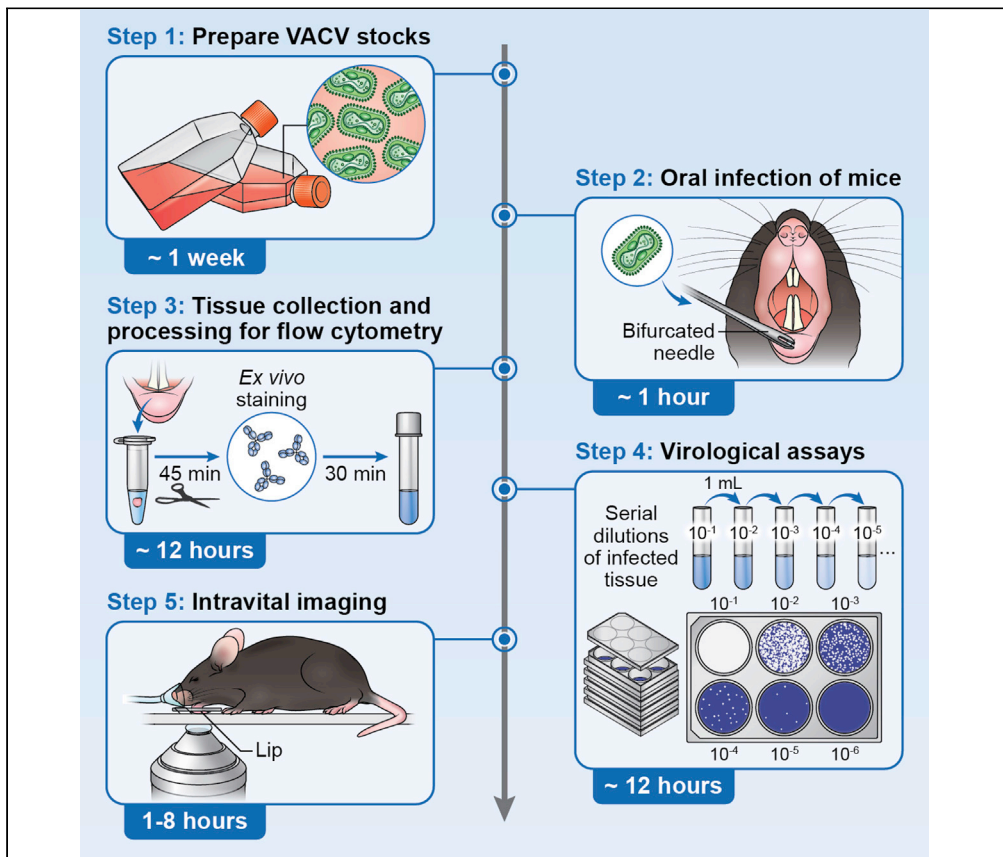


Protocol

Protocol for analyzing and visualizing antiviral immune responses after acute infection of the murine oral mucosa



The oral mucosa is an important site for virus infection and transmission, yet few animal models exist to examine the virology, pathology, and immunology of acute oral mucosal viral infection. Here, we provide a protocol for infecting and imaging the inner lip (labial mucosa) of mice with the poxvirus vaccinia (VACV). Inoculation of the labial mucosa with a bifurcated needle results in viral replication and priming of an adaptive antiviral response that can be imaged using intravital microscopy.

John P. Shannon,
Christian R. Cherry,
Sophia M. Vrba,
Heather D. Hickman

John.Shannon@nih.gov
(J.P.S.)
hhickman@mail.nih.gov
(H.D.H.)

Highlights

The oral mucosa is a critical barrier tissue during viral infection

Vaccinia virus infection provides an acute oral mucosal viral challenge model

Antiviral immune responses can be analyzed using ex vivo and in vivo approaches

The murine inner lip (labial mucosa) can be imaged using intravital microscopy

Protocol

Protocol for analyzing and visualizing antiviral immune responses after acute infection of the murine oral mucosa

John P. Shannon,^{1,2,*} Christian R. Cherry,¹ Sophia M. Vrba,¹ and Heather D. Hickman^{1,3,*}¹Viral Immunity and Pathogenesis Unit, Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA²Technical contact³Lead contact*Correspondence: John.Shannon@nih.gov (J.P.S.), hickman@mail.nih.gov (H.D.H.)
<https://doi.org/10.1016/j.xpro.2021.100790>

SUMMARY

The oral mucosa is an important site for virus infection and transmission, yet few animal models exist to examine the virology, pathology, and immunology of acute oral mucosal viral infection. Here, we provide a protocol for infecting and imaging the inner lip (labial mucosa) of mice with the poxvirus vaccinia virus (VACV). Inoculation of the labial mucosa with a bifurcated needle results in viral replication and priming of an adaptive antiviral response that can be imaged using intravital microscopy. For complete details on the use and execution of this protocol, please refer to Shannon et al. (2021).

BEFORE YOU BEGIN

Investigators should obtain necessary institutional approvals for procedures involving infectious agents and animals. VACV is a biosafety level 2 pathogen and should be handled under appropriate conditions.

Working with vaccinia virus may require vaccination of both laboratory personnel and animal husbandry personnel.

Animal studies must be approved by an institutional animal care and use committee (ACUC) before any experiments begin. All animal studies should be performed in accord with ACUC guidelines.

VACV growth

⌚ Timing: 3 days

1. Plate 1×10^7 143B (human thymidine kinase (TK⁻) cells) in 30 mL of cell culture medium (See [materials and equipment](#) section) per T150 tissue culture flask. Place flasks in humidified tissue-culture incubator at 37°C with 9% CO₂ overnight. All subsequent tissue-culture incubation steps are performed under these conditions unless directly noted.

Note: We culture 143B cells in Dulbecco's Modified Eagle Medium (DMEM) which has a high concentration of sodium bicarbonate. To obtain appropriate pH, higher levels of CO₂ may be used. While CO₂ levels can range from ~5%–12% we have had good luck with 9%. Minimum essential medium (MEM) can also be used to culture cells, if so, we recommend using a lower CO₂ concentration (~5%) to account for lower levels of sodium bicarbonate.



Note: Although passage number can be a concern with immortalized cell lines, we have not detected observable differences in virus growth with older passages of the cells. Allowing the cells to overgrow should be avoided, however, as this can decrease viral titers and impair plaque assays performed with these cells (discussed below). It is advisable to passage cells at least once before growing virus to make sure the cells are healthy and grow well.

2. Confirm each flask has a confluent monolayer of cells and then thaw a VACV stock and calculate the volume of virus needed to infect at the desired multiplicity of infection (MOI) using the formula below. A confluent flask of TK- cells has $\sim 2.5 \times 10^7$ cell and should be infected with a MOI of 0.1–0.5 for best results.

$$\frac{((\# \text{ of cells in confluent T150}) \times (\% \text{ confluency in flask}) \times (\text{desired MOI}))}{\text{virus titer PFU/ml}}$$

For example: $(2.5 \times 10^7 \times 85\% \text{ confluency} \times 0.1 \text{ PFU/cell}) / (2.0 \times 10^8 \text{ PFU/mL}) = 106 \mu\text{s}$ of virus stock added to 10 mls of infection medium

3. Add the volume of virus calculated above to 10 mL of infection medium (See [materials and equipment](#) section).
4. Remove medium from each T150 flask and add 10 mL of infection medium containing virus to each flask. Ensure the entire surface of the flask is covered.
5. Place flasks in humidified tissue culture 37°C incubator with 9% CO₂ for 2 h. Gently rock the flasks every 15–30 min.
6. Add 20 mL of cell culture medium to each flask without removing infection medium containing virus (We typically infect 10–15 flasks for each viral stock which gives us ~ 100 mL of virus at 2E8 PFU/mL). Incubate for 48 h at 37°C with 9% CO₂.

VACV harvest and aliquoting

⌚ Timing: 3.5 h

7. Confirm the presence of cytopathic effect (CPE) in each flask. Cells should be rounded and either floating or lightly adherent to flasks before harvest.
8. Gently tap the flasks to detach cells and transfer contents to a conical bottom 225 mL centrifuge tube.
9. Spin at $870 \times g$ at 4°C for 30 min.
10. Aspirate media and resuspend cell pellet in 10 mL of ice-cold HBSS + 0.1% BSA (See [materials and equipment](#) section).
11. Transfer resuspended pellet into a sterile 50 mL Falcon tube. Rinse 225 mL centrifuge tube with an additional 5 mL of HBSS + 0.1% BSA and transfer to 50 mL Falcon tube, the final volume will be ~ 15 mL.
12. Freeze/thaw resuspended pellet three times on dry ice and then in a 37°C water bath, respectively.
13. Carefully pour mixture into a sterile 15 mL Dounce homogenizer and place on ice.
14. Slowly homogenize by moving the pestle up and down while keeping on ice until the solution is uniform.

⚠ CRITICAL: The freeze/thaw and homogenization lyse cells and releases cell-associated virus. These steps must be followed to obtain maximum virus yield. The freeze-thaw can be performed at room temp or at 37°C in a water bath, pending time.

15. Pipette mixture back into the 50 mL Flacon tube from homogenizer and place on ice.

16. Rinse the homogenizer with an additional 10 mL of ice-cold HBSS + 0.1% BSA and transfer to the 50 mL Falcon tube with mixture from step 15. Bring the volume of the tube up to 50 mL with ice-cold HBSS + 0.1% BSA.
17. Place the 50 mL tube in a cup horn sonicator. Add ice to the cup holder and sonicate for 30 s at 30% power. Repeat process 4 times, equaling a total time of 2 min.
18. Perform a quick spin to remove the cellular membranes. Centrifuge at $870 \times g$ for 5–10 s.

Note: The quick spin process removes most cell debris. Virus may be purified through a sucrose gradient using standard virological approaches if a virus preparation entirely devoid of cell debris is desired.

19. Gently pour the supernatant into a sterile 125 mL media bottle and place on ice.
20. Add 10 mL of ice-cold HBSS + 0.1% BSA to wash and break up pellet, then add another 20 mL of ice-cold HBSS + 0.1% BSA bringing the total volume to 30 mL.
21. Place the 50 mL tube in the sonicator and sonicate for 30 s at 40% power.
22. Repeat the quick spin process, as described in step 18.
23. Pour supernatant into the 125 mL media bottom containing the previous supernatant.
24. Wash the pellet as described in step 20 and repeat the sonication and quick spin steps. Add supernatant to the 125 mL media bottle. The final volume should be ~ 110 mL.
25. Mix virus well and aliquot 1 mL into sterile screw cap 2 mL conical-bottom Sarstedt tubes.

△ CRITICAL: Virus should be aliquoted into single-use aliquots and stored at -80°C . Do not freeze-thaw aliquots as this reduces virus titer.

Virus titration by plaque assay

⌚ **Timing:** 3 days

26. Plate 7.5×10^5 143B cells/well into 6-well plates. Incubate cells overnight at 37°C with 9% CO_2 .
27. Confirm cells are confluent and then thaw a 1 mL vial of virus and sonicate for 45 s at 70% power.
28. Dilute VACV in serial 10-fold dilutions.
 - a. Prepare 8 1.5 mL microcentrifuge tubes and label as 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} .
 - b. Add 1080 μL infection medium (See [materials and equipment](#) section) to each tube.
 - c. Add 120 μL of virus from 1 mL vial to 10^{-1} tube and mix well by pipetting.
 - d. Transfer 120 μL of 10^{-1} to 10^{-2} and mix well by pipetting.
 - e. In the same manner, continue to dilute 1:10 from 10^{-2} to 10^{-8} .
29. Aspirate medium from 6-well plates. Add 500 μL of each virus dilution from 10^{-3} to 10^{-8} per well, performing duplicates for each dilution.
30. Incubate the 6-well plates with virus for 2 h gently rocking the plates every 15 min.
31. After 2 h incubation, add 2 mL of fresh cell culture medium (do not discard infection medium) and incubate for 48 h at 37°C with 9% CO_2 .
32. After 48 h incubation, aspirate media from each well.
33. Stain plates with crystal violet solution (See [materials and equipment](#) section).
 - a. Add 500 μL of crystal violet solution and gently rock plates to cover the entire well.
 - b. Allow plates to stain for 20–30 min at RT then aspirate excess stain and air-dry.
34. After plates are dried, determine viral titer.
 - a. Count the number of distinct plaques in each well (as shown in [Figure 1](#)).
 - b. Calculate the titer as plaque forming units (PFU) per mL using the following formula:

$$\text{PFU} / \text{ml} = \text{Average plaque number} \times \frac{\text{dilution}}{\text{adsorption volume in ml}}$$

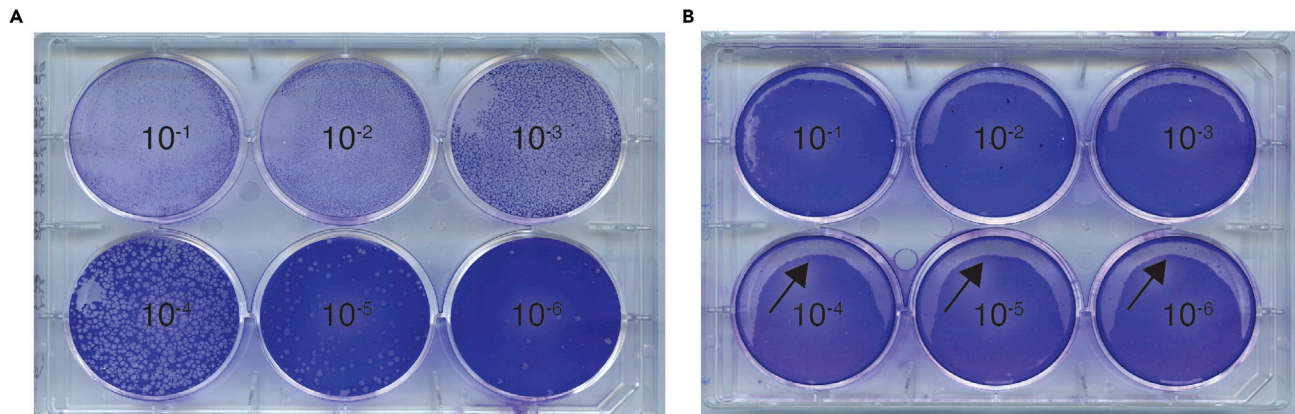


Figure 1. Representative plaque assays to determine VACV titer

(A) Representative plaque assays using 10-fold dilutions (indicated by labels). Viral titers can be determined at the 10⁻⁵ and 10⁻⁶ dilutions.

(B) Representative image of a plaque assay in which cells dried during incubations. Areas of cell death are indicated with arrowheads in the wells in the bottom row.

△ **CRITICAL:** Do not let cells grow past confluency prior to performing the plaque assay. Avoid moving plates during the 48 h incubation step to minimize the potential of satellite colonies. When determining titers by plaque assay, wells with 5–50 plaques should be counted. Counting too few plaques reduces accuracy of quantification; at high densities plaques may merge and be difficult to distinguish.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|-------------|--|
| Antibodies | | |
| Anti-mouse CD45 (30-F11) – 1:200 dilution | eBioscience | Catalog # MCD4528 RRID: AB_10373710 |
| Anti-mouse CD45 (30-F11) – 1:200 dilution | BioLegend | Catalog #103132 RRID: AB_893340 |
| Anti-mouse CD45 (30-F11) – 1:200 dilution | BioLegend | Catalog #103112 RRID: AB_312977 |
| Anti-mouse CD45 (30-F11) – 1:200 dilution | BioLegend | Catalog #103128 RRID: AB_493715 |
| Anti-mouse CD45 (30-F11) – 1:200 dilution | BioLegend | Catalog #103116 RRID: AB_312981 |
| Anti-mouse CD8 (53–6.7) – 1:100 dilution | BioLegend | Catalog #100712 RRID: AB_312751 |
| Anti-mouse CD3 (17A2) – 1:100 dilution | BioLegend | Catalog # 100204 RRID: AB_312661 |
| Anti-mouse CD3 (17A2) – 1:100 dilution | BioLegend | Catalog # 100220 RRID: AB_1732057 |
| Anti-mouse NK1.1 (PK136) – 1:100 dilution | BioLegend | Catalog #108708 RRID: AB_313395 |
| Anti-mouse NK1.1 (PK136) – 1:100 dilution | BioLegend | Catalog #108720 RRID: AB_2132713 |
| Anti-mouse CD4 (GK1.5) – 1:100 dilution | eBioscience | Catalog #100406 RRID: AB_312691 |
| Anti-mouse CD49b (DX5) – 1:100 dilution | BioLegend | Catalog #108908 RRID: AB_313415 |
| Anti-mouse CD49b (DX5) – 1:100 dilution | BioLegend | Catalog #108910 RRID: AB_313417 |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|--|---|
| Anti-mouse CD49a (Ha31/8) – 1:25 dilution | BD Biosciences | Catalog #564862 RRID: AB_2734135 |
| Anti-mouse Eomes (Dan11mag) – 1:100 dilution | eBioscience | Catalog #25-4875-82 RRID: AB_2573454 |
| Anti-mouse T-bet (4B10) – 1:100 dilution | BioLegend | Catalog # 644810 RRID: AB_2200542 |
| Anti-mouse CD31 (MEC13.3) – 1:100 dilution | BioLegend | Catalog #102516 RRID: AB_2161029 |
| Anti-mouse CD69 (H12.F3) – 1:100 dilution | BioLegend | Catalog #104513 RRID: AB_492844 |
| Anti-mouse CD103 (2F7) – 1:100 dilution | BioLegend | Catalog #121432 RRID: AB_2566552 |
| Anti-mouse CD103 (2F7) – 1:100 dilution | BioLegend | Catalog #121422 RRID: AB_2562901 |
| Bacterial and virus strains | | |
| Vaccinia virus wild type (CR19) | Laboratory of H.D.H, NIAID, NIH | N/A |
| Vaccinia virus WRvFire (expressing firefly luciferase) | Laboratory of Bernard Moss, NIAID, NIH | N/A |
| Chemicals, peptides, and recombinant proteins | | |
| BSA | Sigma-Aldrich | A3059-500G |
| DNase I | Sigma-Aldrich | DN25-5G |
| FBS | HyClone | SH30070.03 |
| Paraformaldehyde | Electron Microscopy Sciences | 15714-S |
| Collagenase (type I) | Worthington Biochemicals | LS004216 |
| Collagenase P | Sigma-Aldrich | 11249002001 |
| Crystal Violet | Sigma-Aldrich | C0775-100G |
| Formaldehyde | Sigma-Aldrich | 252549-500ML |
| Gentamicin (50mg/mL) | Sigma-Aldrich | G1397-10ML |
| Vetbond adhesive | Santa Cruz Animal Health | sc-361931 |
| XenoLight D-Luciferin - K+ Salt Bioluminescen Substrate | PerkinElmer | 122799 |
| Experimental models: Cell lines | | |
| 143B | ATCC | CRL-8303 |
| Experimental models: Organisms/strains | | |
| Mouse: C57Bl/6 (6- to 12-week-old female mice) | Taconic Farms | Mouse strain: C57Bl/6NTac |
| Mouse: Rag1 ^{-/-} (6- to 12-week-old female mice) | Taconic Farms – NIAID exchange | Mouse strain: Tac 146 |
| Software and algorithms | | |
| Prism software | GraphPad | RRID:SCR_002798 |
| Imaris | Oxford Instruments | RRID:SCR_007370 |
| FlowJo | Tree Star | RRID:SCR_008520 |
| Other | | |
| BD Fortessa Flow Cytometer | BD Biosciences | RRID:SCR_019600 |
| Leica SP8 Multiphoton Microscope | Leica Microsystems | RRID:SCR_018169 |
| SomnoFlow isoflurane anesthesia machine | Kent Scientific | RRID:SCR_021629 |
| Sorvall X4R Pro 100V centrifuge | Thermo Fisher | RRID:SCR_021628 |

MATERIALS AND EQUIPMENT

Cell line

143B (ATCC CRL-8303)

DMEM cell culture medium

| Reagent | Amount |
|--------------|---------------|
| DMEM | 500 mL |
| FBS | 40 mL |
| Total | 540 mL |

Store at 4°C until manufacturer's expiration date

DMEM infection medium

| Reagent | Amount |
|--------------|---------------|
| DMEM | 500 mL |
| FBS | 10 mL |
| Total | 510 mL |

Store at 4°C until manufacturer's expiration date

RPMI media

| Reagent | Amount |
|--------------|---------------|
| RPMI | 500 mL |
| FBS | 40 mL |
| Total | 540 mL |

Store at 4°C until manufacturer's expiration date

HBBS + (0.1%) BSA

| Reagent | Amount |
|--------------|---------------|
| HBSS | 500 mL |
| BSA | 0.5 g |
| Total | 500 mL |

Store at 4°C until manufacturer's expiration date

Crystal violet solution

| Reagent | Final concentration | Amount |
|---------------------|---------------------|---------------|
| Ethanol (200 proof) | 13.6% | 47.5 mL |
| Milli-Q water | 0.7% | 2.5 mL |
| Formaldehyde (37%) | 85.7% | 300 mL |
| Crystal violet | 4.3% w/v | 1.5 g |
| Total | | 350 mL |

Store at 20°C–25°C for up to 1 year

△ **CRITICAL:** This formulation of crystal violet solution contains formaldehyde (a carcinogen). We handle formaldehyde in a fume hood; consult the institutional biosafety committee for proper handling and disposal.

Tribromoethanol (avertin)

| Reagent | Final concentration | Amount |
|-----------------------------|---------------------|--------------|
| PBS | 98.0% | 50 mL |
| 2,2,2-tribromoethyl alcohol | 2.0% w/v | 1 g |
| tert-amyl alcohol | 2.0% | 1 mL |
| Total | | 51 mL |

Store at 4°C in the dark for up to one week.

△ **CRITICAL:** A stock of 100% avertin is prepared by dissolving 10 g of 2,2,2-tribromoethyl alcohol in 10 mL tert-amyl alcohol by heating to 50°C on a magnetic stir plate while keeping protected from light. 100% stock solutions are diluted to a 2% working solution in PBS as described in the table above. For the most consistent dosing, make fresh avertin for each experiment.

Mice

The mouse strain selected will be dependent on the experimental question. We have encountered good success with 6- to 12-week-old C57Bl/6 mice as well as various transgenic and knock-in reporter mouse strains including *Rag1*^{-/-}, *Rag2*^{-/-}*IL-2rg*^{-/-}, *Perf*^{-/-}, *Ifng*^{-/-}, *Ncr1-gfp*, and T-bet-ZsGreen mice.

All animal studies were approved by and performed in accordance with the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases.

STEP-BY-STEP METHOD DETAILS

Labial mucosal infection of mice with VACV

⌚ Timing: 1–2 h

1. Prepare VACV inoculum:
 - a. Thaw a 1 mL vial of virus on ice and sonicate for 45 s at 70% power.
 - b. Transport virus to vivarium on ice.
2. Infection of the oral mucosa:
 - a. Anesthetize mice with approved inhaled or injectable anesthetic (such as isoflurane or avertin).
 - b. Once the mouse is sedated, using a pipette tip, place a small drop of virus (~ 10 μ L) at the desired concentration (PFU/mL, see below) inside the lower lip (labial mucosa), which forms a small pocket (see Notes).
 - c. Gently poke the mucosa with a bifurcated needle. Alternate: Instead of placing a drop of virus on the lip, the bifurcated needle can be dipped in the virus stock (see [Methods video S1](#)) and then the virus held by the needle is gently poked into the labial mucosa.

Note: The bifurcated needle was designed for human inoculation with the live-virus smallpox vaccine. The needle is dipped into the reconstituted vaccine (containing infectious VACV). One dose of the vaccine is then held in a thin layer between the needle's prongs (see [Methods video S1](#)). The needle is then used to poke the skin of vaccinees 15 times.

Note: While we have inoculated mice using a dipped needle, we most commonly inoculate mice by placing a small drop of virus in the lip and gently poking 5–10 pokes through the drop of virus with a bifurcated needle (see ([Shannon et al., 2021](#)) for viral titers over time with this inoculum of virus). Viral titers can be increased or decreased with more or fewer pokes. More pokes will result in more morbidity and mice should be carefully monitored ([Figure 2](#)).

- d. Wipe the inner lip with sterile gauze to remove any remaining VACV.
- e. Monitor mice until they recover fully from anesthesia.

Note: Thus far we have tested the lip (labial mucosa), tongue and cheek (buccal mucosa) for permissible infection. We have also successfully infected the murine labial mucosa with herpes simplex virus (HSV)-1 strain SC16.

Note: Although we have not found dramatic differences in the protocols between strains, we cannot rule out strain- or sex-specific differences, particularly in anesthesia requirements. Mouse age and weight do impact infection and viral titers (with young mice having higher viral titers), so this should be taken into consideration.

⚠ **CRITICAL:** Two steps are critical when infecting the labial mucosa: 1) ensure that viral inoculum is not inhaled by the mouse during the initial infection (See [Figure 3](#)) and 2) poke the

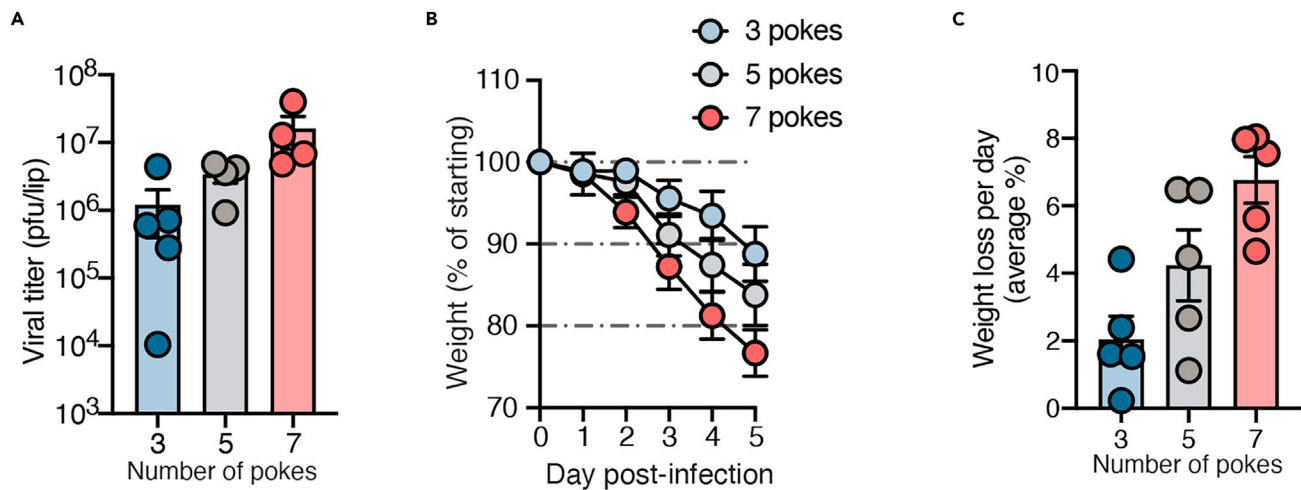


Figure 2. Increasing the number of pokes increases viral inoculum and titers in the labial mucosa

(A) Viral titers in the labial mucosa on day 5 post infection of C57Bl/6 mice. Mice were infected with a starting viral titer of $\sim 2 \times 10^8$ PFU/mL with a 10 μ l droplet inside the lip. The number of pokes with a bifurcated needle is indicated. Dots represent different animals. Error bars = SEM.

(B) Weight loss of mice inoculated in (A). Dots show means. Error bars = SEM.

(C) Average weight loss/day of mice inoculated in (A). Dots represent different animals. Error bars = SEM.

mucosa only gently to prevent skin infection or excess bleeding. We have found that diluting the initial viral stock to 10^4 PFU/mL allows labial mucosal infection without lung infection due to inhalation.

Tissue collection and enzymatic digestion of labial mucosal tissue

⌚ Timing: 2–4 h

3. Prepare collection tubes.
 - a. Appropriately label a screw cap 2 mL conical-bottom Sarstedt tube for each sample.
 - b. Thaw a 1 mL collagenase P aliquot in a 37°C water bath and add to 9 mL of RPMI medium (See [materials and equipment](#) section).
 - c. Pipette 1 mL of diluted collagenase P (final concentration of 1 mg/mL) to each 2 mL tube.

Note: The working concentration of collagenase P is 1 mg/mL. We recommend preparing a stock solution at 10 mg/mL in RPMI without FBS and aliquoting to avoid freeze/thaw cycles. Stock aliquots are stored at -20°C .

4. Harvesting labial mucosa.
 - a. At the desired day post infection, euthanize mice and remove the lower lip using dissection scissors and forceps and place in a 2 mL tube with diluted collagenase P.
5. Generate a single-cell suspension of labial mucosa.
 - a. Finely chop dissected lips with scissors in diluted collagenase P.
 - b. Place in 37°C water bath for 45 min.
 - c. Vigorously pipette up and down with a 1000 μ L pipette until sample appears fully digested and homogenous.

Note: It is essential to slowly pipette up and down to minimize the risk of the sample sticking to the inside of the pipette. We generally set the pipette to ~ 750 μ l, and in our hands we have found minimal sample loss. Depending on experimental question proceed to either

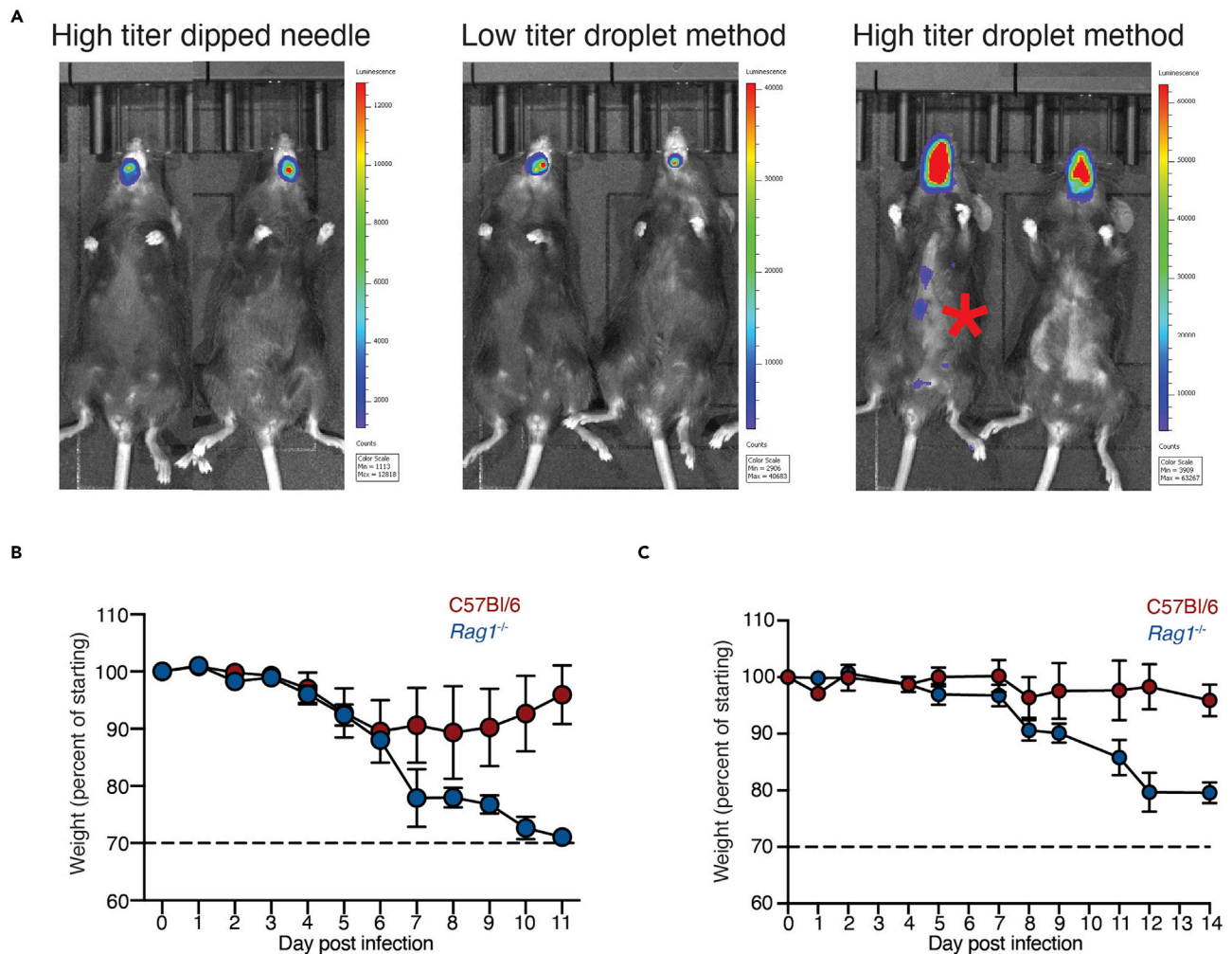


Figure 3. Inoculation using stock virus with high viral titer can result in infection outside the lip and increased morbidity

(A) In vivo imaging system (IVIS) images after infection with a recombinant VACV expressing Luciferase. (Left panel) Mice were infected with virus at $\sim 8 \times 10^8$ PFU/mL with the bifurcated needle dipped into the virus. (Middle panel) Mice were inoculated with 10 pokes of a bifurcated needle through a droplet (10 μ l) of virus at 1×10^4 PFU/mL applied to the lip. (Right) Mice were inoculated with 5 pokes of a bifurcated needle through a droplet of 10 μ l of virus at $\sim 8 \times 10^8$ PFU/mL. An * denotes viral dissemination on day 5 post infection.

(B) Weight loss after inoculation of wild-type C57Bl/6 mice (red dots) or Rag1^{-/-} mice (blue dots) with 5 pokes through a droplet of VACV at a titer of $\sim 2 \times 10^8$ PFU/mL. Dots show averages with 3–5 mice/group. Error bars = SEM.

(C) Weight loss after inoculation of wild-type C57Bl/6 mice (red dots) or Rag1^{-/-} mice (blue dots) with 10 pokes of the bifurcated needle through a droplet of virus at a titer of $\sim 1 \times 10^4$ PFU/mL. Dots show averages with 3–5 mice/group. Error bars = SEM.

“Analyzing oral mucosa tissue by flow cytometry” step or “Determining VACV titers in the oral mucosa” following sample digestion.

Enzymatic tissue digestion to process labial mucosal tissue

⌚ Timing: 4–6 h

⚠ **CRITICAL:** After sample collection, tissue processing should begin immediately in order to avoid compromising flow results. Collected samples should be kept on ice until processing. The ideal digestion parameters should be established for each experiment. In a study of

lymphocyte recovery in the oral mucosa comparing dispase, collagenase I, collagenase II, collagenase III, and collagenase P, we found the greatest recovery of lymphocytes with collagenase P digestion, however some cell surface markers (such as CD4) can be cleaved by collagenase P. In order to analyze CD4⁺ cells, we recommend labial mucosal digestion with collagenase I (10000 units/mL) for 45 mins at 37°C.

6. Filter digested sample through a 70 μm cell strainer into a 5 mL tube.
7. Rinse filter with 3 additional mL of RPMI medium and centrifuge cells at 870 \times g at 4°C for 5 min.
8. Discard the supernatant and resuspend cells in 250 μL of RPMI medium and filter through a 60 μm 96-well filter plate. (Alternatively, any 70 μm cell strainer can be used).
9. Transfer filtered samples to a labeled 96-well round-bottom plate and centrifuge cells at 870 \times g at 4°C for 2 min.
10. Discard the supernatant and resuspend cells in 250 μL of RPMI medium.
11. Count cells and determine viability using Trypan Blue exclusion.

Note: Typically, the number of cells obtained from the oral mucosa of a single mouse is only enough to stain a single well for flow cytometry (with counts falling between 8×10^5 – 2×10^6 cells/well). Thus, the cells do not need to be counted and diluted into different wells as one might do for a larger organ, like the murine spleen.

12. Centrifuge cells at $\sim 870 \times$ g at 4°C for 2 min.
13. Discard supernatant and resuspend in diluted antibody solution.
14. Extracellular staining of samples.
 - a. Prepare antibody dilutions in HBSS + 0.1% BSA or other buffer compatible with flow cytometry (See [materials and equipment](#) section) and resuspend each sample in 50 μL volume. A fixable viability dye can also be included at this point.

Note: The dilution of each antibody depends on fluorophore and company. We have successfully stained using the antibodies mentioned in the [key resources table](#), however we recommend performing antibody titrations prior to starting. Depending on the goal of the experiment, a FcR block can be added prior to extracellular staining.

- b. Protect samples from light and incubate for 30 min on ice.
 - c. Add 200 μL of cold PBS to each well and centrifuge $\sim 870 \times$ g at 4°C for 2 min.
15. Fixation of samples.
 - a. Prepare 3.2% PFA from a 16% PFA stock by diluting 1:5 with PBS.

Note: Samples are fixed in PFA to kill any infectious virus present in the sample before running through the cytometer. Diluted PFA should be made fresh just prior to fixation step. We recommend preparing PFA during the antibody staining incubation period. PFA is light sensitive. Any excess PFA should be disposed of according to institutional safety of-fice guidance.

- b. Discard supernatant and add 100 μL / sample of 3.2% PFA and pipette each sample to ensure resuspension.
 - c. Protect samples from light and incubate for 15 min at RT.

▣▣ Pause point: Fixed samples can be stored at 4°C protected from light for at least 24 h. For longer storage, the effect on fluorescence should be empirically determined. We have not tested freezing cells for later analysis.

16. Preparing samples for flow cytometric analysis.
 - a. Centrifuge samples from step 15c at $\sim 870 \times$ g at 4°C for 2 min.

- b. Discard supernatant and resuspend in 250 μ L of HBSS + 0.1% BSA + 0.1% EDTA.
17. Analyze via flow cytometry.

Note: This flow cytometry protocol has been used successfully to stain for a variety of extracellular markers including but not limited to CD45, CD8, CD4, CD49a, CD49b, CD103, CD69, and NK1.1. Gating for different cell populations will depend on the experiment and the desired cell populations. It can also be used for standard intracellular cytokine/protein staining as described for the transcription factor Eomes in (Shannon et al., 2021).

Determining VACV titer in the labial mucosa

⌚ Timing: 3 days

Note: Digested samples from step 5c can be stored at -80°C .

⚠ **CRITICAL:** When titering animal tissues, an antibiotic must be added to the dilution tubes. We generally use 1 mL of gentamicin solution (at 50 mg/mL) in 500 mL of infection medium.

18. Plate 7.5×10^5 143B cells/well into 6-well plates. Incubate cells overnight at 37°C with 9% CO_2 .
19. Freeze-thaw digested samples three times.
20. Serially dilute samples ten-fold.
 - a. Prepare 6 1.5 mL microcentrifuge tubes and label as 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} .
 - b. Add 1080 μ L infection medium to each tube.
 - c. Add 120 μ L of digested sample from 1 mL vial to 10^{-1} tube and mix well by pipetting.
 - d. Transfer 120 μ L of 10^{-1} to 10^{-2} and mix well by pipetting.
 - e. In the same manner, continue to dilute 1:10 from 10^{-2} to 10^{-6} .
21. Follow steps 29 – 34 of viral titration above (see “virus titration by plaque assay” section).

Visualizing the labial mucosal immune response using intravital microscopy

⌚ Timing: 1–8 h

22. Prepare mouse for intravital microscopy.
 - a. Collect mouse from vivarium at desired time post-infection.
 - b. Place animal in induction chamber and induce anesthesia using 2% isoflurane. Alternatively, an injectable anesthetic can be used for initial anesthesia (we use avertin).
 - c. Sterilize the outer lip by alternating scrubs of Betadine and 70% ethanol, taking care not to disturb the labial mucosa.
 - d. Make two small incisions (approximately 2 mm) at the angles of the mouth using dissecting scissors. Limit the size of the incisions to prevent bleeding.
 - e. Using tweezers, very gently pull the lip through a mini-stage and attach using either Vetbond or cyanoacrylate adhesive. Manipulate the lip gently to avoid bruising this tissue. The lip should be placed as flatly as possible or the mucosa will be out of focus.

Note: Different mini-stages can be used to stabilize the lip. We have a pre-made stainless steel mini-stage; however, a thin, double-sided razor blade trimmed of its sharp edges can also be used.

- f. Place a water-based lubricating gel on the inner lip and turn mouse so that the lip makes stable, firm contact with a glass cover slip. Any air bubbles will prevent imaging.
- g. Tape both sides of the mini-stage firmly to the microscope stage to prevent movement and ensure contact with the coverslip.

- h. Gently attach a nosecone to the mouse with surgical tape so as not to disturb the lip/mini-stage. Secure nosecone and tubing to microscope stage using surgical tape. Maintain anesthesia at a concentration of 1.75% or as otherwise determined.

Note: Illustrations of the mini-stage and mouse preparation can be found here ([Shannon et al., 2019](#)).

23. Image the labial mucosal immune response using intravital microscopy.
 - a. Place the prepared mouse on the microscope in the heated environmental chamber. We typically use a Leica DMI8 inverted five-channel confocal microscope equipped with an environmental chamber and dual multiphoton lasers: a MaiTai and an InSight DeepSee. We use ultrasensitive hybrid detectors and a 25 X water-immersion objective.
 - b. Maintain continuous, steady heat throughout the experiment (using a heated environmental chamber).
 - c. The signal generated using second harmonic generation of collagen can be used to determine the lamina propria versus the mucosal epithelium.
 - d. Mice may be imaged for multiple hours using this setup. Ensure an appropriate amount of water-based lubricant is applied such that the lip does not dry.

Note: One advantage of using VACV as a model of acute viral infection is the availability of numerous recombinant and deletion viruses. We visualize VACV-infected cells using fluorescent-reporter viruses ([Hickman et al., 2013](#)). Various reporter mice can be used to image the immune response. We have encountered good success imaging both GFP and ZsGreen expression in the oral mucosa. To visualize blood vessels in the lamina propria, fluorescently labeled CD31 antibody can be injected intravenously prior to surgical preparation.

EXPECTED OUTCOMES

Infection of wild-type mice with VACV as described in the protocol should result in a robust infection of all animals that is controlled by the adaptive immune response within 14 days. Immunodeficient animals may succumb to infection.

LIMITATIONS

We have experienced variability in infections based on the location of mucosal infection; sometimes the location of infected cells prevents easy imaging. As mice are euthanized at the end of imaging periods before recovering from anesthesia, this method does not allow sequential imaging of the same animal at different points in time. As with any viral infection, the initial dose of the virus is critical and mouse age and inoculum should be consistent between groups.

TROUBLESHOOTING

Problem 1

Wild-type mice succumb to oral mucosal VACV infection. (See [labial mucosal infection of mice with VACV](#), step 2).

Potential solution

Take care to avoid inhalation of the viral inoculum (leading to unintended lung infection). Use older mice or a lower number of pokes of virus.

Problem 2

Weak staining of specific cell surface antigens (markers) by flow cytometry. (See [enzymatic tissue digestion to process labial mucosal tissue](#), step 17).

Potential solution

Try a different digestion method or decrease incubation period during digestion. Titrate antibodies between lots and with different fluorophores.

Problem 3

Inconsistent or low viral titers from harvested tissues. (See [determining VACV titer in the labial mucosa](#)).

Potential solution

Change bifurcated needle after use on 5 mice (or fewer) to prevent the needle from dulling, thus impeding tissue penetration. Sonicate virus stock before infection.

Problem 4

Mice die during the intravital imaging process. (See [visualizing the labial mucosal immune response using intravital microscopy](#).)

Potential solution

Reduce the percentage of isoflurane or use an injectable anesthetic (such as avertin). However, if using an injectable, it will require repeated administration of anesthetic to consistently keep mice under.

Problem 5

Cell motility is decreased or stops during intravital imaging period. (See [visualizing the labial mucosal immune response using intravital microscopy](#).)

Potential solution

Ensure that the heated chamber (recommended) or blanket is set to appropriate temperature. If using a heated chamber, it takes ~ 2 h to reach the desired temperature.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Heather D. Hickman (hhickman@mail.nih.gov).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100790>.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the NIAID, NIH. Graphical abstract was drawn by Ethan Tyler, NIH Medical Arts Branch.

AUTHOR CONTRIBUTIONS

Conceptualization, H.D.H. and J.P.S.; methodology, H.D.H. and J.P.S.; investigation, H.D.H., J.P.S., C.R.C., and S.M.V.; writing, H.D.H. and J.P.S.; funding acquisition, H.D.H. All the authors critically reviewed, edited, and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Hickman, H.D., Reynoso, G.V., Ngudiankama, B.F., Rubin, E.J., Magadan, J.G., Cush, S.S., Gibbs, J., Molon, B., Bronte, V., Bennink, J.R., and Yewdell, J.W. (2013). Anatomically restricted synergistic antiviral activities of innate and adaptive immune cells in the skin. *Cell Host Microbe* 13, 155–168.

Shannon, J.P., Kamenyeva, O., Reynoso, G.V., and Hickman, H.D. (2019). Intravital imaging of vaccinia virus-infected mice. *Methods Mol. Biol.* 2023, 301–311.

Shannon, J.P., Vrba, S.M., Reynoso, G.V., Wynne-Jones, E., Kamenyeva, O., Malo, C.S., Cherry, C.R., McManus, D.T., and Hickman, H.D. (2021). Group 1 innate lymphoid-cell-derived interferon-gamma maintains anti-viral vigilance in the mucosal epithelium. *Immunity* 54, 276–290.e5.