorescent Streptavidin conjugate to the slides.

4.11. Counterstain nuclei with DAPI *(Dapi)* at 20 micrograms per milliliter for 5 minutes **[1]** and mount the slides using antifade mounting medium **[2]**.

4.11.1. Talent pipetting DAPI solution over the tissue section.

4.11.2. Talent applying antifade medium and sealing with coverslip.

Results

5. Results

5.1. This figure illustrates the histopathological differences in lung fibrosis between single and repetitive bleomycin administration, showing hematoxylin and eosin-stained sections at 14 days post-injury **[1]**.

5.1.1. LAB MEDIA: Figure 1B.

5.2. Notably, the repetitive bleomycin model exhibited persistent and more extensive fibroblastic foci **[1]** than the single-dose group **[2]**, marked by dense fibrotic clusters **[3]**.

5.2.1. LAB MEDIA: Figure 1B. *Video Editor: Highlight the two images at 14 dpi repetitive column.*

5.2.2. LAB MEDIA: Figure 1B. *Video Editor: Highlight the two images at 14 dpi single column.*

5.2.3. LAB MEDIA: Figure 1B.

5.3. Lung sections from repetitive bleomycin-treated mice at 60 days post-injury showed more extensive fibrotic remodeling **[1]** compared to the single-dose group **[2]**, with significant distortion of alveolar structure **[3]**.

5.3.1. LAB MEDIA: Figure 1C. *Video Editor: Highlight the two images at 60 dpi repetitive column.*

5.3.2. LAB MEDIA: Figure 1C. *Video Editor: Highlight the two images at 60 dpi single column.*

5.3.3. LAB MEDIA: Figure 1C.

5.4. Masson's trichrome staining revealed increased collagen deposition in the repetitive model **[1]** relative to the single-dose group **[2].**

5.4.1. LAB MEDIA: Figure 1D. *Video Editor: Highlight the Repetitive image.*

5.4.2. LAB MEDIA: Figure 1D. *Video Editor: Highlight the single image.*

5.5. Immunohistochemistry showed elevated expression of α-smooth *(alpha-smooth)* muscle actin and hydroxyproline in the repetitive group, indicating persistent

myofibroblast activity and matrix accumulation **[1]**, along with a marked increase in keratin 5-positive epithelial cells **[2]**.

5.5.1. LAB MEDIA: Figure 1E. *Video Editor: Highlight the two images at α-SMA column.*

5.5.2. LAB MEDIA: Figure 1E. *Video Editor: Highlight the two images at KRT5 column.*

5.6. Immunofluorescence revealed ΔN-p63-positive *(Delta-N P-sixty-three positive)* basal cell clusters exclusively in the repetitive model, absent in single-dose lungs **[1]**.

5.6.1. LAB MEDIA: Figure 1F.

5.7. Additionally, honeycomb-like cystic structures were observed in the repetitive model, resembling hallmark features of idiopathic pulmonary fibrosis **[1]**.

5.7.1. LAB MEDIA: Figure 1G.

5.8. Bronchiolization of alveolar epithelium was also evident in the repetitive injury group **[1]**.

5.8.1. LAB MEDIA: Figure 1H.