Live Imaging of the Lung

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ABSTRACT

Live imaging is critical to determining the dynamics and spatial interactions of cells within the tissue environment. In the lung, this has proven to be difficult due to the motion incurred by ventilation and cardiac contractions. In this chapter, we report protocols for imaging ex vivo live lung slices and the intact mouse lung. *Curr. Protoc. Cytom.* 60:12.28.1-12.28.12. © 2012 by John Wiley & Sons, Inc.

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INTRODUCTION

A majority of the analytical methods used to study experimental systems are end-point assays that provide a snap-shot view of biological processes. While much useful information can be gleaned from these assays, there is often a need to visualize the dynamics and spatial interactions of cells and their environment in real time. This data can only be obtained from live imaging, and here we present the basic protocols for live imaging in the mouse lung.

Basic Protocol 1 details the methods needed to image live lung slices from the mouse. This protocol has the advantages of superior image stabilization and imaging depth, increased imaging durability, and ready access to the lung parenchyma for immunostaining of cells. Basic Protocol 2 provides the methods for intravital imaging in the mouse lung. This protocol relies on image stabilization with a thoracic suction window, and is ideal for time-lapsed imaging of the lung microcirculation and alveolar spaces. Both of the protocols require familiarity with mouse surgery and advanced microscopy.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

LIVE IMAGING OF LUNG SLICES

The use of lung slices provides access to the lung parenchyma as well as airways of varying sizes. Previous studies have used this method to examine airway smooth muscle contraction (Bergner, et al., 2002), but it is also a valuable tool to understand immune reactions that contribute to ongoing immune responses. By choosing sections of varying depth, one can obtain a real-time picture of cellular behavior in most compartments in the lung. The tradeoff for access to the deepest parts of the tissue is that blood and lymph flow to and from the organ is absent. Therefore, findings using live imaging of lung slices should be verified as much as possible with the use of lung intravital imaging (Basic Protocol 2).

Materials

Transgenic mouse of interest
Anesthetic: e.g., 2.5% (w/v) Avertin
70% (v/v) ethanol
2% (w/v) low-melting-temperature agarose (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), 4°C
Vetbond (3M)
RPMI 1640 medium without phenol red

Dissection board
Dissection instruments
Suture: 3–0 silk
Plastic sheath from 18-G catheter (Exel Safelet Cath, Fisher Scientific)
1-ml syringe without a needle
Vibratome
Plastic cover slips (Fisher)
Microscope
Perfusion system: peristaltic pump, in-line heater, and heated imaging chamber (Warner Instruments)

Additional reagents and equipment for intraperitoneal injection of mice (Donovan and Brown, 2006)

Preparation of lungs for section live imaging

1. Inject the mouse i.p. (Donovan and Brown, 2006) with a lethal overdose of an anesthetic permitted by IACUC (e.g., 1 ml of 2.5% Avertin). Wait for the mouse to stop breathing.

   Cervical dislocation and carbon dioxide euthanasia can detrimentally affect lung viability and should be avoided.

2. Sterilize the mouse with 70% ethanol and immobilize on a dissection board.

3. Open the peritoneum and cut the descending aorta so that blood pools in the abdomen and not the chest cavity.

4. Open the chest cavity by cutting up through the diaphragm. Perform a sternotomy by cutting from the xyphoid process through the top of the sternum. Continue to cut through the skin and connective tissue over the trachea.

   Care must be taken to not damage the lungs or to cut through the trachea.

5. Expose the trachea and separate it from the surrounding connective tissue, being careful not to cut the trachea itself. Thread the 3–0 suture material under the trachea and tie in a loose knot.

6. Snip a small opening in the exposed trachea parallel to the cartilaginous rings, as close to the larynx as possible, taking care not to cut completely through. Cut the tip of the plastic sheath from an 18-G catheter at a 45° angle to form a beveled end. Gently insert the catheter sheath into the trachea. Tighten the suture material around the trachea to secure the catheter in place (Fig. 12.28.1A).

   Insert the catheter only 2 to 3 mm. You should be able to see the end of the catheter through the trachea. By inserting too deeply, you may traumatize the carina or only inflate one side of the lungs.

7. Using a syringe, without a needle, containing 1 ml of 37°C 2% agarose (taken directly from a bottle in a constant temperature bath), slowly instill the agarose through the catheter into the lungs.
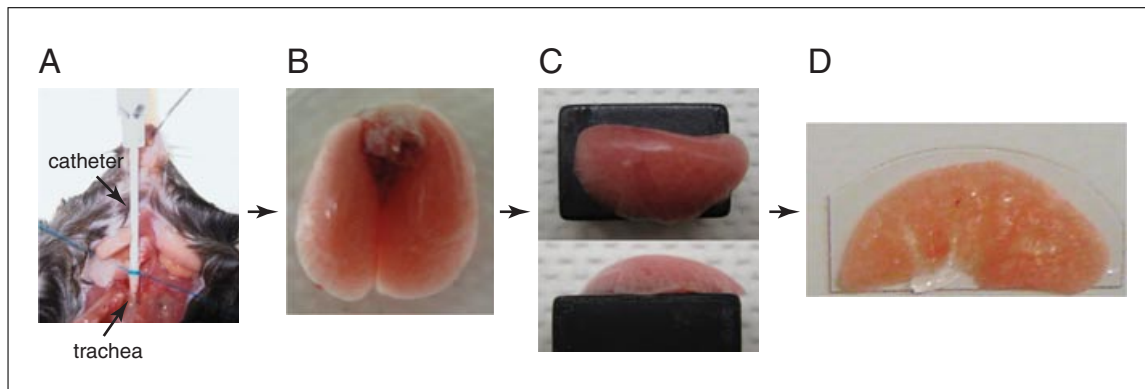
Figure 12.28.1 Protocol for preparing lungs for slice imaging. (**A**) Catheter inserted in the trachea is stabilized with suture. (**B**) Lungs filled with 1 ml low melting temperature agarose. (**C**) Left lobe mounted on a Vibtratome block with Vetbond. (**D**) 300-μm section immobilized on a plastic coverslip.

8. Once the lungs are fully inflated, pour approximately 50 ml of 4°C PBS over the inflated lungs. Allow the lungs to cool for 1 min to set the agarose. Remove the catheter sheath and tighten the suture material around the trachea to prevent any non-solidified agarose from leaking.

9. Holding onto the trachea with forceps directly above the suture, cut through the trachea. Gently pull the trachea up, pulling the lungs out of the chest cavity. Cut away the connective tissue and esophagus until the lungs are separated from the mouse (Fig. 12.28.1B).

10. Place lungs in cool RPMI medium without phenol red for transport (between 4°C and room temperature). Do not place lungs on ice.

Vibratome sectioning and mounting for live imaging

11. Fill the Vibratome half with room temperature and half with 4°C PBS to achieve a temperature of ∼16°C.

12. Separate the left lobe from the other lobes/heart/thymus by gently separating it with your fingers and cutting the mainstem bronchus.

13. Place the lobe on a Kimwipe and pat gently until the surface is almost dry.

14. Mount the lobe bronchus–side-down with Vetbond so that the largest surface area possible is in contact with the Vibratome slug (Fig. 12.28.1C).

15. Section the lung into 300-μm slices.

16. Mount slices on plastic cover slips cut to the size of the imaging chamber, using Vetbond (Fig. 12.28.1D).

17. Maintain slices at room temperature in RPMI without phenol red, until imaging.

Imaging lung sections

18. Set up perfusion system (consisting of a peristaltic pump, in-line heater, heated stage, and vacuum suction) on the microscope with oxygenated RPMI without phenol red so that the medium in the chamber measures as close to 37°C without going over.

    Warner Instruments sells all of the components for heated perfusion systems, which can be adapted to most microscope set-ups.

19. Place the section of interest into the chamber and allow it to equilibrate to the chamber temperature (about 5 min).

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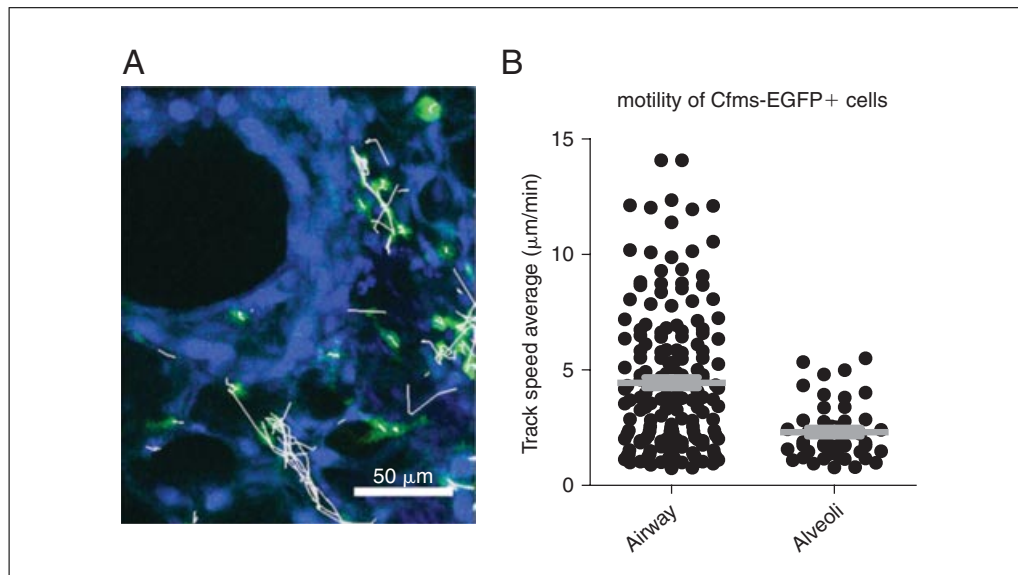
Figure 12.28.2 Motility of c-fms-EGFP$^+$ cells in lung sections. (**A**) Actin-CFP c-fms-EGFP lung slice shows motility of cells within the tissue with overlaid tracks. (**B**) Quantification of c-fms-EGFP cells shows increased motility near the airway.

20. Use the microscope eyepieces and magnification adjustment to locate the surface of the lung section. For two-photon microscopy, adjust laser power, wavelength output, and photomultiplier tubes to optimally excite and capture images for the chosen fluorophore (Figure 12.28.2).

STAINING LUNG SECTIONS WITH FLUORESCENT ANTIBODIES

Despite the large number of available fluorescently-labeled transgenic mice, one marker is often not sufficient to fully understand what specific cell type is being visualized. In addition, labeling certain surface molecules such as CD31 (blood vessels) and Lyve-1 (lymphatics) can provide context for the interactions occurring within the tissue (Fig. 12.28.3). Lung sections can be labeled with fluorescently tagged antibodies that are commonly used for FACS. By staining after sectioning and incubating at room temperature, the entire section can be stained without impacting cell behavior.

Materials

Lung sections (Basic Protocol 1, step 15)
RPMI 1640 medium without phenol red
Primary antibodies: Lyve-1, unconjugated (R&D) and PE-conjugated CD31 (BioLegend)
Secondary antibody: anti-goat Dylight 649 for Lyve-1 primary (Jackson ImmunoResearch)

24-well cell culture plate
Shaking platform

1. Place sections into 300 µl of RPMI without phenol red in a 24-well cell culture plate.

2. Add antibody to each well at two times the concentration normally used for FACS: 1 to 2 µg for Lyve-1 (to stain lymphatics) and/or CD31 (to stain blood vessels).

3. Incubate the sections at room temperature on a shaking platform for 2 hr in the dark.

4. Wash each section three times, each time for 5 min, with 1 ml of RPMI.

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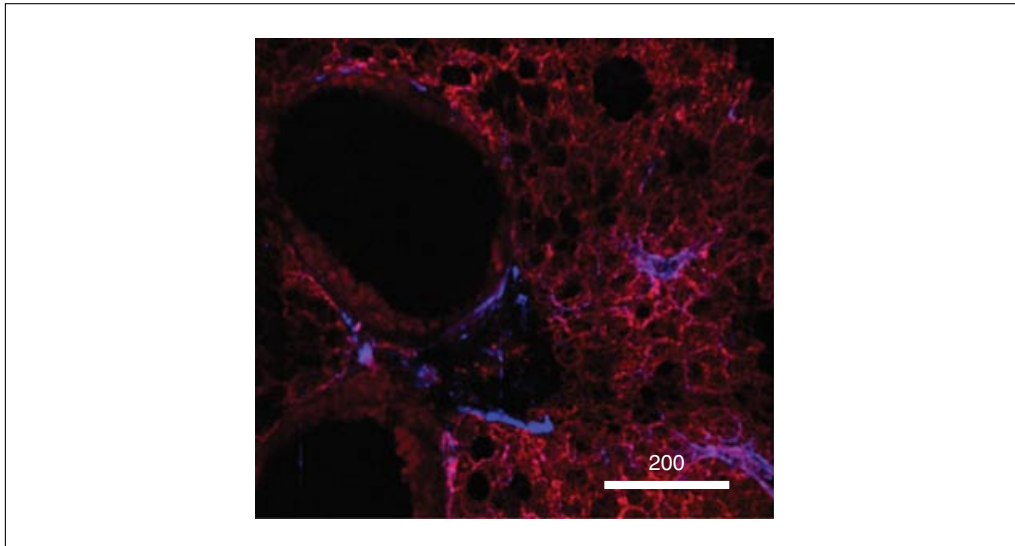
Figure 12.28.3 Staining of Lyve-1 and CD31 in lung slices. Lung sections stained as in Support Protocol 1 show Lyve-1 (blue) and CD31 (red). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy1228.*

5. If using an unconjugated antibody, add a secondary antibody (6 µg anti-goat Dylight 649 for Lyve-1 primary).

6. Incubate the sections at room temperature on the shaking platform for 2 hr in the dark.

7. Wash each section three times, each time for 5 min, with 1 ml of RPMI.

8. Mount sections and image as in Basic Protocol 1.

LIVE IMAGING IN THE MOUSE LUNG

Intravital imaging has been routinely applied to many tissues including the brain, cremaster, liver, and lymph nodes. A common feature shared by these tissues is easy accessibility and stabilization, both of which are problematic with lungs. To gain access to the surface of the lungs, the pleural cavity must first be breached, which leads to the loss of negative pleural pressures and lung collapse. Therefore, mechanical ventilation is needed to provide positive pressures that inflate the exposed lung. Remaining, however, is the major problem of motion both from the respiring lung and from cardiac contractions. To overcome the motion obstacle, we have applied a technique of using a thoracic suction window to gently immobilize the lung and allow for stable visualization (Looney, et al., 2011).

Materials

Mice (procedure is best performed in adult mice >20 g in body weight)
Anesthetics: ketamine, xylazine, isoflurane
Compressible gases (21%, 100% oxygen)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Alcohol swabs

Two-photon microscope with motorized and heated stage
Rectal thermometer
Adhesive tape
Suture: 3–0 silk
Fiber optic illuminator (Cole Parmer) for surgery

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Surgical tools for tracheotomy and thoracotomy

PE-90 tubing (Intramedic)

Mechanical ventilator (e.g., Kent Scientific or Harvard Apparatus)

Isoflurane vaporizer (Molecular Imaging Products, cat. no. AS-01-0007; *http://www.mipcompany.com/*)

Customized thoracic suction window (see annotation to step 14, below)

Micromanipulator (Thorlabs; *http://www.thorlabs.com*)

12-mm glass coverslips

Suction regulator and tubing

Additional reagents and equipment for intraperitoneal injection of mice (Donovan and Brown, 2006)

Anesthesia and mechanical ventilation

1. Anesthetize mouse with i.p. injections (Donovan and Brown, 2006) of ketamine (80 to 100 mg/kg) and xylazine (5 to 10 mg/kg).

   Alternative anesthetics may be used with the goal of achieving 10 to 30 min of anesthesia prior to initiating isoflurane anesthesia via the mechanical ventilator.

2. Place mouse on a heated microscope stage to achieve a rectal temperature of 37°C.

3. Position mouse in the supine position by taping the limbs to the heated stage. Flatten the neck and head by looping 3–0 silk suture around the incisors and taping the suture to the stage. Illuminate surgical area with fiber-optic illuminator.

4. Once adequate anesthesia has been induced (paw pinch), use small scissors and forceps to dissect the trachea and make a small horizontal incision with fine scissors in the anterior trachea. Insert trimmed PE-90 tubing into the trachea and secure with 3–0 silk suture (see Basic Protocol 1).

5. Turn on mechanical ventilator and adjust settings.

   Both volume- and pressure-regulated mechanical ventilators can be used to deliver positive-pressure breaths. For volume-regulated ventilators, we suggest using tidal volumes of 8 to 10 μl/g body weight with respiratory rates of approximately 120 breaths per min. For pressure-regulated ventilators, peak inspiratory pressures of 10 to 15 cm H_2O can be used. It is important to calibrate the mechanical ventilator for the tidal volume being delivered at the end of the ventilator tubing. This is best done using a glass 1-ml syringe.

   Depending on the experimental procedure, either room air (21% oxygen) or higher fractions of oxygen can be used.

   Positive-end expiratory pressure (PEEP) (approximately 3 cm H_2O) should be applied to the exhalation circuit before exhaled gases are scavenged with activated charcoal.

6. Connect tracheotomy tube (PE-90 tubing inserted in step 4) to the mechanical ventilator tubing and observe for inward and outward displacement of the thorax.

7. Dial isoflurane vaporizer to deliver 1% to 2% gas.

Thoracotomy and placement of thoracic window

8. Reposition mouse in on its right side (right lateral decubitus position). Place a capped needle or similar sized object under the front shoulders of the mouse to lift the left thoracic cavity. Re-tape the mouse to the heated stage.

9. Administer an i.p. injection (Donovan and Brown, 2006) of PBS (0.5 to 1.0 ml).

   This injection is given to replace surgical and insensible losses that occur during the experiment. A repeat injection of 0.3 ml PBS can be given at 2 hr, and hourly thereafter.

10. Reassess depth of anesthesia with a paw pinch.
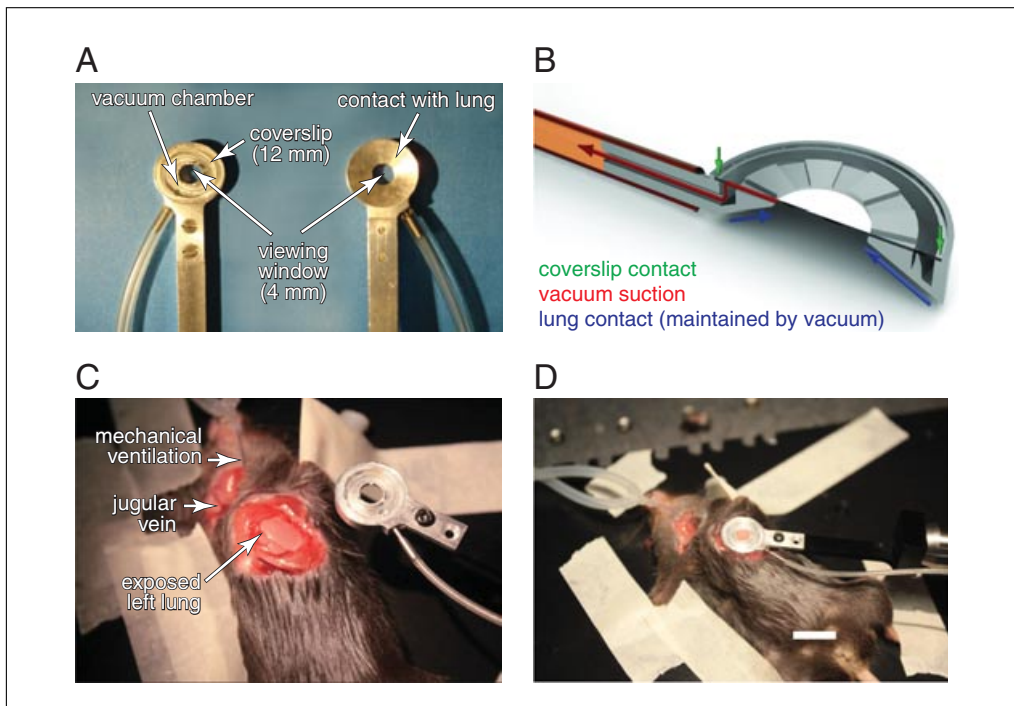
Figure 12.28.4 Surgical preparation for lung intravital microscopy. (**A**) Anterior and posterior views of the thoracic suction window fitted with a coverslip. (**B**) Side-view rendering of the suction window showing suction chamber, cover slip (green arrows), and vacuum flows (blue arrows near tissue, red arrows toward suction regulator). (**C**) Surgical preparation of left thorax with exposed left lung. (**D**) Suction window in situ. Scale bars, 5 mm (**B**), 10 mm (**C,D**) (Looney et al., 2011). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy1228*.

11. Use an alcohol swab to wet the mouse fur overlying the left thoracic cavity.

    Alternatively, small clippers can be used to shave the mouse fur.

12. Use fine scissors and forceps to remove mouse fur and underlying subcutaneous tissue overlying the left thoracic cavity. Delicately dissect until the ribs are visualized. Remove all tissue overlying approximately four anterior ribs.

13. Use fine scissors and forceps to transect and remove three anterior ribs overlying the left lung lobe (Fig. 12.28.4).

    The left lung is chosen for imaging as it is one large lobe without fissures in the mouse. Extreme caution must be used to not touch the surface of the lung with surgical instruments or with the transected ribs.

14. Attach thoracic suction window (Fig. 12.28.4) to strut and use a micromanipulator to adjust height and horizontal plane. Use vacuum grease to seal a 12-mm glass coverslip onto the suction window.

    We have posted a Solid Works design of the thoracic suction window at http://pathology.ucsf.edu/BIDC/. Click on Biomicroscopy Wiki. Then, on the left-hand side under "Instruments," click on "Design Repository."

16. Apply 20 to 25 mmHg suction to the thoracic window and lower onto the left lung; the lung will then enter the thoracic window and be stabilized for imaging. Lower the two-photon microscope objective to just above the glass coverslip and apply PBS to form a meniscus between the suction window coverslip and the microscope objective.

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Use as little suction as necessary for the lung to enter the thoracic window. It is imperative that the suction window be absolutely horizontal to avoid tangential imaging (i.e., imaging through a tilted coverslip).

17. Locate the top (pleural surface) of the lung and use *z*-steps to scan to the deepest lung depth.

18. For two-photon microscopy, adjust laser power, wavelength output, and photomultiplier tubes to optimally excite and capture images for the chosen fluorophore.

INTRATRACHEAL AND INTRAVASCULAR INSTILLATIONS

For selected experiments using Basic Protocol 1 or 2, intratracheal or intravascular instillations may be desired. Intratracheal or intravascular instillations of cells, proteins, or labeling agents may facilitate experimental protocols. Here we describe basic methods and materials for these procedures.

Additional Materials (also see Basic Protocols 1 and 2)

> Dextran-conjugated dyes (Invitrogen; also see recipe)
> Fluorescent dyes or microbeads (Invitrogen; also see recipe)
>
> PE-10 tubing (Intramedic)
> 30-G needle (or the smallest you can find)
> Syringes (0.5 to 1 ml sizes)

1. For intratracheal instillations, direct, noninvasive, visualized instillations can be done as previously described (Su et al., 2004). Alternatively, once the tracheotomy tube has been inserted, instillations can be done through this tube using PE-10 tubing attached to a small syringe (0.5 to 1.0 ml) and inserted into the PE-90 tracheotomy tube.

2. Mice can be positioned by manipulating the microscope stage to selectively instill into the left lobe.

3. Instillation of small (1 μm) fluorescent microbeads is especially useful to delineate the alveolar spaces in the imaged lung.

4. For intravascular injections, a variety of entry sites can be utilized including the lateral tail vein, jugular vein, femoral vein, or retro-orbital venous plexus. Attach a small (e.g., 30-G) needle to a 0.5- or 1.0-ml syringe. Depending on the site of injection, blood can be aspirated to verify proper placement. Heparinized saline (4 U/ml) can be loaded into to the syringe and tubing to avoid blood clotting.

5. Dextran-conjugated dyes or fluorescent microbeads are useful to identify intravascular spaces.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A;** *for suppliers, see* **SUPPLIERS APPENDIX.**

Fluorescent dextran conjugates

> Invitrogen is an excellent source for fluorescent dextran conjugates in many different colors and sizes (*http://probes.invitrogen.com/media/pis/mp01800.pdf*). The dextran conjugates are reconstituted, aliquotted, and frozen at $-20°C$.

Fluorescent microspheres

Unmodified polystyrene FluoSpheres (1 μm, Invitrogen) can be used for blood flow experiments. The color should be chosen to fit into the scheme with other markers of interest. Beads are very bright so they should be tested to be sure they will not bleed into other channels.

Low melting point agarose, 2% in PBS

To make 20 ml of solution, weigh 0.4 g of agarose (SeaPlaque GTG Agarose, Lonza), add to 20 ml phosphate-buffered saline (PBS; APPENDIX 2A), and heat to dissolve.

Agarose will solidify at room temperature, so maintain in a 37°C water bath until used for intratracheal instillation.

COMMENTARY

Background Information

Lung slice imaging

Lung slices have been used to assess smooth muscle contraction (Dandurand et al., 1993). The technique has evolved to provide for optimal contractility and the analysis of calcium fluxes within the tissue (Bergner and Sanderson, 2002). These advances in physiology can also be applied to immunology. Live imaging has allowed the field of immunology to move from snapshots of the immune system to following the elaboration of an immune response. While FACS and fixed section staining are useful tools to understand what cells are present in the tissue, interactions between cells cannot be adequately assessed by these methods. Early work with lymph node explants demonstrated that in vivo imaging is not always necessary to see interactions of interest (Miller et al., 2002). Slice imaging allows the measurement of cell behaviors deep within the lung tissue that would not be accessible through any other method. Despite the advantage of imaging deep into the lung parenchyma, the ex vivo conditions result in loss of blood and lymph flow. Live imaging is required in cases where ingress and egress are necessary for the study; however, there are caveats in depth of imaging when lungs are filled with air due to the numerous air-water interfaces. Future studies using the slice method will help to define the complicated field of lung immunology. Data that identify which cells actively contribute to pathogenic immune responses in viral infections, asthma, acute lung injury, and other lung-related diseases can lead to improved and directed treatments.

Lung intravital imaging

Intravital microscopy is an ideal method when examining biological processes that can-

not be studied with static or end-point measurements. With intravital microscopy, we can peer into tissues and record the screenplay of the living host under basal and injury conditions. Intravital imaging has been successfully performed in larger animals, such as the dog, rabbit, and rat. Stabilization with a thoracic suction window has been applied to the canine pulmonary circulation (Wagner, 1969), and we have reported its use in the mouse lung (Looney et al., 2011).

Lung intravital microscopy is particularly suited to biological processes that are fast-moving. With the ability to record at video-rate (30 frames per sec), perfusion velocities of plasma and cells can be measured. We have used two-photon microscopy in the lung to measure the perfusion velocities of fluorescent microspheres, neutrophils, and naïve and activated lymphocytes (Looney et al., 2011). Transgenic mice expressing fluorescent proteins, or adoptive transfer of fluorescently-labeled cells, facilitate these measurements. Several transgenic strains can be bred together producing mice with multiple colors that can be imaged with two-photon microscopes that may have the capability to excite the fluorophores with more than one laser source. Combining fluorophores enables the visualization of spatial interactions between different cell types, such as immune cells and stromal cells in the lung (see Fig. 12.28.2).

Limitations of our method for lung intravital microscopy include the thoracic window itself and the inability to penetrate deep into the lung for imaging. The thoracic window must be surgically placed, and there is of course concern that the normal lung physiology will be perturbed. We have measured perfusion velocities in the lung subtended by the thoracic window, and our gentle immobilization does

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not alter normal perfusion or lead to abnormal immune cell recruitment (Looney et al., 2011). We have so far been unable to image deeper structures of the lung, such as larger blood vessels or airways. The air-filled alveoli scatter the two-photon laser, and beyond 125 μm the signal is impaired.

Alternative approaches to lung intravital imaging include using Vetbond to glue a coverslip to the lung surface (Kreisel et al., 2010), clamping of the ipsilateral bronchus to interrupt the motion from ventilation (Hasegawa et al., 2010), and coordinated acquisition of images by timing image capture with inspiration/expiration or by temporarily stopping ventilation altogether (Tabuchi et al., 2008). The ex vivo isolated and perfused lung preparation is yet another alternative that allows ventilation and perfusion and provides optimal image stabilization (Kuebler et al., 1999; Kiefmann et al., 2008).

Until intratracheal fiber-optic imaging in the mouse is optimized, intravital imaging can be combined with the lung slice method to image both subpleural alveolar structures and deeper into the lung parenchyma.

Critical Parameters and Troubleshooting

Microscope set-up

The multitude of biological questions that can be answered by live imaging requires a variety of different imaging set-ups to acquire the desired data. In order to test how a cell is circulating through the vasculature or extravasating into the tissue, video-rate imaging is required to capture the events of interest. In contrast, imaging interactions between tissue-resident cells may only require a timepoint every 30 sec. These considerations should be taken into account when choosing a microscope and again during acquisition.

The laser power that reaches the sample has the ability to easily cause injury to the lung tissue. It is therefore critical to have the appropriate settings in place before beginning an experiment. Several parameters can be adjusted to achieve optimal illumination without damage to the tissue. First, the amount of laser light that reaches the sample, the number of frames averaged, and photomultiplier tube (PMT) gain should all be optimized to allow for the best signal-to-noise ratio while keeping the laser power and dwell time to a minimum. In order to test the settings, a transgenic line that marks motile cells, such as CD2-RFP or c-fms-EGFP, should be tested. The cells of interest should

be motile throughout the imaging session, and the sample should not dim or bleach at all. Cell motility and maintenance of fluorescence are important indicators of the health of the tissue. If either is impaired, the settings should be reconfigured and tested again.

For two-photon imaging with water-dipping lenses, one should periodically assess the meniscus formed by PBS applied to the coverslip or medium in the imaging chamber. Both should be added or replaced as needed. Ambient light should be carefully screened from the microscope and PMTs. For in vivo imaging, monitoring of the mouse should be done in between timepoints and with as little intervention as possible.

Imaging reagents

Fluorescently tagged mouse stains and staining reagents require different excitation light for two-photon microscopy than for single-photon imaging. A general rule of thumb is that protein dyes such as GFP are excited with 910-nm light. Chemical dyes such as CFSE are generally excited with 810-nm light. A notable exception is mCherry, which is excited below 810 and above 1000 nm. Emission of fluorophores is the same in two-photon imaging as with single photon. When designing an experiment, it is important to know that all of the fluorophores of interest are excited by the same wavelength of light or to obtain a system with two lasers for simultaneous excitation with two wavelengths of light. Pilot experiments should be performed to verify that all fluorophores are excited as expected and to determine the bleed-over between all the channels.

Mouse surgery and anesthesia

Surgical preparation is the key to optimal lung imaging. Great care must be taken to avoid touching the surface of the lung during the thoracotomy and placement of the thoracic suction window. After the thoracic window is placed, it can be easily removed and replaced by turning off the suction. A rim of atelectasis may appear after the window is released, but a gentle recruitment maneuver performed by slightly pinching the expiratory limb of the ventilator circuit will resolve the atelectasis.

Depth of anesthesia is also a critical parameter. Mice must be deeply anesthetized with isoflurane during the imaging to obtain quality movies. This can be periodically assessed with a paw pinch, and the isoflurane flow rate can be adjusted. Neuromuscular blockade is a

potential option to avoid any motion artifact, but in our hands it is not needed.

When using mechanical ventilation in mice, it is easy to mistakenly deliver either inappropriately high or low tidal volumes. In either scenario, there are untoward physiologic and structural consequences. We cannot emphasize enough the proper calibration of delivered tidal volumes, which is best done using a 1-ml glass syringe attached to the Y-connector at the end of the ventilator tubing. Because of the dead space of the compressible tubing used in the experimental set-up, the tidal volume that is set on the ventilator may be very different than the tidal volume actually delivered to the animal. If needed, arterial blood gas sampling can be done to verify appropriate ventilation.

Lung and section viability

When imaging over an extended period of time, it is important to periodically assess the health of the tissue. One simple and fast way to assess the health of lung sections is to look for ciliary beating along the large airways. Only the first few branches are ciliated, but these airways continue beating throughout imaging. Cell motility can also act as a "canary in the coal mine," since motility is very sensitive to temperature, oxygen levels, and phototoxicity. If cells slow down or do not move during an imaging session, the imaging set-up should be checked for any of these three critical parameters. Placing lungs or lung sections on ice can also negatively affect the motility of some immune cells. Video 12.28.1 (*http://www.currentprotocols.com/protocol/cy1228*) shows appropriate motility of c-fms-EGFP cells in an actin-CFP lung.

In intravital lung imaging, each preparation should be tested for viability at the beginning of an experiment. Dextran leak and neutrophil accumulation can be used as measures of tissue damage. 70-kD dextran should not leak from a good surgical preparation. Neutrophils (injected i.v. or in a LysM-GFP mouse) should not accumulate during imaging and will make for a good control population to assess motility (see Video 12.28.2 at *http://www.currentprotocols.com/protocol/cy1228*).

Anticipated Results

Slice lung imaging can allow for imaging of cell-cell interactions within the lung over the course of several hours. Although blood and lymph flow are interrupted, comparisons between airways of different sizes within the same lobe can be made by imaging sequential sections. With antibody labeling, the number of detectors on the microscope is the only limit to how many cell surface markers can be identified.

With care and diligence, lung intravital microscopy can be successfully performed, but it takes the coordinated involvement of the following skills: anesthesia, thoracic surgery, and advanced microscopy and image acquisition. Mice can be imaged for several hours with proper anesthesia, warming to 37°C, and fluid replacement. We are currently optimizing an optional surgical approach using a custom, miniaturized thoracic window that can be inserted between ribs spread by a retractor. This option would allow for the ribs to be closed at the end of the experiment and for the mouse to be recovered, extubated (mouse could be orally intubated instead of the tracheotomy), and perhaps re-imaged at a later timepoint.

Time Considerations

Imaging lung slices. Preparation of lung tissue for imaging takes between 30 min and 1 hr. Sectioning an entire lobe can take as little as 15 min. As with all protocols, it takes repetition to achieve the optimal timing. Lung slices can be imaged for 8 hr without changes in immune cell behavior, but any imaging after that timepoint should be validated.

Intravital lung imaging. Set-up of ventilator, suction apparatus, and microscope should take approximately 20 min. Anesthesia, tracheotomy, thoracotomy, and placement of the thoracic suction window should take an additional 30 min. Image acquisition can proceed for several hours, depending on the experimental conditions.

Acknowledgements

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Key References

Looney et al., 2011. See above.

This reference describes in detail the methods needed for two-photon, intravital microscopy in the lung.