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Imaging precision cut lung slices to visualize leukocyte localization and trafficking

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Abstract

Pulmonary dendritic cells (DCs) are potent antigen-presenting cells that can activate both naïve and memory/effector T cells. However, very little is known of how movements and localization of DCs contribute to these events. To study this, we have developed new procedures that combine precision cut lung slices with cell staining using fluorescently-tagged antibodies to detect individual cell types. In this chapter, we describe these methods in detail and show how they can be used to study the localization of not only DCs, but also other leukocytes of interest, as well as structural cells within the lung.

Keywords

live cell imaging; precision cut lung slices; lung; dendritic cells; macrophages; antigen-presenting cells; localization; confocal microscopy; lymphatics; epithelial cells; airway epithelium

1. Introduction

Dendritic cells (DCs) span the interface between innate and adaptive immunity and have several unique features that distinguish them from other cell types. They acquire proteins from the extracellular environment, including the airway lumen and parenchymal tissue, and digest them into peptides that are presented on the cell surface in context of major histocompatibility complex (MHC) class II [1]. Pulmonary DCs, which are located within the airway epithelium, lung parenchyma, and alveolar spaces, acquire inhaled antigens and migrate to regional mediastinal lymph nodes (LNs) to activate CD4⁺ T cells, thereby initiating adaptive immune responses to those inhaled proteins [2]. Despite the well-established ability of DCs to migrate to regional LNs, not all DCs appear to do this. Indeed, many antigen-bearing DCs remain in the lung for several months after acquiring antigen [3,4]. It seems likely that these resident DCs can interact with memory/effector T cells that re-enter the lung from the circulation, but it is unclear whether the functions of resident DCs are similar or different than those of DCs that migrate to regional LNs.

Flow cytometry is a powerful technique for quantitative analysis of cell surface proteins on a variety of immune cell types, but it provides no information on the precise localization and migration patterns of these cells into, within, and out of the lung. Likewise, *in vitro* chemotaxis assays can assess the ability of cells to respond to chemotactic factors *in vitro*, but these assays do not reproduce the complex, three-dimensional environment of the intact lung. Here, we describe how precision-cut lung slices (PCLS) can be generated, stained with fluorescently-tagged antibodies, and visualized by confocal microscopy to study the location and movement of individual cell types within the lung. This procedure has multiple advantages, including retention of the three-dimensional architecture of the lung slices, the ability to visualize an area as large as a cross section of an entire lobe, as well as the opportunity to use video microscopy visualize cell movements and interactions in the lung slices at 37 °C. We have used this protocol to successfully visualize the localization of a wide variety of immune cell types including different types of dendritic cells, macrophages, neutrophils, T cell and B cells, as well as structural cells such as lymphatic, endothelial, and epithelial cells.

2. Materials

2.1 Lung Preparation

1. Sodium pentobarbital: 250 mg/kg body weight
2. Cork board or polystyrene foam base
3. 1 mL syringe
4. 1.5 inch, 20 gauge (G) needle
5. Polyethylene tubing (BD Diagnostic Systems): 0.86 mm inside diameter, 1.27 mm outside diameter
6. PBS: (Mg⁻ Ca⁻) pH 7.4; Ice-cold
7. 2 % agarose: 2 % low melting point agarose (GeneMate Sieve GQA); warmed to 40 °C
8. 40 °C water bath
9. Refrigerator or cold room (4 - 8 °C)

2.2 PCLS preparation

1. Compressstome™ VF-300 (Precisionary Instruments) or equivalent instrument with metal cooling block, plunger and metal syringe
2. Double edge stainless razor blade (Electron Microscopy Sciences)
3. Super glue (Krazy Glue): All-purpose, no-run gel
4. Forceps
5. Thin spatula or paintbrush
6. 24-well plate

2.3 PCLS staining

1. Phosphate buffered saline (PBS): (Mg^{2+} , Ca^{2+}) pH 7.2-7.4
2. Leibovitz-10 medium: Leibovitz's L-15 medium containing 10 % fetal bovine serum
3. Normal mouse serum
4. Normal rat serum
5. Fc blocker: anti-CD16/32 mAb (clone 2.4G2) 5 μ g/mL purified mAb or 10 % hybridoma culture supernatant
6. Antibody combinations designed to detect specific cell types [5]

Dendritic cell panel:

CD324/E-cadherin – Alexa Fluor 488 (AF488) (clone DECMA-1)

CD88/C5Ra1 – Phycoerythrin (PE) (clone 20/70)

CD11c/ α X integrin – Brilliant Violet 711 (BV711) (clone N418)

CD103/ α E integrin – Allophycocyanin (APC) (clone 2E7)

Structural cell panel:

CD90.2/Thy1.2 – eFluor 450 (eF450) (clone 53-2.1)

CD324/E-cadherin – AF488 (clone DECMA-1)

CD31/PECAM-1 – Alexa Fluor 647 (AF647) (clone MEC13.3)

Myeloid cell panel:

CD324/E-cadherin – Alexa Fluor 488 (AF488) (clone DECMA-1)

CD88/C5Ra1 – Phycoerythrin (PE) (clone 20/70)

CD11c/ α X integrin – Brilliant Violet 711 (BV711) (clone N418)

CD172a/Sirp1a – Allophycocyanin (APC) (clone P84)

7. Antibody cocktail for static imaging: 1 - 5 μ g/mL antibodies added to 1 mL PBS containing 5 % normal mouse serum, 5 % normal rat serum, and Fc blocker (5 μ g/mL mAb or 10 % supernatant)

Antibody cocktail for live cell imaging: 5 μ g/mL mAbs or 10 % supernatant of hybridoma added to 1 mL Leibovitz-10 containing 5 % normal mouse serum, 5 % normal rat serum, and Fc blocker (5 μ g/mL mAb or 10 % supernatant)

2.4 Confocal Microscopy of PCLS

1. 35-mm glass bottom microwell dishes (MatTek Corporation)
2. 15 mm coverslip, No. 1.5 glass thickness (MatTek Corporation)
3. ProLong Gold antifade mountant (ThermoFisher Scientific)

4. Confocal microscope: e.g. Zeiss 880 multi-photon laser-scanning microscope
5. Plan-Apochromat 20x/0.8 M27 objective lens (Carl Zeiss)
6. ZEN: Image processing software (Carl Zeiss)

2.5 Live cell imaging of PCLS

1. Cold room (4-8 °C)
2. Incubator, 37 °C
3. Leibovitz-10 Matrigel: Growth factor reduced, basement membrane matrix, Phenol Red-free, *LDEV-free (Corning)
4. Nunc Lab-Tek chambered coverglass (8 slots)
5. Bare platinum wire: wire bent into 5 - 8 mm long “L” shape

3. Methods

3.1 Lung preparation

1. Euthanize mouse by intraperitoneal injection of 5 mg sodium pentobarbital. Pin the animal to a polystyrene foam base.
2. Open the abdominal cavity and cut the inferior vena cava to drain blood away from lungs. Carefully puncture the diaphragm to allow the rib cage to expand.
3. Remove salivary glands and muscle to expose the upper trachea. Make a small incision on the anterior side of the thickest band of cartilage using fine forceps or scissors, being careful not to cut all the way through the trachea. The incision should be just large enough to allow a 20 G needle to pass through (Fig. 1).
4. Insert a 20 G needle into a section of the polyethylene tubing, leaving approximately 1 cm of tubing beyond the end of the needle. Cut the tubing at an angle of approximately 45° to bevel the end, and attach the needle to a 1 mL syringe. Load the syringe with 0.8 mL of warm (40 °C) 2% agarose, immediately place the end of the tubing into the incision in the trachea, and slowly inject the agarose into the lungs. The lungs should be seen inflating within the chest. Without moving the needle or syringe, tape the syringe to the polystyrene foam base to ensure the needle remains in the trachea (Fig. 1) [Note 1].
5. Place the mouse, with base and syringe still inserted in the trachea, at 4 °C for at least 10 min to allow the agarose to completely solidify. If necessary, the animal can be kept here for up to several hours.
6. Carefully excise the agarose-inflated lungs and place them in a 35-mm dish with ice cold PBS. Keep on ice.

¹After injection, the lungs will be larger and inflated within the chest. If only one lobe expands, the needle has been inserted too deeply (past the bronchus), and it will be necessary to pull it out slightly before proceeding. This part of the procedure needs to be done relatively quickly, before the agarose starts to solidify.

7. Any lobe of the lung can be used to make lung slices, but we prefer the right superior lobe. Ensure that the interior part of the lung (where it connects to trachea) faces down in the dish. The lobe used and its orientation on the plunger will determine the size of vessels and airways that will be visible. The orientation described here is ideal for visualization of large airways and parenchyma (Fig. 2A) [Note 2].

3.2 PCLS preparation

1. To prepare lung slices, use a Compressstome, metal cooling block, plunger and metal syringe, or similar, according to the manufacturer's instructions. Place a small drop of super glue onto the plunger and spread the glue in a circular motion (Fig. 2B) [Note 3].
2. Immediately after covering the plunger with super glue, gently grasp the excised lung lobe with forceps with the trachea side down, dab it on a Kimwipe paper to remove excess liquid, and carefully place it on top of the plunger. Trim off any extra tissue extending beyond the edge of the plunger (Fig. 2C).
3. Move the plunger down so that the sides of the metal syringe move up and over the tissue, creating a well with the lung glued at the bottom, no more than several centimeters deep. Tape around the bottom of the metal syringe to hold this position in place (Fig. 2D).
4. Carefully pour 2 % agarose (40 °C) into the well until the agarose just covers the top of the lobe.
5. Surround the metal syringe with the ice cold chilling block and cool the lobe and 2% agarose for 1-2 minutes. A shorter cooling time than this might cause disintegration of the agarose during slicing, which could lead to unevenly cut PCLS.
6. Load the specimen syringe into the tissue slicer and fill the buffer tank with ice cold PBS. Align the step motor drive with the specimen syringe and remove the piece of tape from the syringe. Turn the switch to the fast forward (FF) position until the step motor drive just touches the back of the plunger. Turn off the motor when the drive touches the plunger.
7. Align the fresh blade with the specimen syringe. Set the Compressstome settings to desired slice thickness (150 μ m, continuous cutting, oscillation: 9 and speed: 3-4).
8. Fill buffer tank with cold PBS, and turn on the power.

². Mice have one large left lobe and four smaller lobes on the right. For PCLS imaging experiments that involve introduction of allergens into the lungs by oropharyngeal or intranasal aspiration, we use the right superior lobe due to a consistent and dispersed exposure to inhaled reagents. However, other lobes also can be studied as long as the same lobe is used when comparing strains or treatments.

³. Super glue should not touch the sides of the metal syringe. If the super glue touches the sides of the syringe, it will glue the syringe and plunger together.

9. During slicing, PCLS separate from agarose and fall into the buffer tank. Using a thin spatula or paintbrush, collect the PCLS one at a time as they fall into the buffer tank and place them in a 24-well plate containing ice cold PBS. It is important to keep the slices in the order that they are cut to maintain consistency between experiments. Although individual tissue samples are different due to age, gender and weight, the 10th slice usually yields reproducibly sized airways.

3.3 PCLS staining

1. Design a panel of fluorescently tagged antibodies based on the cell types of interest, taking into account the potential for fluorescent spectral overlap and the detection capabilities of the microscope. Fluorescence spectral viewer tools such as FluoroFinder (<https://fluorofinder.com>) and BioLegend Fluorescence Spectra Analyzer (<http://www.biolegend.com>) are useful to design panels. The panel described here is useful to detect DCs.
2. Depending on the method, prepare the antibody cocktail for static imaging or antibody cocktail for live imaging with a desired final concentration, which needs to be optimized for each individual antibody (usually 1-5 µg/mL). Transfer the antibody cocktail solution to 35-mm dish, and keep on ice in the dark until ready to use.
3. Choose a PCLS having an anatomical area of interest, such as large airways or periphery of the lung, and place in 35 mm dish containing 1 mL antibody cocktail.
4. Stain in the dark, on ice, rocking slowly for 20 - 60 minutes. A shorter incubation time is recommended for live cell imaging.
5. Remove the antibody solution from the dish, and rinse the slices twice with 1 mL ice cold PBS for static PCLS imaging, or cold Leibovitz-10 for live cell imaging. Proceed to either step 3.4 for confocal microscopy of whole PCLS or step 3.5 for live cell video imaging.

3.4 Confocal microscopy of PCLS

1. Pipette 50 µL PBS onto a 35-mm round glass-bottom dish. Using a spatula or paintbrush, place the stained PCLS onto the drop, gently manipulate the slice until it is flat and spread out. Remove the PBS with a pipette (Fig. 3A and B). The slice should lie as flat as possible. The above procedures should be done quickly to avoid photobleaching
2. Place 1 drop of room temperature ProLong Gold antifade mounting medium onto the slice and gently place a round glass coverslip into the well of the plate (Fig. 3C).
3. When viewing under a confocal microscope using ZEN software, use the “tile” tool, or a similar setting, to locate and mark the edges of the tissue in the “convex hull” setting. This will help reduce the time of the tile scan.

4. While setting the z-stack range, verify at multiple areas of the slice, especially along the edges, that the set ranges are appropriate. The z-range will likely need to be greater than the thickness of the tissue itself because it is very difficult to get the tissue to lay completely flat. For example, with a 150 μm thick PCLS, the z-stack range will need to be set from 150-250 μm to accommodate curves in the slice. Depending on the z-stack range and the size of the tissue, each full lung scan will take between 7-12 hours. Each lung is unique and the settings must be adjusted for every experiment.

3.5 Live cell imaging of PCLS

1. Pipette 50 μL Leibovitz-10 into one slot of a Lab-Tek chambered coverglass. Use a spatula to place the PCLS onto the medium, gently manipulating it until the slice is flat and spread out. Remove the 50 μL medium using a pipette (Fig. 3A and B). It is acceptable if the edges of the slice are laying vertically against the wall of the chamber.
2. Using forceps, carefully drop “L” shaped platinum wire weights on top of the slice. We recommend using two weights that form a square with the four sides of the two “L” shaped wires to be used as a viewing window under the microscope (Fig. 4A and B)
3. In a cold room, mix 100 μL of Matrigel with 100 μL Leibovitz-10 in a microtube. Use pre-cooled pipette tips for this procedure, or the Matrigel will solidify in the tip. This ratio of Matrigel and medium (1:1) creates a gel dense enough to hold the PCLS down, and maintains cell viability.
4. In the cold room, gently pipette the Leibovitz-10 Matrigel to mix, and carefully pipette 100 μL on top of the PCLS, making sure that the gel goes on top of the lung slice, and that the slice does not float up on top of the gel. The gel should work as a weight, anchoring the PCLS down onto the bottom of the chamber (Fig. 4B and C).
5. Carefully transfer the chambered coverglass to a 37 °C incubator and let the Leibovitz-10 Matrigel solidify for ~10 minutes.
6. Transfer the entire chambered coverglass onto the pre-warmed stage (37 °C) of a confocal microscope. Sample should be maintained at 37 °C throughout imaging [Note 4].
7. Set the z-stack range and tile range based on the desired interval between frames. 4×4 tiles and 10 z-stacks will generally result in 1 frame/2 min. Ensure the auto-focus function is activated before starting the experiment to prevent x-y and z-drift.

⁴.CO₂ is not essential as long as Leibovitz's medium is used during imaging and incubation.

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Figure 1. Lung Inflation Preparation.

(A) A 1-mL syringe attached to 20-G needle with beveled polyethylene tubing slid onto the tip has been inserted into the trachea, agarose injected, and the syringe taped in place. (B) Higher magnification image of the hole cut on the anterior side of the thickest band of cartilage that is large enough to insert a 20-G needle. (C) Higher magnification image of fully inflated lungs in the chest cavity.

Figure 2. Orientation of Lung Attachment onto Plunger.

Diagram of the preferred orientation of the lung glued onto the plunger. (A, B) The lung, resting trachea side down in a dish, is dabbed dry, then placed gently onto the wet super glue on the plunger in the same orientation. (C) The lobe is trimmed as necessary when hanging over the edge of the plunger. (D) The plunger is moved down to create a space to add agarose. Four to six mm from the top of the tissue to the syringe top is optimal.

Figure 3. Flattening of lung slice for static imaging.

(A) PBS (50 μ L) is placed onto a 3-cm round glass-bottom dish. A lung slice is placed onto the drop using a spatula or paintbrush, and gently manipulated until it spreads out. (B) Remove the PBS with a pipette. The slice should not have any wrinkles or bumps. (C) Place 1 drop of mounting medium onto the slice and gently place a round glass coverslip into the well of the plate. The lung slice should be as flat as possible at this point.

Figure 4. Flattening of lung slice for live cell imaging.

(A) After placing a PCLS on a drop of Leibovitz-10 in a chambered coverglass, the medium is removed with pipette as depicted in Fig. 3A and 3B. Two L-shaped platinum wire weights are carefully placed on top of the slice to form a square imaging window. (B) Leibovitz-10 Matrigel is carefully pipetted directly on top of the lung slice. (C) Final PCLS set-up for live-cell imaging. The matrigel and wire weights are anchoring the PCLS down, and the slice should lie flat at this point. Once solidified, the matrigel holds PCLS down throughout imaging.