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METHODS USED TO STUDY RESPIRATORY VIRUS INFECTION

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Abstract

This unit describes protocols for infecting the mouse respiratory tract, and assaying virus replication and host response in the lung. Respiratory infections are the leading cause of acute illness worldwide, affecting mostly infants and children in developing countries. The purpose of this unit is to provide the readers with a basic strategy and protocols to study the pathogenesis and immunology of respiratory virus infection using the mouse as an animal model. The procedures include: (i) basic techniques for mouse infection, tissue sampling and preservation, (ii) determination of viral titers, isolation and analysis of lymphocytes and dendritic cells using flow-cytometry, and (iii) lung histology, immunohistochemistry and in situ hybridization.

Keywords

respiratory viruses; pathology; immunohistochemistry; immunology; respiratory syncytial virus; influenza; murine γ -herpesvirus 68; lung; lymphoid organs; T lymphocytes; dendritic cells

INTRODUCTION

The respiratory tract is remarkable for its extensive surface area (70 m² in adult humans) which is in continuous contact with the external environment. The lung samples approximately 10,000 L of air every day and is exposed to a vast array of foreign particles. As a consequence, the respiratory system is a major portal by which microorganisms enter the body. When studying infection and immunity in this tract, the structurally and functionally distinct compartments must be appreciated as each compartment has distinct populations of both immune and parenchymal cells. The nasopharynx, or uppermost airway, is lined by both respiratory and olfactory epithelium. The respiratory epithelium of the mouse nasopharynx is columnar to cuboidal with cilia, and scattered goblet cells. The tracheal lining is similar but, unlike the human trachea, has a high percentage (50–60%) of non-ciliated, secretory Clara cells. The right lung of the mouse is divided into 5 lobes; the left lung is not divided. The lower respiratory tract is comprised of the branching conducting airways, which extend from the trachea to the terminal bronchioles, which open into the alveolar ducts. The bronchi and bronchioles of the mouse are lined predominantly by Clara cells, which are relatively rare in the human airway (Sternberg, 1997). This altered cell distribution must be kept in mind as it will alter the pattern of infection with some viral respiratory pathogens. Goblet cells are not found in the uninfected mouse lung. The bulk of the lung volume is made up of alveoli where gas exchange occurs. The alveolar ducts and spaces are lined primarily by flat type I pneumocytes overlying capillaries. The rare, plump type II cells produce surfactant and are thought to be the source of new type I cells (Maronpot et al., 1999).

Because efficient gas exchange requires that the alveoli be relatively free of fluid and inflammatory cells, the respiratory mucosal immune system must maintain a fine balance between eliminating pathogens and inhibiting inflammatory pathology. This requires discriminating between innocuous airborne antigens and pathogen-associated antigens, and inducing tolerance or immunity, respectively. Surveillance is maintained by a layer of dendritic cells underlying the mucosa of the larger conducting airways (Vermaelen and Pauwels, 2005; Weslow-Schmidt et al., 2007), and alveolar macrophages present within the air spaces of the lung. Additional inflammatory cells are recruited by chemokines secreted by epithelial and immune cells when pathogens are detected. The mouse model is permissive for some but not all human respiratory pathogens, and among those viruses which will infect both mouse and man, disease in the mouse may not necessarily mirror disease in the human host. This is an important consideration when undertaking infection experiments to model human diseases in the murine host.

This unit describes protocols for infecting the mouse respiratory tract, and assaying virus replication and host response in the lung. The reader is reminded that all procedures involving viral pathogens and infected tissues must be carried out in a biosafety cabinet prior to fixation.

INTRANASAL INFECTION OF MICE (basic protocol 1)

Most intranasal infection protocols use relatively large volumes (30–100 μ l) of inoculum, resulting in intratracheal delivery of virus. In this section we will discuss intranasal inoculation using both small and large volumes. Choice of route will vary with the virus studied, and the interest of the investigator in upper versus lower airway infection.

Materials

Avertin anesthesia (See **REAGENTS** section)

1 ml syringes and needles (25G^{2/8} to 27G) (BD)

Viral inoculum diluted in PBS or HBSS (Cellgro) to a volume of 30–100 μ l/dose

Positive displacement pipet and tips (Rainin)

1. Restrain the mouse in dorsal recumbency by picking it up by the scruff of the neck between index and thumb. Hold the tail against your hand with your little finger.
2. Insert the needle in the caudal 2/3 of the right side of the abdomen, taking care to avoid internal organs. After the needle is inserted, draw back. If anything is aspirated, you have likely hit the viscera. Withdraw and get a new needle before trying again. Inject Avertin (200–250 μ l, 25 mg/kg), pausing briefly before withdrawing the needle so that liquid does not seep out.
3. Monitor anesthesia by testing the mouse toe pinch reflex.
4. When the mouse is fully anesthetized, inoculate the mouse by placing the pipet tip containing the inoculum at the opening of the nares. Slowly expel the virus preparation from the tip, checking that the mouse is inhaling (not swallowing) the solution. Inadequate anesthesia increases the probability that the mouse will sneeze or swallow the virus.

The use of a positive displacement pipette prevents aerosol contamination and isolates the virus sample from the pipette body. This precaution is extremely important if different virus mutants, strains or virus species are being used. You can change the titer of the inoculum by altering the

amount of stock virus used and/or by adjusting the volume of diluent accordingly. We normally use volumes between 30 and 100 μ l for mice 6 weeks of age or older, smaller volumes may be needed for younger animals.

Let the mouse recover for 5–10 minutes. It will have an increased respiratory rate for some minutes after intranasal inoculation. If a proper dose of anesthesia has been used, the mouse will regain consciousness and breathing will return to normal within 15 – 30 minutes.

INFECTION OF THE UPPERMOST AIRWAY (Alternate Protocol)

This is a modification of the above intranasal infection. In this method, an inoculum is delivered specifically to the upper airway by using a smaller volume and administering the inoculum over several minutes (Gitiban et al., 2005; Visweswaraiah et al., 2002). Materials required are listed above except that the viral inoculum will be prepared such that each dose is contained within a 20 μ l. volume. A timer is also required for this protocol.

1. Anesthetize mice with Avertin as above.
2. Place anesthetized animals on a flat surface, in a dorsal recumbent position with the nose facing you.
3. Administer 2 μ l of inoculum to each naris of a mouse at 0, 2, 7, 9, and 11 minutes.

DETERMINING VIRUS TITER (basic protocol 2)

The optimal method for determining virus titer will depend upon the virus you are using. In this section we outline generally applicable techniques for obtaining samples for testing, and examples of titrating methods used for two viruses commonly used in our laboratory, RSV and influenza A virus.

1) Obtaining lung tissue

Materials—2L nalgene beaker

Styrofoam insert (3–5 cm. in thickness, equal to the beaker in circumference)

Dry ice

Parafilm

70% ethanol or water

Dissecting scissors, forceps

Sterile plastic snap-cap tubes, preweighed, one for each sample

1. Assemble an asphyxiation chamber by placing dry ice at the bottom of the 2L beaker, and covering this with a Styrofoam insert constructed from packing materials. Cover the beaker with parafilm until enough gas is generated such that the parafilm begins to bulge outward. Place the mouse to be sacrificed onto the Styrofoam platform and recover the beaker. The mouse will lose consciousness within ~ 2 minutes.

This primitive CO₂ generation chamber is convenient as it can be sterilized after use, is portable, and can be placed within the biosafety cabinet.

2. Place the unresponsive animal in a supine position, wet the fur over the thorax with 70% ethanol, open the skin and remove the chest plate. The lungs, which are salmon pink in color, can be removed from this approach. If the trachea is to be collected, the neck as well as the chest must be opened. The trachea with its distinctive cartilaginous rings sits just below the esophagus which can be peeled off and discarded. To avoid contamination of samples, clean and disinfect your dissecting tools between animals. The chemical sterilization method outlined below can be used in the biosafety cabinet.

Lung tissue (and adjacent lymph nodes if desired) harvested in this manner can be used for many purposes, but must be processed or frozen quickly to maintain its integrity. For determination of virus titers or cytokine levels, lungs should be placed in a sterile, plastic snap-cap tube (without liquid) and frozen immediately on dry ice. These samples can later be transferred to a -80°C freezer for storage.

2) Tissue homogenization

Materials—Tissue homogenizer (E.g. PowerGen model 125, Fisher Scientific)

Decarbonated Dulbecco's Modified Eagle Medium (dcDMEM).

1. Reweigh the tared tubes containing your samples to determine the tissue mass, so that titers can be expressed in terms of pfu/gm of tissue.
2. Add carefully measured dcDMEM (approximately 5–10 ml/g tissue), and homogenize each sample. Sterilize the homogenizer between samples as directed below.
3. Centrifuge 5 min at $2000 \times g$ to clarify. Carefully remove the clear supernatant, avoiding pellet and flocculent material at the bottom of the tube, and any insoluble fatty material floating on top.

It is important that samples not undergo repeated freeze-thaw cycles before virus titers are determined. You may homogenize your samples the day they are collected, then freeze (-80°) for assay at a later time. Or, freeze the samples as they are being collected, preparing homogenates and then setting up the plaque assay the same day. Viruses differ with respect to their ability to tolerate freezing, some pathogens may lose as much as a log of infectivity with each freeze-thaw cycle.

3) Chemical sterilization between samples

It is important to assure that biologically active organisms and molecules be reliably eliminated from all surfaces that will contact subsequent samples. In processing any significant number of samples, sterilization by autoclaving is impractical. The procedure described below will clean the apparatus and destroy any trace residual biologically active protein, nucleic acid or lipid.

Materials—Sterile 50 ml tissue culture tubes

0.1% SDS, 0.006 M sodium hypochlorite (commercial chlorine bleach diluted 1:100).

0.1% SDS, 0.001% coomassie blue

70% Ethanol

PBS

Run the homogenizer with the following solutions, taking care that the liquid covers all surfaces that will potentially contact subsequent samples.

1. Run two cycles with the 0.1% SDS, 0.006 M sodium hypochlorite solution; making sure any visible tissue residue has been removed. (Note: for stainless steel homogenizers, prolonged contact with hypochlorite will be corrosive. However, at the specified dilution, followed immediately by the rest of the cleaning cycle, corrosion is not significant.)
2. Run one cycle with 0.1% SDS, 0.001% coomassie blue. The dye is included as a sensitive indicator for residual hypochlorite; if the dye is bleached, the cycle should be repeated.
3. Run two cycles with 70% ethanol to remove residual SDS. The dye serves as a visible proxy for the detergent; make sure all traces of dye have been eliminated.
4. Rinse with sterile PBS to remove any residual ethanol, which may damage the next sample. After the last sample, however, omit the PBS wash to avoid salt precipitation during storage of the apparatus.

4) Plaque assay

Any virus recovered from mouse respiratory tract will, presumably, replicate to some degree in mouse cells. Different viruses, however, vary widely in the degree to which they produce “plaques” (countable lesions) on a cell monolayer. RSV, for instance, normally produces no obvious cytopathology in cultured mouse cells, and has historically been assayed on monolayers of human HEp2 cells or monkey Vero cells.

This procedure is for RSV; specifics will vary for other viruses.

Materials—1% Methylcellulose in MEM

10× Minimal Eagle’s Medium (Invitrogen)

10× Earle’s Balanced Salt Solution (Sigma-Aldrich)

Overlay medium

HEBSA

Susceptible cell line

24 well plates

Fixative/stain

1. Prepare a $\sim 5 \times 10^5$ cells/ml suspension in DMEM + 10 mM HEPES + 5% Fetal Calf Serum.
2. Plate 0.4 ml into each well of a 24-well plate.
3. Allow the cells to adhere and spread at 37°, 5% CO₂, for 2–16 hours. (Monolayers should be >80% confluent; sensitivity to virus infection declines as the monolayer ages).
4. Serial dilutions of virus samples are conveniently carried out in a multiwell plate. Prefill the appropriate number of wells with 0.180 ml HEBSA. Dilute 0.020 ml

homogenate or BAL into the first well; mix well. Transfer 0.020 ml from that well into the next well, etc.

It is important that the dilution series be done as accurately as possible, since systematic errors will be amplified exponentially by the process of serial dilution. For this reason, care must be taken to avoid bubbles, and pipet tips changed between transfers. Care should be taken that the pipet tips should not carry any extra liquid as drops clinging outside of the pipet tip. HEBSA should be at room temperature, since a temperature differential between pipet tip and medium introduces a systematic inaccuracy. A multichannel pipettor can be used, but the above listed cautions must be observed with respect to each of the pipet tips at each step!

5. Aspirate* the medium from each well, and replace it with serially diluted lung homogenate or BAL fluid diluted in HEBSA, 0.100 ml/well.
6. Incubate the plate at 37° for two hours in a humidified incubator, keeping the liquid distributed over the monolayer* by periodic (at least every 15 minutes) shaking of the plate.
7. Aspirate* the inoculum and replace it with 0.4 ml/well of the overlay solution.
8. Incubate at 37°, 5% CO₂ for 5 days.
9. Aspirate* medium; replace with fix/stain (~0.5 ml/well); after 30 minutes, room temperature, rinse the plate briefly in cold tap water.
10. Count plaques with a dissecting microscope.

*In all these steps, it is important to prevent the monolayers from drying out. Aspirate with a minimum of suction; avoid exposing the cells to rushing air; remove/replace medium from no more than a few wells at a time.

Calculations—The concentration of infectivity (commonly, but not quite correctly, referred to as “titer”) is estimated as:

(Number of plaques/well / Volume of inoculum/well) × Dilution.

E.g., the well inoculated from the 4th well in the dilution series above shows 35 plaques.

Inoculum volume = 0.100 ml

Dilution = 10⁴

Concentration of infectious virus = (35/0.100)×10⁴ = 3.5 × 10⁶.

Estimation of uncertainty—The standard error is estimated as the ratio as the square root of the total number of plaques counted. The relative standard error (i.e. expressed as a fraction of the concentration) is therefore the inverse of the square root of the number of plaques counted. In the above example, the standard error is (35)^{-1/2} = 16.9% If duplicate wells had been inoculated, yielding 34 and 36 plaques, for example, the estimated concentration would be the same, but the error would be reduced to (70)^{-1/2} = 12.0%

5) Fluorescent focus assay

Many viruses (e.g. influenza virus, parainfluenza virus 5, Newcastle disease virus) do not produce visible plaques. Estimation of the infectivity of such viruses can be done by following the procedure given above, but staining the infected monolayers with fluorescent or enzymatically tagged antibodies. The procedure for immunofluorescent detection is outlined below. The procedure for enzyme-linked antibodies is similar, but details vary with the specific enzyme used.

Materials—Inverted fluorescent microscope

Primary antibody against viral antigen (primary antibody, e.g. rabbit anti-influenza)

Fluorophore-tagged secondary antibody (e.g. FITC-goat anti-rabbit IgG)

PBSTA

1. After the infection has been allowed to progress (often 24 hours will suffice for fluorescent staining, since infection of a single cell is detectable, eliminating the need to allow time for spread to adjacent cells), fix the monolayer as for plaque assay (above), but without dye.
2. After fixation (30 minutes, room temperature) wells are washed twice with PBSTA. The detergent in this solution permeabilizes the cells, exposing intracellular antigens to recognition by antibody.
3. Dilute primary antibody against in PBSTA. (Optimal dilutions are determined empirically, as the dilution giving an optimal foreground/background ratio; typically in the range of 200 – 2000-fold). Add a sufficient volume of diluted antibody to cover the monolayer, ~0.15 ml/well of a 24-well plate).
4. Remove the primary antibody, wash wells twice with PBSTA.
5. Add the secondary antibody tagged with fluorophore, again diluted in PBSTA, and incubate 1 hour at room temperature. Exposure to light should be minimized for fluorescently tagged antibodies.
6. Remove the secondary antibody, and replace it with PBS. Fluorescent foci are counted using the fluorescent microscope. Concentration of infectivity is estimated as in the plaque assay, but reported as “fluorescent focus forming units/ml” (ffu/ml).

LEUKOCYTE ISOLATION (basic protocol 3)

This section outlines the procedures for sampling and processing mouse tissues to obtain single cell suspensions that can be used for flow cytometry or other assays (ELISpots, migration assays, proliferation assays, and purification of cell subsets). The method used to process the organs differs depending on the cell population of interest. For the analysis of T cell lymphocytes the authors use mechanical digestion which is quick and convenient. For the analysis of dendritic cells, collagenase digestion is recommended. This procedure is necessary to separate dendritic cells from the extracellular matrix, and is followed by a short incubation in an EDTA solution, which helps to disrupt multicellular complexes.

Materials

Avertin solution

Assorted syringes and needles (BD)

Forceps, scissors

Terumo Surflo i.v. catheter $186 \times 1^{1/4}$ "

70% ethanol

15 ml and 50 ml centrifuge tubes (BD)

Serological pipettes (Costar)

Pipet-Aid (Drummond Scientific)

Tissue culture dishes (Falcon)

70 μ m cell strainers (BD Falcon)

HBSS no $MgCl_2$, $MgSO_4$, or $CaCl_2$ (Cellgro)

Gey's solution

Centrifuge (such as Sorvall Legend RT)

Hemocytometer (Hausser Scientific)

Trypan blue (MP Biomedicals)

Microscope (such as Zeiss Axiostar plus)

Mouse dissection

1. Anesthetize the mouse.
2. Wet the animal down using 70% ethanol. Place the mouse in dorsal recumbency on 2–3 layers of paper towels.
3. Bleed the mouse.

You can draw blood from the heart by sticking the needle of a 1 ml syringe into the apex of the left ventricle or by axillary bleed using a Pasteur pipet. Transfer the blood into a tube containing 5 ml HBSS/heparin.

4. Bronchoalveolar lavage (BAL).

With your forceps, hold of the skin of the neck and cut along the neck from the chin to the thorax. Expose the trachea and make a small incision using sharp scissors or a needle with a beveled edge. Insert only the plastic sleeve of a catheter in the trachea, and using a 1 ml syringe flush lungs 3 times with 1 ml HBSS. Repeat twice more using a total of 3 ml HBSS. Work slowly to avoid collapsing the lungs. As a general rule, you will pool the lavage from the mice that belong to the same experimental group due to the limited number of lymphocytes found in the airways. If the BAL specimen is to be used for cytokine assays, use only 1 ml of lavage fluid to maximize the concentration of mediators present.

5. Harvest lung and draining lymph nodes.

Cut the skin of the mouse from the abdomen to the top of the thorax. Open the abdominal wall below the ribcage. Lift the sternum with tweezers and cut the diaphragm. Then cut away the lower part of the ribcage to expose the heart and lungs. Using fine tweezers harvest the lymph nodes. The mediastinal lymph nodes are located ventral to the trachea at the level of the thymus. The bronchial lymph nodes are situated at the bifurcation of the trachea. Next, harvest the lung. Place each organ in a labeled tube containing 5 ml HBSS.

6. Harvest spleen, which lies adjacent to the greater curvature of the stomach. Place it in a labeled tube containing 5 ml HBSS.

Obtaining a single cell suspension

7. Process the tissues.

If you are interested in analyzing lymphocytes a simple mechanical disruption of the tissues is enough. The spleen, lymph nodes and lung are gently pressed through a cell strainer mesh with a syringe plunger to achieve single cell suspensions. The strainers are repeatedly rinsed with HBSS to recover as many lymphocytes as possible. If you are interested in analyzing dendritic cells you will need to perform an enzymatic digestion of the organs. We mince the organs in a solution of 5 mg/ml collagenase A in MEM in a small dish and incubate them for 30 min at 37°C (see support protocol). The BAL samples do not need processing. All the samples are transferred to 15 ml conical tube if they are not already in one.

8. Centrifuge all single cell suspensions at 375 g for 10 minutes at 8°C.
9. The supernatant is then removed from the cell pellet via aspiration. The cell pellets are flicked to break up cell clumps.

Erythrocyte lysis

10. Add 3 ml of Gey's solution (see recipe) to the spleen and lung cells, 1 ml to the lymph nodes and BAL samples and 10 ml to the peripheral blood cells. Mix gently.
11. Incubate at 37°C for 3–5 minutes in a water bath.
12. Top off tubes with cold HBSS to achieve a final volume of 10. Centrifuge at 375 g for 10 minutes at 8°C.
13. Discard the supernatants, disrupt the cell pellets, and resuspend cells in HBSS.

We use 5 ml for spleen and 1 ml lymph nodes and BAL. Lung cells need to be further purified using a gradient. Thus, lung cells are resuspended in 1.0 ml. of 80% Percoll (see support protocol 1).

Count cells

14. While the lung cells are spinning count cells in the rest of the samples using a hemacytometer and trypan blue exclusion or using an automatic cell counter. Count lung cells when the gradient is done.

Samples with low cell numbers such as BAL and lymph nodes have to be counted using the hemacytometer. This is a tedious and time consuming process, but it is essential in order to calculate absolute

numbers of cells in each tissue using the frequencies obtained after flow cytometry analysis.

PERCOLL GRADIENT FOR LUNG TISSUE (support protocol 2)

Percoll solutions cannot be filtered, so be extra careful and keep them sterile. We use the same gradient to process liver cells.

Materials

(in addition to basic protocol 3)

Percoll (GE Health Care)

10× HBSS no MgCl_2 , MgSO_4 , or CaCl_2 (Cellgro)

HBSS no MgCl_2 , MgSO_4 , or CaCl_2 (Cellgro)

1. Let percoll solutions warm up at room temperature.
2. Resuspend the lungs cells in 2 ml 80% isotonic percoll. Vortex 3 sec.
3. Carefully layer 2 ml of 40% isotonic percoll on top and centrifuge at 600 g for 25 min at 20°C.
4. Transfer the cells from the 80%-40% percoll interface using a Pasteur pipet and place into a 50 ml conical tube filled with HBSS.
5. Centrifuge all single cell suspensions at 375 g for 10 minutes at 8°C.
6. Resuspend in 1 ml HBSS and count cells.

COLLAGENASE DIGESTION (support protocol 3)

Materials

(in addition to basic protocol 3)

Collagenase A solution

HBSS/2 mM EDTA solution

Razor blade

1. Place organ (spleen, lung) in a small tissue culture dish with 5 ml collagenase solution.
2. Inject the organs with collagenase using a 1 ml syringe.
3. Mince the organs with a razor blade.
4. Pass the tissue through a 3 ml syringe to break it up.
5. Incubate the tissues for 30 min at 37°C in a tissue culture incubator. Pass the tissues through the syringe every 5–10 min.
6. Transfer the cells to a 50-ml tube through a cell strainer. Use HBSS and a cell scraper to recover cells attached to the plastic.
7. Centrifuge at 375 g for 10 minutes at 8°C.
8. Resuspend the pellets in 10 ml HBSS/2mM EDTA.
9. Incubate 10 min at room temperature.

10. Centrifuge at 1200 rpm for 10 minutes at 8°C.
11. Resuspend pellet in HBSS or percoll solution as needed and follow the tissue processing protocols.

If you need to digest lymph nodes, transfer the organs to a 5 ml to a FACS tube with 200 μ l collagenase solution, smash it up with a 1 ml syringe plunger, and incubate as indicated above.

FLOW CYTOMETRY STAINING AND ANALYSIS OF ANTIGEN-SPECIFIC CD8 T CELL LYMPHOCYTES IN LUNG (basic protocol 4)

This section describes a basic protocol for the staining of tissue lymphocytes to analyze antigen-specific CD8 T cells using flow cytometry. CD8 T cell responses are key for the control of viral infections (Doherty and Christensen, 2000; Wong and Pamer, 2003). The T cell receptor (TCR) on the surface of CD8 T cells recognizes peptides derived from viral antigens complexed with major histocompatibility complex (MHC) class I molecules on the surface of infected cells. CD8 T cells express a broad range of effector mechanisms that mediate resistance to infection: (i) direct cytotoxicity of target cells mediated by perforin, granzymes and Fas, (ii) secretion of cytokines such as TNF α and IFN- γ and (iii) secretion of chemokines that attract inflammatory cells at sites of infection. A prominent characteristic of respiratory virus infections, such as those mediated by influenza or respiratory syncytial virus, is that viral replication is limited to the lung epithelium. Thus, for viral clearance, the immune system must specifically target the mucosal surfaces of the respiratory tract. To accomplish this, antigen presenting cells migrate to the draining lymph nodes where they initiate a program of T cell proliferation and differentiation (Legge and Braciale, 2003; Vermaelen et al., 2001). Newly generated effector T cells return to the respiratory tract, complete their differentiation process, and terminate the infection (Cerwenka et al., 1999; Lawrence and Braciale, 2004). The effector CD8 T cells and memory cells that remain in the lung once infection is cleared are functionally different from their counterparts in lymphoid tissues (Hikono et al., 2006).

Fluorescence-activated flow cytometry is one of the most broadly used technologies in cell biology, immunology, hematology and oncology. It allows the simultaneous and rapid analysis of multiple parameters of each cell in a suspension. This technology provides the means to define and analyze specialized subpopulations of cells that may carry out distinct functions. In addition, the availability of MHC class I-tetrameric reagents (Altman et al., 1996) which can label epitope-specific CD8 T cells allows us to analyze antigen-specific T cells at the single cell level. Immunologists now routinely use “tetramers” to track and analyze T cell responses with a level of detail that was unimaginable 15-years ago.

Materials

Antibodies: Fc-Block (anti-mouse CD16/CD32 Fc II/III), anti-CD8 α (FITC, Alexa 700, APC), anti-KLRG1 FITC (eBiosciences or BD Biosciences)

Tetrameric reagent: γ HV68 ORF_{6487–495} /K^b APC (NIH Tetramer Core Laboratory)

Staining Wash Buffer (SWB)

10% paraformaldehyde

1.1 ml microtubes (National Scientific Supply Co.)

1. Add 10⁶ cells/well in a 96-well, round bottom plate from your single cell suspension derived from lung, spleen, or lymph node. Set up as many single-color

control wells as fluorochromes in your staining panel, and also a negative control well (use spleen cells for the control wells). For the authors staining panel, you will need four control wells.

2. Centrifuge the plate at 450 g for 3 min. Flick and gently vortex the plate.
3. Add 50 μ l Fc-block diluted 1:200 in SWB to each well and incubate 10 min on ice.
4. Add 150 μ l of SWB to each well.
5. Centrifuge the plate at 450 g for 3 min. Flick and gently vortex the plate.
6. Add 50 μ l/well of the appropriate dilution of the tetramer which has been diluted in SWB. Mix by pipetting up and down (never vortex a tetramer).
7. Incubate for 1 h at room temperature in the dark.
8. Add 150 μ l/well of SWB to each well.
9. Centrifuge the plate at 450 g for 3 min. Flick and gently vortex the plate.
10. Add 50 μ l/well of the appropriate dilution of antibodies (anti-CD8 α Alexa 700, anti-KLRG1 FITC) in SWB to the experimental samples. Add control antibodies:

Negative control- SWB

FITC control - anti-CD8 α FITC

APC control - anti-CD8 α APC

Alexa 700 - anti-CD8 α Alexa 700

11. Incubate 20 min on ice in the dark.
12. Add 150 μ l/well of SWB to each well.
13. Centrifuge the plate at 450 g for 3 min. Flick and gently vortex the plate.
14. Add 200 μ l/well of SWB to each well.
15. Centrifuge the plate at 450 g for 3 min.
16. While the plate is spinning, add 20 μ l 10% paraformaldehyde to the appropriate number of microtubes.
17. Flick plate and vortex gently.
18. Add 180 μ l/well of SWB to each well, pipet up and down to resuspend the cells and transfer them into microtubes containing 20 μ l paraformaldehyde solution.
19. Keep the tubes on ice or at 4°C and in the dark until running on a flow cytometer.
20. Analyze data on FlowJo (TreeStar)

The flow cytometry analysis on Figure 1 shows antigen-specific CD8 T cells gated using the tetrameric reagent ORF6₄₈₇₋₄₉₅/K^b, a murine γ -herpesvirus 68 (γ HV68) peptide recognized by CD8 T cells when complexed with the MHC Class I allele K^b. This gate is used to plot histograms showing the expression profile of KLRG1 (killer lectin receptor G1). KLRG1 is a killer inhibitory receptor that is used to define terminally differentiated short-lived effector T cells (SLECs). As observed, the distribution of SLECs is different in lung and spleen: the majority of SLECs concentrate in spleen because by day 14 after γ HV68 infection acute respiratory infection has been cleared and the virus infection is at the peak of latency phase establishment in the spleen in B cells, macrophages and dendritic cells (Flaño et al., 2000).

FLOW CYTOMETRY STAINING AND ANALYSIS OF DENDRITIC CELLS IN LUNG (support protocol 4)

Dendritic cells are professional antigen presenting cells essential for the generation of adaptive immune responses. Dendritic cells develop from bone marrow-derived precursors and are recruited from the blood circulation to peripheral organs, where they continuously sample their environment for foreign substances. Dendritic cells are found through the airways forming a network through the epithelium and also constitute a small fraction of the cells in the BAL (Vermaelen and Pauwels, 2005). They are able to take up and process antigens and migrate to the draining lymph nodes where they contribute to the initiation of adaptive immune responses. Pulmonary dendritic cells are key regulators of the local immune response to airborne pathogens, antigens and allergens (Holt et al., 2008).

In mice, the dendritic cells in the respiratory tract express high levels of MHC class II and CD11c (Wikstrom and Stumbles, 2007). They can be distinguished from the lung macrophages because dendritic cells are less autofluorescent and express higher levels of class II molecules on their cell surface (Jakubzick et al., 2006). As their secondary lymphoid organ counterparts, respiratory dendritic cells can be divided into two main subsets: conventional dendritic cells (CD11c⁺B220⁻) and a very small fraction of plasmacytoid dendritic cells (CD11c⁺B220⁺) (Weslow-Schmidt et al., 2007). Conventional respiratory dendritic cells can be further divided into two subsets on the basis of CD11b expression, with the predominant subset expressing high levels of CD11b. None of these subsets has been characterized extensively, but there is some evidence to suggest that they are functionally and phenotypically distinct (Belz et al., 2004; Julia et al., 2002; von Garnier et al., 2005). Other dendritic cell markers such as CD103, CD205 or CD8 α can also be used to further define these populations.

Figure 2 presents a comparison between dendritic cells from spleen and lung stained with the same panel of fluorochrome-conjugated antibodies. The staining procedure is performed as described in the basic protocol 4 eliminating the steps 6 to 9 that are specific for the tetramer staining. As dendritic cells are a minor fraction of the total cells, the authors staining panel uses a dump channel with a mixture of antibodies (CD3, CD19 and NK1.1) to gate out lineage-positive cells (T cells, B cells and natural killer cells, respectively). The level of class II expression helps to differentiate dendritic cells from pulmonary macrophages. Then, conventional dendritic cells (CD11c⁺) are plotted against plasmacytoid dendritic cells (B220) and further analyzed using expression of CD11b versus CD8 α . CD11b expression defines a subpopulation formerly known as “myeloid” dendritic cells and CD8 α is expressed on “lymphoid” dendritic cells. The distribution of the subpopulations of dendritic cells is different between lung and spleen.

Respiratory dendritic cell staining antibody panel:

- MHC class-II-FITC
- CD3-PE, CD19-PE, NK1.1-PE
- CD8 α -PE-Cy5
- CD11c-APC
- B220-APC-Cy7
- CD11b-Pacific Blue

HISTOLOGY (basic protocol 5)

Examination of infected tissues under the microscope allows the investigator to assess many aspects of inflammation and disease that cannot be discerned from tissue homogenates. Standard hematoxylin and eosin (H&E) stained sections provide information about trafficking and localization of inflammatory cells, as well as the extent of necrosis/apoptosis.

Materials

2L nalgene beaker

Styrofoam insert (3–5 cm. in thickness, equal to the beaker in circumference)

Dry ice

Parafilm

70% ethanol or water

Dissecting scissors, forceps

10% buffered formalin (volume $\geq$ than 10x the volume of harvested tissues) in a closed container (Fisher Scientific)

5 ml. syringe and needles (25G^{2/8} to 27G) (BD)

Tissue cassettes (Fisher Scientific)

No. 2 pencil

1. Open the thorax and remove the lungs as described above. Good lung histology requires that the lung tissue be inflated with formalin at the time of fixation. This can be done using the syringe and small gauge needle. Inject the lung tissue with formalin (gently!) until it is filled, but not taut, and place the inflated lungs or lung lobes into appropriately labeled tissue cassettes. Be sure to label the cassettes with a pencil as many inks or “permanent” markers will wash off during processing.
2. Fix the tissue for at least 4, but not more than 72, hours before it is processed by the histology lab. The processor usually runs overnight, and the next day the histotechnologist will embed the tissue contained in each cassette within a paraffin block. If the tissue orientation is not correct, the block can be melted and the tissue re-embedded at a later time.

Extended formalin fixation will inhibit binding of both antibodies and nucleic acid probes to tissue sections. Once fixed tissue is processed and embedded in paraffin, it is stable for many years at room temperature.

3. H&E stained paraffin sections can be ordered from the histology lab. Additional sections for IHC or ISH can also be requested at the same time, but specify the purpose of these additional sections so that the appropriate glass slides can be used. Slides for IHC or ISH are coated with a polyanion such as poly-L-lysine to increase tissue adherence.

It is best to rely on the histology laboratory for the standard H&E stain as it requires many solutions that must be renewed often.,

An example of an H&E stained lung section is shown in the left-hand panel of Figure 3 and demonstrates the anatomy of the lung infected 24 hours earlier with influenza A virus. At

this low magnification (photomicrograph is taken through a 5× objective) the anatomy of the lung can be clearly seen with large airways branching into smaller airways, and finally opening into the alveolar ducts and space. Very little inflammation can be appreciated at this time point.

BAL CYTOLOGY (basic protocol 6)

You may wish to characterize the different types of inflammatory cells within the airspaces of the lung, and this can be done by using a portion of the BAL fluid, obtained as described above, to make cytopsins.

Cytocentrifuge (Thermo Scientific)

Cytoslides (Thermo Scientific)

Disposable cytofunnels (Thermo Scientific)

TBS

Acetone (Fisher Scientific)

1. Count the leukocytes within the BAL using a hemacytometer taking care that no more than $\sim 10^4$ cells are pelleted onto a single slide. Make sure you have a sample volume of at least 150 μ l before loading each sample. Interpretable data can also be obtained with many fewer than 10^4 cells as only the edges are readable when the cell pellet is thick.
2. Disposable cytofunnels are available from Thermo Scientific. Cytoslides with a raised circle to contain the pelleted cells are attached to the slides following the manufacturer's instructions, and the samples are loaded into individual funnels before they are inserted into the machine. Samples are spun for 6 minutes at 1000 rpm (113 g).
3. If you wish to do a differential cell count to determine the make up of your specimen (that is, % lymphocytes, % eosinophils, % monocytes/macrophages), allow the slides to air dry for several hours and request a Wright-Giemsa stain from the pathology laboratory. This stain is done routinely to examine blood smears in a hospital setting and it is probably best to make use of their expertise given your limited sample size.
4. If you wish to use your slide for IHC, fix in cold acetone for 5 minutes, rinse in TBS for 5 minutes, and continue on to step 3 in the IHC protocol.

IMMUNOHISTOCHEMISTRY (basic protocol 7)

Immunohistochemistry relies upon the availability of specific antibodies which can bind with high affinity to antigens in tissue sections. Basically, this technique involves incubating tissues with a primary, antigen-specific antibody, followed by a conjugated secondary antibody that will bind to the Fc region of the primary antibody. For example, when mouse IgG is used to stain human tissue, a biotinylated secondary antibody recognizing mouse IgG can be used to detect the bound primary antibody. Avidin bound to horseradish peroxidase will specifically bind to biotin, and when chromogen is added it will be deposited at sites where primary antibody is bound.

Materials

4 μ M tissue sections on Plus slides (Surgipath)

65°C oven

Coplin jars (Fisher Scientific)

Xylene

100% ethanol

100% ethanol

3% H₂O₂ (McKesson)

Humidified chamber

Deionized water

TBS

Super sensitive wash buffer (Biogenex)

Superblock (Scytek)

Primary antibody (determined by investigator)

Primary Antibody Dilution Buffer (Biomedica/Fisher Scientific) or use TBS with 1% BSA (Fraction V, Fisher Scientific) (w/v) as an antibody dilution buffer

Pap pen (Biocare Medical)

Biotinylated Secondary Antibody (determined by investigator)

Streptavidin-enzyme and substrate kits (Scytek or Dako)

Crystal/Mount (Biomedica)

Mayer's Hematoxylin (Dako)

0.25% ammonium hydroxide

Permaslip (Alban Scientific)

Coverslips

1. Slides prepared by the histology lab are incubated at 65°C for an hour prior to staining to improve tissue adherence.
2. Sections from conventional paraffin blocks must be deparaffinized and rehydrated before staining. This requires setting up (in a chemical hood) a series of baths in coplin jars, and incubating the slides as follows: two times for 3 minutes in xylene; once for 5 minutes in xylene; three times for 2 minutes in 100% ethanol; once for 1 minute in 95% ethanol. Finally, rinse in deionized water.
3. Place slides in 3% H₂O₂ for 15 minutes to reduce background staining, and then rinse with deionized H₂O for 2 minutes.
4. Circle tissue section to be stained with the pap pen so that the staining reactions can be carried out in a small volume, within the circle, while slides sit within a humidified chamber. Such chambers can be purchased, or made from a Tupperware

container holding moist paper towels and spacer to hold the slides away from the wet surface. The slides are incubated for 5 minutes at room temperature with Superblock, and then rinsed with TBS.

5. The appropriate dilution of each primary antibody must be determined empirically and may vary from 1:50 to 1:5000. Tissue sections are incubated with the primary antibody solution for 2 hours at room temperature or overnight at 4° C.
6. Wash 10 minutes in TBS, and apply the biotinylated secondary antibody, incubating 10–20 min at room temperature. Following this incubation, wash a second time with TBS.
7. Staining involves additional incubations, first with Streptavidin-enzyme reagents, followed by substrate. Kits are available from a number of companies; we generally use the Streptavidin/ HRP system from Scytek, and follow the manufacturer's directions.
8. Rinse slides with TBS, then water. Counterstain in hematoxylin (15 seconds to 1 minute, depending on the batch of stain) followed by bluing (just a few dips) in 0.25% ammonium hydroxide. Wash with deionized water.
9. Once the slide is dry, cover the tissue with Crystal/Mount, incubating at 65°C until dry. Use Permaslip to attach the cover slip.

This protocol is the most flexible, allowing you to use many different sources of primary and secondary antibody. Nonetheless, a more sensitive methodology is coming into use where, instead of a biotinylated secondary antibody and enzyme-conjugated avidin, an enzyme-linked polymer conjugated to a secondary antibody is used. The EnVision reagents from DAKO make use of this system, and although compatible reagents are not yet available for all primary antibodies, enhanced foreground and decreased background are routinely obtained with this technique.

MAXIMIZING SIGNAL (support protocol 7)

Here we include a number of approaches to increasing the signal obtained with a particular primary antibody. As reagents are often limited when working with rodent models, it is often necessary to optimize results obtained with the antibodies available. For a detailed discussion of options available it is best to consult a manual devoted to the subject (Renshaw, 2007)

1) Antigen Retrieval

If a primary antibody gives a poor signal using the standard IHC protocol outlined above, “antigen retrieval” using a pressure cooker to heat the tissue sections may increase the foreground.

Materials—Coplin jars (Fisher Scientific)

0.5 M Tris at pH 10

Pressure cooker (any)

1. After the sections are deparaffinized, as in steps 1 and 2 above, place the slides in a coplin jar filled with 0.5 M Tris at pH 10, and place the jar in a pressure cooker with sufficient boiling water to heat, but not leak into, the container. Heat for 3 – 15 minutes at a pressure of 15–17 psi.

2. Cool the pressure cooker by running under cool tap water for 10 minutes. Remove the coplin jar and allow the contents to cool for 30 minutes at room temperature.
3. Wash the slides in TBS, then water, and return to step 3 of the IHC protocol.

These conditions work for many antigens, but some will require less heat or more acidic conditions for optimal unmasking. Optimal conditions for a particular antigen must be worked out empirically.

2) IHC Using Frozen Sections

Sometimes it is possible to use monoclonal antibodies to stain frozen tissues even when they are unable to detect antigen in fixed tissue sections.

Materials—5 ml syringe (BD)

Terumo Surflo i.v. catheter 186 × 1^{1/4}"

Plus Slides (Surgipath)

Tissue Tek Cryo-OCT embedding compound (Fisher Scientific)

Coplin jar (Fisher Scientific)

Acetone (Fisher Scientific)

Cryostat

Cryostat "chuck" (obtained from cryostat manufacturer)

TBS

1. When harvesting lung tissue for this purpose, it is still necessary to inflate the lung tissue as much as possible, but this must be done using a water soluble embedding compound (Cryo-OCT) which is very thick and difficult to manipulate. The viscosity is temperature dependent, so heat an aliquot to 37° C before using to inflate the lungs. This can best be done by cannulating the trachea as described for the BAL, and instilling 0.5 ml OCT as gently as possible, then separating individual lung lobes for individual embedding. Expect a mess.
2. Cryostats are used to section frozen tissues, but also to freeze and embed them. Place a thin layer of OCT medium onto a cold "chuck", but before it completely solidifies add a tissue section and cover it quickly with additional OCT. These sections can be cut as soon as the medium is completely frozen, or store embedded samples in a sealed bag at -70°C.
3. Cutting 4 – 7 micron frozen sections is something of an art, and you may want to ask a histotechnologist for help. Place the sections on the Plus slides, and allow to dry for 1 – 3 hours at room temperature. Fix in cold acetone for 5 minutes, and store with desiccant at -70°C if the slides are not stained immediately.
4. Rinse in TBS for 5 minutes, and continue on to step 3 in the IHC protocol.

IN SITU HYBRIDIZATION (basic protocol 8)

This method describes the use of a non-radioactive RNA probe to detect mRNA in paraffin-embedded tissue sections. For this procedure you will need a probe that is labeled with digoxigenin (DIG). We use the DIG RNA Labeling Kit (Roche) to make the riboprobe and

test its labeling efficiency. The following procedure takes two days to complete. For the first day all reagents and containers must be RNase free. Either treat all solutions with DEPC or make up the solutions with DEPC-treated water.

Materials—Slide warmer

Shaking/orbital rocker

Water bath

Incubator/Hybridization oven

Humidified chamber

Coplin jars (Fisher Scientific)

Kimwipes

Pap pen (Biocare Medical)

Diethylpyrucarbonate (DEPC)-treated water

PBS

100 mM glycine (Fisher Scientific)

Triton-X100 (Sigma)

Proteinase K (Sigma)

4% paraformaldehyde (Electron Microscopy Sciences)

100 mM Triethanolamine pH 8

Acetic Anhydride (Sigma)

20× SSC

Digoxigenin labeled riboprobe

Prehybridization Buffer

Hybridization Buffer

ISH Buffers 1, 2, 3

Newborn calf serum

Anti-DIG alkaline phosphatase Fab fragment (Roche)

NBT/BCIP tablets (Roche)

Fast green FCF (Fluka)

1. Deparaffinize and rehydrate tissue sections as described for IHC, but using DEPC-treated ddH₂O.

2. Wash slides twice, for 5 minutes each wash, in PBS.
3. Incubate slides twice, 5 minutes for each incubation, in 100mM glycine.
4. Incubate the slides in PBS containing 0.3% Triton X-100 for 15 minutes.
5. Wash slides twice, 5 minutes for each wash, in PBS.
6. Encircle tissue section using a Pap pen. Permeabilize with 5µg/ml proteinase K in PBS at 37°C for 30 minutes, followed by two 5 minute washes in PBS.
7. Post-fix tissue section in cold (4°C) 4% paraformaldehyde in PBS for 5 minutes, followed by two 5 minute washes in PBS.
8. Acetylate tissue section with two 5 minute incubations in fresh 100mM triethanolamine, pH 8, containing 0.25% acetic acid anhydride.

Add the acetic anhydride to the TEA buffer just before use, and use a clean coplin jar for the 2nd incubation.
9. Incubate sections in 4× SSC/50% deionized formamide (pre-hybridization buffer) for at least 10 minutes at 37°C in a humidified chamber.
10. During the incubation in pre-hybridization buffer add 60 ng. of DIG- labeled riboprobe, made and tested according to the manufacturer's instructions, to 100 µl of hybridization buffer. Denature riboprobe at 80°C for 10 minutes then place on ice.
11. Blot off the pre-hybridization solution from each slide by placing the end of the slide on absorbent paper towels, and re-encircle the tissue section with the Pap pen.
12. Add the hybridization solution with the DIG-labeled riboprobe to the encircled tissue. Incubate the slide in a humidified chamber at 42°C overnight.
13. Blot off the probe from each slide taking care not to disturb the tissue section. Wash the tissue sections in 2× SSC for 5–10 minutes at room temperature, in 2× SSC for 15 minutes at 37°C, in 1× SSC for 15 minutes at 37°C, and in 0.1× SSC for 30 minutes at 37°C with shaking.
14. Incubate slides with Buffer 1, two 10 minute incubations.
15. Placing slides flat in the humidified chamber, block with 100µl of Buffer 1 containing 0.1% Triton-X and 2% newborn calf serum for 30 minutes at room temperature.
16. Blot off the blocking solution, and cover the tissue section with 100µl of a 1:500 dilution of anti-DIG-alkaline phosphatase in Buffer 1 containing 0.1% Triton-X and 1% serum. Incubate for 2 hours at room temperature.
17. Wash twice in a coplin jar filled with Buffer 1, 10 minutes for each wash, followed by two 10 minute incubations in Buffer 2.
18. During the 2nd incubation in Buffer 2 prepare the substrate solution by placing half of a NBT/BCIP tablet in 5 ml of ddH₂O in a 15ml conical tube. Place slides in the humidified chamber, and cover each tissue section with 200µl of substrate solution. Incubate from 1–24 hours in the dark, monitoring the color development of positive controls to determine when the reaction should be stopped.
19. Stop the reaction by placing slides in a coplin jar containing Buffer 3 for 5 minutes.
20. Dip slides briefly in water, counterstain tissue section in 0.02% fast green FCF for 1 minute, then wash in water for 5 minutes.

21. Blot the excess water from each slide, and add enough Crystal/Mount to cover the section. Dry at 65°C and then coverslip.

REAGENTS AND SOLUTIONS

Avertin

Avertin (tribromoethanol 20 mg/ml) is a short-acting anesthetic that is not available commercially and must be prepared. Sterile preparation procedures are essential. If Avertin is improperly prepared or stored in the light or at room temperature, it will break down within 24 hours into dibromoacetic acid and hydrobromic acid which are lethal. Freshly mixed solutions are strongly recommended for safe use. The solution can be kept as long as 4 months if it is stored in the dark at 4°C. The solution should be tested to ensure that it has a pH >5.

Materials—Glass Erlenmeyer flask

Magnetic stirrer

Hot plate with stirrer

Bottle-top filter 0.22 μ m (Millipore)

tert-amyl-alcohol (Fisher)

2,2,2-tribromoethanol (Sigma)

Work in the fume hood.

Add 10 ml tert-amyl-OH into glass bottle

Add 10 g 2,2,2-tribromoethanol to the alcohol

Stir until the solid goes into solution room temperature

Transfer to a hot plate on low heat

Very slowly add 250 ml distilled water to flask while stirring. Make sure that salts stay in solution before you add more water. This process takes several hours.

Bring solution up to 500 ml with distilled water.

Turn off the hot plate when all of the solid has gone into solution. Cover flask with aluminum foil to protect from the light, and stir overnight.

Check that the 2,2,2-tribromoethanol has not precipitated overnight. If necessary, return the flask to the hot plate and stir again on low heat until the solid has gone back into solution.

Adjust pH if necessary.

Bottle-top filter (0.22 μ m).

Aliquot into 50 ml glass bottles, of darkened glass or foil-covered.

Store in the refrigerator.

Test it on mice.

The effective dose in mice is on the order of 25 mg/kg (approx 200–250 µl/animal) but this will vary by strain with 129 mice being very susceptible and requiring a lower dose, and C57BL6 mice requiring a higher dose. The optimal dose is best decided empirically with each new batch of Avertin made, and each mouse strain used.

Collagenase A, 5 mg/ml

Collagenase A (Roche)
Culture medium (MEM, Cellgro)
Sterile filter
5 ml aliquots
Store at –20°C

Decarbonated Dulbecco's Modified Eagle Medium (dcDMEM)

The standard formulation of DMEM is buffered with bicarbonate, and therefore becomes alkaline when exposed to ambient atmosphere. For this reason, the medium used for the extended manipulations called for in these protocols is not supplemented with bicarbonate (Invitrogen). Adjust pH to neutral with sodium hydroxide).

D5HS

DMEM buffered with 25 mM HEPES (Invitrogen catalog #12320)
supplemented with 5% fetal calf serum.

Fast Green FCF 0.02% (w/v)

0.01g fast green
50 ml ddH₂O
Vortex
Filter to remove particulate matter

Fixative/Stain

0.003% crystal violet in 5% (v/v) formalin, 50 mM sodium phosphate, pH 7.4

Gey's solution (buffered ammonium chloride)

24.9 g NH₄Cl
3 g KHCO₃
3 ml 0.5% Phenol Red
ddH₂O to a final volume of 3 liters
Sterile filter
Store at 4°C

Hybridization buffer for ISH

40% deionized formamide

10% dextran sulfate
1× Denhardt's solution
4× SSC
10mM DTT
1 mg/ml yeast tRNA
1mg/ml denatured and sheared salmon sperm DNA
ddH₂O

Hybridization solution can be made up without salmon sperm DNA and stored at −20°C. When making up the hybridization buffer, dissolve the dextran sulfate in the water and Formamide before adding the other components.

ISH buffer 1

100mM Tris-HCl pH 7.5
150mM NaCl

ISH buffer 2

100mM Tris-HCl pH 9.5
100mM NaCl
50mM MgCl₂

ISH buffer 3

10mM Tris-HCl pH 8.1
1mM EDTA

1% Methylcellulose in MEM

Place 5 grams of methylcellulose (Sigma-Aldrich cat. #M0512) in an autoclavable bottle

Add 435 ml distilled water, preheated to > 70° C. (Important! Methylcellulose will form intractable “clots” in cold water!)

Before it cools, sterilize by autoclaving.

Cap tightly and keep the bottle shaking, e.g. by automatic rotator, as it cools for at least 60 minutes at room temperature. The room temperature cooling is to lower the temperature to the point where immersion in ice (see below) will not crack the glass bottle. The shaking is to prevent the (still insoluble) methylcellulose from settling.

Transfer the bottle to an ice bath, and keep shaking it until the methylcellulose dissolves, forming a clear viscous solution.

Add 50 ml of 10× MEM, and mix thoroughly. (It will take some time and effort, since the solution is very viscous.)

Add 14 ml of sterile 7.5% Sodium Bicarbonate.

Clear the solution of insoluble material by centrifugation at 3000x gravity, 30 minutes.

The solution should be orange to cherry-red. If the pH becomes too high (fuchsia colored) it can be adjusted by gassing the bottle with (sterile) CO₂.

Overlay medium

Mix equal volumes of growth medium (DMEM + 25mM HEPES +5% Fetal calf serum + antibiotics*) and 1% methylcellulose in MEM. Mix thoroughly by repeated pipetting, to eliminate pockets of low viscosity.

Since the source of the virus – a live animal – cannot be “sterile”, there is a possibility of bacterial and fungal contamination. Antibacterial agents (e.g. penicillin/streptomycin) and antifungal agents (e.g. amphotericin B) may be added to prevent these from overgrowing the cultures.

4% Paraformaldehyde pH 7 (200 ml)

8g paraformaldehyde (Electron Microscopy Sciences)

20ml 10× PBS

2N NaOH

ddH₂O

Heat ddH₂O in glass beaker with stirrer on hot plate to 60°C in fume hood.

Add paraformaldehyde powder and keep at 60°C.

Add 2 drops 2N NaOH, and the solution should clear within a few minutes.

Remove from heat and let cool to room temperature.

Add 20ml 10× PBS and bring volume up to 200ml with ddH₂O.

Filter and store at 4°C.

PBS pH 7.4

140 mM NaCl

2.7mM KCl

10mM Na₂HPO₄

1.8mM KH₂PO₄

Adjust pH to 7.4 with HCl

Store at room temperature

PBSTA

PBS with

0.1% Triton X-100

0.1% bovine serum albumin

0.1% sodium azide)

Percoll solutions

100% isotonic Percoll: 90 ml Percoll and 10 ml 10x HBSS

80% isotonic Percoll: 80 ml of 100% isotonic Percoll and 20 ml HBSS

40% isotonic Percoll: 40 ml of 100% isotonic Percoll and 60 ml HBSS

Store at 4°C

Prehybridization buffer for ISH

50% deionized formamide in 4× SSC

20× SSC

3M NaCl

300mM sodium citrate

Adjust pH to 7.4 with HCl

Staining Wash Buffer (SWB)

60 ml 10× PBS

6 ml 10% Sodium Azide

12 ml newborn calf serum

522 ml ddH₂O

0.5 M Tris at pH 10

60.57 gm. Tris base

900 ml ddH₂O

Adjust pH with 1M NaOH

Make up to final volume of 1L with ddH₂O

TBS pH 7.6

0.05 M Tris-HCl

0.15 M NaCl

Adjust pH to 7.6

COMMENTARY

Background Information

The study of virus infections *in vivo* requires the analysis of many parameters by a large number of different methods. Because a single unit cannot encompass all of these methods in detail, we have focused on providing the reader with protocols for harvesting the relevant samples. Our suggested methods for detection of infectious virus, flow cytometry, and histology are given as examples of how the samples might be assayed. The assay techniques outlined here are relatively general, as we have chosen the most widely applicable techniques and reagents. It is up to the individual investigator to tailor these assays to meet their specific needs.

Critical Parameters

Measuring cytokines and chemokines—Although not explicitly described in this chapter, cytokine assays can be carried out using either BAL specimens (described in basic protocol 4) or lung homogenates. The same homogenate can be used to assay both virus titers and cytokines, although we have found that BAL samples generally have a lower background for cytokine measurement than do whole lung homogenates. ELISA assays are commercially available for many mouse cytokines and chemokines. A biological assay for

IFN- α or - β activity is described in detail by Vogel et al. in Current Protocols in Immunology (Vogel et al., 1991), and this method can be used to assay lung BALs and homogenates, as well as serum samples.

Flow-cytometry—Successful flow cytometry analysis of a given population requires the knowledge and experience to calibrate the instrument, standardize procedures and settings, and to analyze the data with a software package. However, the most important requirements are a good single cell suspension and a proven staining panel/protocol. The correct choice of reagents requires certain knowledge of the characteristics of each fluorochrome and of the antigens of interest. However, the basic approach described here can be used for multiple extracellular antigens, cell subpopulations and tissues with minor modifications.

Histology—High quality H&E sections are best obtained from a pathology core or hospital laboratory as production of formalin-fixed, paraffin-embedded tissue ‘blocks’ and slides from these blocks require a major investment in equipment and training which is not appropriate for most research laboratories. Nonetheless, if fixed tissue samples are submitted to the histotechnologist in tissue cassettes, taking care to explain the orientation needed for optimal slide interpretation, high quality H&E stained sections can easily be obtained. Additional sections for immunohistochemistry (IHC) or *in situ* hybridization (ISH) studies can also be prepared from these tissue blocks. IHC using virus-specific antibodies allows the investigator to visualize the extent of virus spread, and the cell type(s) targeted by a particular virus. This method can also be used to visualize the appearance of immune cell types in infected tissues over time, although this type of experiment may require the use of unfixed, frozen tissue sections. ISH is an alternative method if specific antibodies are not available, or the investigator wishes to determine the source of a secreted protein.

Immunohistochemistry—For human tissues there are a large number of commercially available monoclonal antibodies that can serve this purpose, and work well for staining formalin-fixed, paraffin-embedded tissue sections. Unfortunately this is not the case for mouse tissues, as there is a relatively small demand for these reagents. Many monoclonal antibodies that work well for staining cell suspensions prior to FACS analysis will not work well for staining tissue sections. The company selling the antibody may specify this, but usually this must be determined empirically. In some cases this can be overcome by using frozen sections rather than paraffin sections of fixed tissues, however frozen tissues are difficult to cut and the resulting histology is often suboptimal. In addition to the problem of low foreground signal, most monoclonal antibodies are made in mice, and background staining results when the anti-mouse secondary antibody recognizes endogenous antibody bound to mouse tissues or B cells. “Mouse to mouse” blocking reagents are available from a number of companies, but do not always solve the background problems. Our best results in mouse tissue have been obtained using polyclonal antisera raised in a non-mouse species such as hamster, goat, or rabbit. Fortunately, many such antisera, specific for a large number of viruses, are available from Millipore, and can be used to visualize infected cells in tissues using basic protocol 7. The right hand panel of Figure 3 demonstrates the utility of this technique with evidence of many infected airway lining cells (seen as red) following infection 24 hours earlier with a high dose (10^6 pfu) of influenza A virus. This is a demonstration of robust infection prior to the onset of visible pathology at early times post-inoculation.

Troubleshooting

We have included here a variety of different protocols to study respiratory virus infections. In addition, because of the many manipulations that are required in these protocols and the diverse technologies used, there are many different things that can go wrong. Thus, the

investigator should refer to the specific protocols or to manual specific to each subject for more detailed information.

Anticipated Results

In these protocols, as with any *in vivo* experimental model, there will be variability between experiments and between individual animals at a given time point. As this is unavoidable, it is important to use sufficient numbers of animals such that a meaningful statistical analysis can be done. We typically use between 5 and 10 animals for each data point, and this is important for both objective measures such as cytokine production, as well as the more subjective evaluation of histopathology. When comparing more than 2 cohorts of animals, a simple student-t test is often not sufficient, and an analysis of variance must be performed to determine significance. For histologic analysis, differences between groups of animals will often be a matter of degree rather than a $\pm$ situation, so it is important to examine the slides blindly. We typically handle this by labeling each slide with only a number, and asking an unbiased examiner to determine whether the samples appear to segregate into groups. When no clear differences are present between 2 groups, we conclude that those are not significantly different. Although this system has worked well for us, it is important to use both qualitative and quantitative measures whenever possible, e.g. comparing BAL cytology and/or FACS analysis with the pattern of inflammation seen on tissue sections at each time point.

Time Considerations

In planning an infection experiment, it is important to determine in advance which samples can be frozen (e.g. lungs for virus titers or BALs for ELISA), and which must be analyzed on the day that they are harvested. Tissues for histology must be fixed immediately, but cannot be processed until they have been fixed in formalin for several hours, but no more than 2 days. Lymphocyte analysis must be done immediately. Isolating immune cells from tissue samples and setting up a FACS-staining will take from 4 to 12 hr. depending on the number of tissue samples and the complexity of the staining. The samples can be run at the end of the day or, if fixed, during the following day. An ISH analysis will take at least one week, including the time needed to harvest, fix, process, and section tissues, with 2 days needed for staining.

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Figure 1.

Flow cytometry staining of spleen and lung cells from a γ HV68 infected mouse on day 16. Antigen-specific CD8 T cells are gated using the tetrameric reagent ORF6₄₈₇₋₄₉₅/K^b to show the expression profile of KLRG1. KLRG1 is a killer inhibitory receptor that is used to define terminally differentiated short-lived effector T cells.

Figure 2.

Analysis of dendritic cell subpopulations in lung and spleen of a γ HV68 infected mouse on day 7. Lineage-positive cells are gated out using a dump channel (CD3, CD19 and NK1.1). Conventional dendritic cells (cDC) are defined as class II⁺CD11c⁺B220⁻ and further divided into subsets: CD11b⁺ (population 1), CD8a⁺ (population 2) and CD11b⁻CD8a⁻ (population 3). Plasmacytoid dendritic cells (pDC) are class II⁺CD11c⁺B220⁺.

Figure 3.

Much information can be gained from examining the histology of infected lungs. Panel A is a standard H&E stained section photographed at low power (50×). The large conducting airways branching into the smaller bronchioles and finally alveolar spaces can be clearly seen. This animal was infected 24 hours previously with a high dose (10^6 pfu) of influenza A virus, but no evidence of pathology is appreciable at this early time point. In panel B, a higher magnification image (100×) shows that, despite the lack of inflammatory cells at this early time point, many airway lining cells (shown as red) already show evidence of infection. The slide shown in panel B was stained using polyclonal rabbit serum raised against whole virus, and the method described in basic protocol 6. This level of staining was obtained without antigen retrieval.