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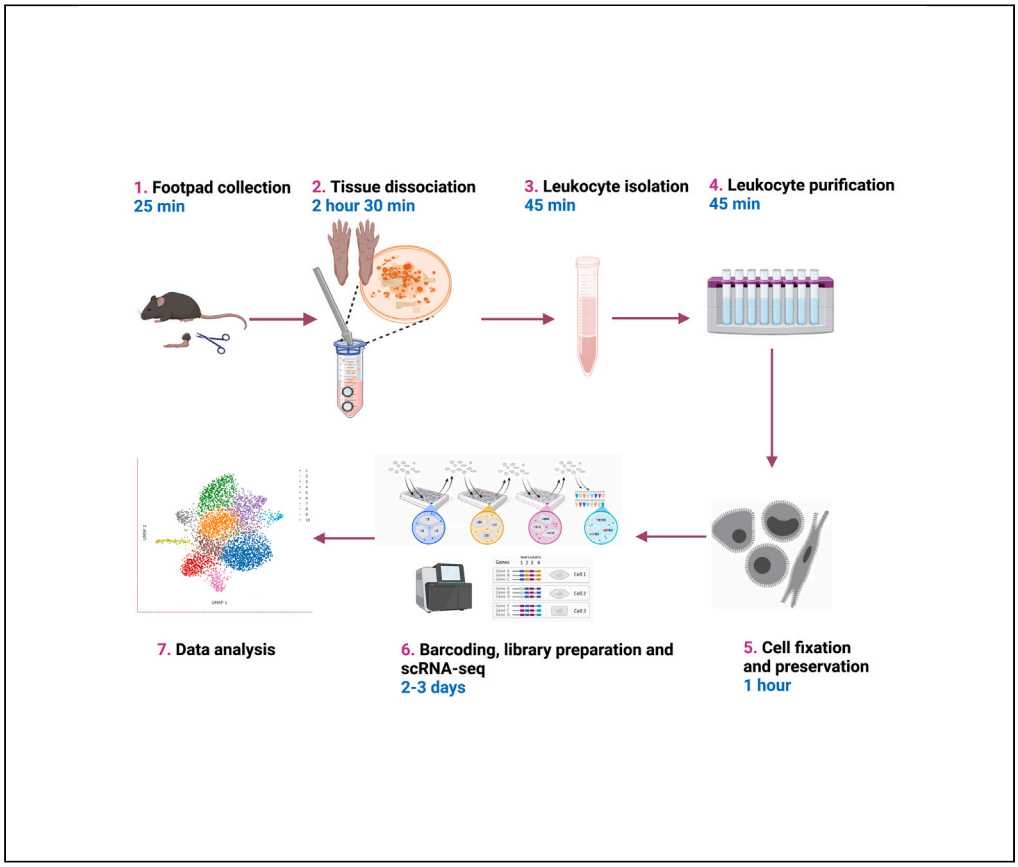
Optimized protocol for mouse footpad immune cell isolation for single-cell RNA sequencing and flow cytometry



Single-cell RNA sequencing (scRNA-seq) requires the preparation of a highly viable single-cell suspension to get reliable sequencing results. Here, we present a protocol for isolating mouse footpad leukocytes while maintaining high viability. We describe steps for footpad collection, enzymatic tissue dissociation, leukocyte isolation and purification, and cell fixation and preservation. We then detail combinatorial barcoding, library preparation, scRNA-seq, and data analysis. Cells can be used to generate a complete molecular atlas at the single cell level.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Muddassar Hameed, Pallavi Rai, Melissa Makris, James Weger-Lucarelli

weger@vt.edu

Highlights

Isolation of immune cells from mouse footpads with high viability

Purify immune cells using the CD45-positive selection kit

Fix immune cells for scRNA-seq

Perform scRNA-seq to generate a complete molecular atlas at the single-cell level

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Optimized protocol for mouse footpad immune cell isolation for single-cell RNA sequencing and flow cytometry

Muddassar Hameed,[1,2] Pallavi Rai,[1,2] Melissa Makris,[1] and James Weger-Lucarelli[1,2,3,4,*]

[1]Department of Biomedical Sciences and Pathobiology, VA-MD Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24060, USA

[2]Center for Zoonotic and Arthropod-borne Pathogens, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, USA

[3]Technical contact

[4]Lead contact

*Correspondence: weger@vt.edu
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SUMMARY

Single-cell RNA sequencing (scRNA-seq) requires the preparation of a highly viable single-cell suspension to get reliable sequencing results. Here, we present a protocol for isolating mouse footpad leukocytes while maintaining high viability. We describe steps for footpad collection, enzymatic tissue dissociation, leukocyte isolation and purification, and cell fixation and preservation. We then detail combinatorial barcoding, library preparation, scRNA-seq, and data analysis. Cells can be used to generate a complete molecular atlas at the single cell level.

BEFORE YOU BEGIN

This protocol describes the step-by-step procedure for mouse footpad collection, skin removal, tissue dissociation to isolate single cells, isolation of leukocytes with positive selection of $CD45^+$ cells, and subsequent processing for scRNA-seq and flow cytometry. Isolated cells should be processed immediately to avoid cell death or transcriptional changes. Key reagents and equipment should be prepared before starting the experiment, which is critical to maintain high cell viability. All experiments were performed with the approval of Virginia Tech's Institutional Animal Care & Use Committee (IACUC) under protocol number 21-041.

Background

The protocol describes leukocyte isolation and characterization from mouse footpads. Footpad inoculation of various pathogens, including several alphaviruses like chikungunya, Ross River and Mayaro viruses (MAYV) leads to prominent footpad swelling, consistent with clinical disease observed in humans; thus, understanding the immunological response to infection in the footpad is critical to identify therapeutic strategies. Here, we collected MAYV infected animals' footpads at peak swelling (7 dpi),[1–4] and processed the cell for scRNA-seq and flow cytometry. While this protocol is validated for mouse tissues, it can be adopted for tissues from other species, including humans.

Prepare before beginning the experiment

1. Flow cytometry staining (FACS) buffer.

Phosphate buffered saline (PBS) supplemented with 2% heat inactivated fetal bovine serum (FBS).

2. 10% Roswell Park Memorial Institute 1640 media

Roswell Park Memorial Institute 1640 (RPMI-1640) media supplemented with 10% FBS.

3. 2 mM EDTA FACS buffer.

FACS buffer containing 2 mM ethylenediaminetetraacetic acid (EDTA).

4. Digestion media.

10% RPMI-1640 media with 2.5 mg/mL Collagenase I and 17 μg/mL DNase I.

5. 4% Formalin.

4% formalin in autoclaved distilled water.

6. Zombie aqua dye.

Prepare Zombie aqua dye according to manufacturer's instructions (Zombie Aqua Fixable Viability Kit (biolegend.com)). Briefly, add 100 μL DMSO into the lyophilized reagent and vortex to mix. Spin briefly and store in aliquots at −20°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified rat anti-mouse CD16/CD32 antibody 1/100	BD Biosciences	Cat. No 553142
PerCP/Cyanine5.5 anti-mouse/human CD11b antibody 1/200	BioLegend	AB_893233 (Cat. No. 101227)
Alexa Fluor 700 anti-mouse CD45 antibody 1/200	BioLegend	AB_493714 (Cat. No. 103127)
APC anti-mouse Ly-6G antibody 1/100	BioLegend	AB_2227348 (Cat. No. 127614)
PE anti-mouse CD3 antibody 1/100	BioLegend	AB_312663 (Cat. No. 100206)
PerCP/Cyanine5.5 anti-mouse CD4 antibody 1/100	BioLegend	AB_2563023 (Cat. No. 116012)
FITC anti-mouse CD8a antibody 1/200	BioLegend	AB_312745 (Cat. No. 100706)
PE anti-mouse Ly-6C antibody 1/400	BioLegend	AB_1186133 (Cat. No. 128007)
Chemicals, Peptides, and Recombinant Proteins		
Collagenase I	Worthington Biochemical Corporation	Cat. No. LS004196
DNase I	Worthington Biochemical Corporation	Cat. No. LS006333
RPMI 1640	Genesee scientific	Cat. No. 25-506N
PBS	Genesee scientific	Ref. No. 25-507B
Heat inactivated fetal bovine serum	R&D systems	Cat. No. S11150H
Bovine serum albumin (BSA)	Thermo Fisher Scientific	Cat. No. FERB14
16% formaldehyde solution (w/v)	Thermo Fisher Scientific	Ref. No. 28908
Zombie aqua fixable viability kit	BioLegend	Cat. No. 423101
1× RBC lysis buffer	Thermo Fisher Scientific	Cat. No. 00-4333-57
Mono-Poly medium	MP Biomedicals	Cat. No. 091698049
SUPERase·In	Thermo Fisher Scientific	AM2694
Evercode cell fixation V2 kit	Parse Biosciences	ECF2001
Evercode whole transcriptome - up to 48 samples and 100k cells/nuclei per experiment	Parse Biosciences	EC-W01030

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Isoflurane (FLURISO)	Vet one	NDC 13985-528-60
0.5 mL insulin syringe	BD	Ref. No.329461
5 mL polycarbonate tubes	Genesee Scientific	Cat. No. 24-285
15 mL Conical tubes	Genesee Scientific	Cat. No. 28-103
50 mL tubes	Genesee Scientific	Cat. No. 28-106
Advanced cell strainers (70 μm)	Genesee Scientific	Cat. No. 25-376
PrimeFlow 96 well plates (V bottom)	Thermo Fisher Scientific	Ref. No. 44-17005-46
Ultracomp ebeads	Thermo Fisher Scientific	Cat. No. 01-2222-42
Trypan blue stain 0.4%	Thermo Fisher Scientific	Ref. No. 15250-061
Hausser Scientific Bright-line counting chamber	Thermo Fisher Scientific	Cat. No. 02-671-51B
Evos 5000 imaging system	Thermo Fisher Scientific	Cat. No. AMF5000
Heraeus multifuge x3R	Thermo Fisher Scientific	Ref. No. 7500-4516
Incubator	Shel Lab	Model No. SC06AD
EasySep Mouse CD45 Positive Selection Kit	STEMCELL Technologies	Cat. No. 18945
FACSAria Fusion Flow cytometer	BD Biosciences	
Benchtop shaker Model 55S	Thermo Fisher Scientific	SER. No. 07M1102MS
Glass beads (3 mm)	Thermo Fisher Scientific	Cat. No. 11.312A
EasyEight EasySep magnet	STEMCELL Technologies	Cat. No. 18103
Bacterial and Virus Strains		
Infectious-clone derived Mayaro virus (MAYV)	Chuong et al.[5]	Strain: TRVL 4675
Experimental Models: Organisms/Strains		
C57BL/6J	The Jackson Laboratory	Strain: C57BL/6J, Male, 4–6-week-old
Software and Algorithms		
BioRender	Created with BioRender.com	https://www.biorender.com/
FlowJo™ v10	BD Biosciences	https://www.bdbiosciences.com/en-us

MATERIALS AND EQUIPMENT

Preparation for mouse footpad collection

Timing: 10 min

Prepare the following equipment and reagents:

- 1 mL 10% RPMI-1640 in 2 mL Eppendorf tubes.
- Isoflurane vaporizer.
- Sterilized surgical scissors and forceps.
- 70% ethanol.
- Institution approved bags for waste and carcass disposal.
- Tube rack for Eppendorf tubes.

STEP-BY-STEP METHOD DETAILS

Collection of footpads

Timing: 15 min (time varies depending on the number of mice)

Note: All steps should be performed in the biosafety cabinet.

1. Anesthetize mice with isoflurane.
2. Sacrifice mice by cervical dislocation.
3. Cut foot above the ankle (just below the calf muscle) and remove skin (Figures 1A and 1B).

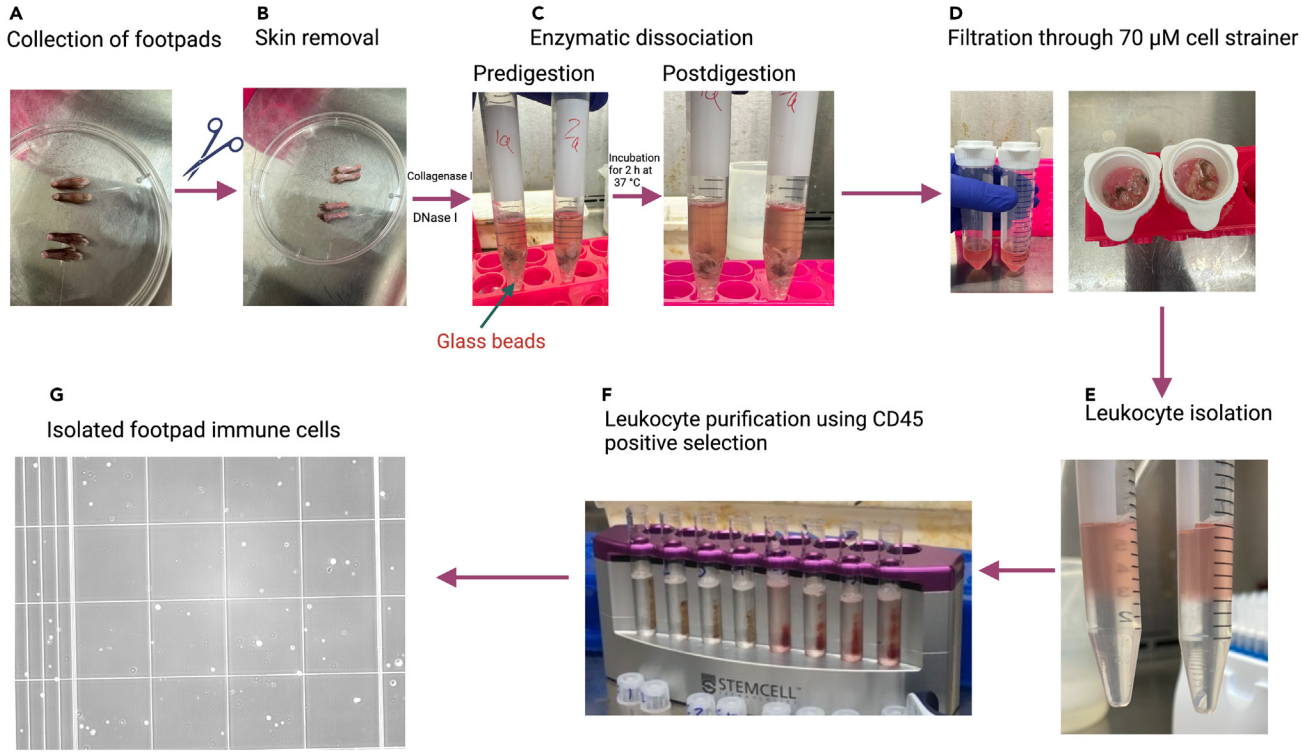

Figure 1. Isolation of single cells from mouse footpad

(A) Mouse footpads collected in RPMI-1640 medium supplemented with 10% FBS.
(B) Remove skin from footpads.
(C) Transfer deskinned footpad in 15 mL conical tube containing digestion media.
(D) Filter dissociated tissues through 70 µM cell strainer.
(E) Leukocyte isolation using Mono-Poly medium.
(F) Leukocyte purification using CD45 positive selection kit.
(G) Isolated footpad immune cells.

Tissue processing

Timing: 2:30 h

4. Aliquot 5 mL freshly prepared digestion media into a 15 mL conical tube containing glass beads and wrap parafilm outside of cap to avoid leakage (*5 footpads in a single 15 mL tube, each foot pad cut into 3 pieces, 5 glass beads/tube*).
   a. Optional: Add SUPERase·In (4 units per mL) to block RNase activity if preparing samples for scRNA-seq.

   Note: We have added glass beads to promote physical disruption of footpad tissues.

5. Shake vigorously for 2 h at 37°C in a benchtop shaker (>180 rpm).

   Note: Manual shaking can be performed every 15 min if a shaker is not available.

6. Pass tissue digests through a 70 µm cell strainer by pouring mixture from the 15 mL conical tube directly onto the strainer fitted in a 50 mL tube (Figure 1D).

   Note: The foot will still be relatively intact, but cells have been digested out of the tissue.

7. Wash strainer with 10 mL cold 10% RPMI-1640 media.
8. Pellet cells by centrifugation (400 × *g*, 5 min, 4°C) and remove supernatant.
9. Resuspend cell pellet into 3 mL 10% RPMI-1640 media (this media should be at room temperature, because we will isolate cells by using a mono-poly medium).

Protocol for footpad leukocyte isolation using mono-poly medium

Timing: 45 min

10. Immediately before use, mix mono-poly medium (M-P M) well by inverting at least 3–5 times and add 3 mL M-P M to a sterile 15 mL conical tube.
    a. Carefully layer 3 mL footpad cell solution onto the M-P M (Place tube at a slight vertical angle and slowly add media containing cells).
    b. Centrifuge at 300 × *g* for 30 min in a swinging bucket rotor at room temperature (20°C–25°C) (Figure 1E).

Note: Set the centrifuge machine brake at zero.

11. Collect cell layers between RPMI-1640 media and M-P M to isolate mononuclear and polymorphonuclear cells and add to a 15 mL conical tube containing 10 mL cold FACS buffer. There will be a thick layer at the RPMI-1640 and M-P M interface. The second layer is often small or not visible. The first layer mostly contains mononuclear cells, while the second layer contains polymorphonuclear cells.

Note: For CD45 positive selection, FACS buffer should be supplemented with 2 mM EDTA.

12. Centrifuge at 400 × *g* for 10 min at 4°C.
13. Resuspend in 1 mL FACS buffer.
    a. For scRNA-seq, proceed for CD45 purification.
    b. For flow cytometry, wash cells with FACS buffer, perform cell counting and proceed with staining in V bottom 96 well plate.

CD45 positive selection for leukocyte purification

Timing: 45 min

Note: We have performed CD45 based selection because we had many samples, and it was more convenient to all samples at a time with this kit to maintain higher viability of the isolated cells. But users can choose other purification methods such as fluorescence activated cell sorting (FACS) depending on sample size, the required number of cells and viability percentage.

14. Add sample to a 5 mL (12 × 75 mm) polystyrene round-bottom tube (Figure 1F)
15. Prepare Selection Cocktail from EasySep Mouse CD45 Positive Selection Kit. For each 1 mL sample, prepare 100 μL of cocktail (50 μL of component A + 50 μL of component B).

Note: Selection Cocktail must be prepared fresh before each use.

16. Add Selection Cocktail to sample. 100 μL/mL of sample, mix with pipette and incubate at room temperature for 5 min.
17. Take out Vortex RapidSpheres™ from EasySep Mouse CD45 Positive Selection Kit and vortex for 30 s.

Note: Particles should appear evenly dispersed.

18. Add 50 μL of RapidSpheres™ per 1 mL of sample, mix with pipette and incubate at room temperature for 3 min.
19. Add 2.5 mL FACS buffer to the tube. Mix by gently pipetting up and down 2–3 times. Place the tube (without lid) onto the magnet and incubate for 5 min at room temperature (Figure 1F).
20. Pick up the magnet, and in one continuous motion invert the magnet and tube into a waste container. Leave the magnet and tube inverted for 2–3 s, then return upright. Do not shake or blot off any drops that may remain hanging at the lip of the tube while pouring off the supernatant.
21. Repeat steps 19 and 20 three more times. During each separation period, cells need to be in 2.5 mL FACS buffer in the upright position for 5-min (total of 4 × 5-min separations).
22. Remove the tube from the magnet; this tube contains the isolated cells.
23. Resuspend cells in 1 mL FACS buffer. Make sure to collect cells from the sides of the tube.

Isolated cells are ready to use for scRNA-seq. We can process these cells directly for single cell barcoding, library preparation and sequencing. In addition, we can fix these cells to store at −80°C and process later for scRNA-seq.

In the present study, we fixed cells using the Parse fixation kit and preserved them at −80°C. Then we sent samples on dry ice from Virginia Tech to the University of Michigan for library preparation and sequencing. We followed the standard fixation protocol provided by Parse biosciences with some minor modifications such as: increased centrifuge speed from 200 × *g* to 400 × *g* and resuspended cell pellet by flicking after removing the supernatant but before adding cell buffer; this will result in better resuspension of the pellet after step 28 www.parsebiosciences.com/products/evercode-whole-transcriptome.

Preparation of cells for scRNA-seq

Timing: 10 min

24. **Cell counting**: Take 10 μL suspended cells + 10 μL 0.4% trypan blue, mix and use microscope or automated cell counter to count cells and determine viability percentage.

Note: We recommend use of manual cell counting for scRNA-seq as we found it to be more consistent (Figure 2).

25. Prepare the following items before starting the cell fixation:
    a. Coat 15 mL conical tubes with 1% BSA.

Note: Each sample will require one additional 15 mL conical tube during the fixation workflow.

    i. Make 1% BSA solution in sterile water.
    ii. Add 15 mL 1% BSA solution to each tube.
    iii. Incubate for 30 min at room temperature.
    iv. Pour BSA out of the tubes and use a P1000 pipette to remove the remaining BSA in the bottom of the tube.
    v. Air dry for 30 min with the uncapped tubes in the biosafety cabinet.
    vi. Proceed to fixation or store capped BSA coated tubes at 4°C for up to 4 weeks.

Note: We recommend preparing these tubes in advance.

    b. Fill ice in a bucket large enough to hold all samples and reagents.
    c. Bring your swinging-bucket centrifuge to 4°C.
    d. Prepare 10% w/v BSA in PBS according to samples (50 μL required for one sample); this will be used to prepare cell buffer for use in step 28.

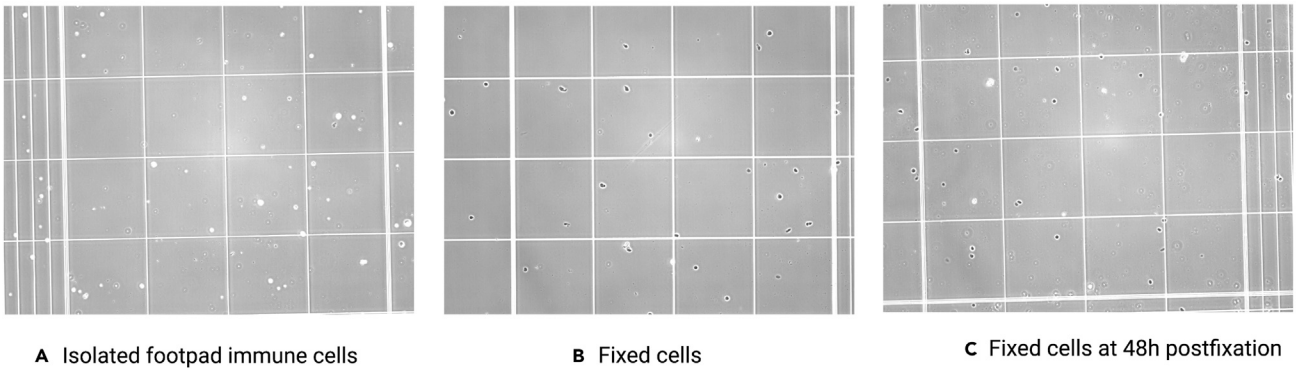

Figure 2. Immune cells isolated from mouse footpads and fixed with Evercode cell fixation V2 kit (images were taken with 10X lens)
(A) Freshly isolated immune cells.
(B) Fixed immune cells.
(C) Fixed cells at 48-h post fixation.

e. To store fixed samples at −80°C, take out a "Mr. Frosty" or similar device which enables cooling of a sample at around −1°C/min. Ensure it is at room temperature.

Note: Only proceed if you have taken out items listed in step 25. All items that require thawing should be fully thawed before starting this section. It is important that all fixation kit solutions (except DMSO) are kept on ice after thawing.

Cell fixation

Timing: 50 min

26. Transfer up to 4 million cells into a BSA coated 15 mL conical tube.

Note: No more than 4 million cells should be used for any single sample. Starting with more cells may result in elevated doublet rates. A fixed volume is not required for this step; however, it should fit into a 15 mL conical tube.

27. Centrifuge tubes in a swinging bucket centrifuge at 4°C for 400 × *g* for 10 min.

Note: During centrifugation, prepare 1 mL of cell buffer (provided in Evercode cell fixation V2 kit) containing 0.5% BSA per sample. Do not add BSA directly to the cell buffer tube as you will need a cell buffer without BSA for later use. Prepare cell buffer + BSA in a separate tube and keep the original tube on ice.

28. Carefully remove the supernatant and fully resuspend pellet in 750 μL of cold cell buffer containing 0.5% BSA.
    a. Note: Resuspend pellet by flicking after removing the supernatant but before adding cell buffer; this will result in better resuspension of the pellet. Failure to fully resuspend cells may lead to elevated doublet rates.
29. Pipette cells through a 40 μM strainer (provided with Evercode cell fixation V2 kit) into a new 15 mL conical tube. Place cells back on ice.

Note: To ensure that all the liquid passes through the strainer, gently press the tip of the pipette against the filter and press the pipette plunger down steadily. All the liquid should pass through the strainer in ~1 second.

30. Add 250 μL of cold fixation solution and mix immediately by pipetting up and down exactly 3 times (with the pipette still set to 250 μL).

Note: Do not perform additional mixing at this step.

31. Wait 10 min with cells on ice.
32. Add 80 μL of cold permeabilization solution provided with Evercode cell fixation V2 kit. Mix by pipetting up and down 3 times with a P1000 set to 250 μL. Wait 3 min with cells on ice.

Note: Do not vortex the neutralization buffer provided with Evercode cell fixation V2 kit. To mix, invert the tube five times.

33. Add 4 mL of cold neutralization buffer and gently invert the tube once to mix. Place cells back on ice.
34. Centrifuge tube in swinging bucket centrifuge at 4°C set to 400 × *g* for 10 min. Aspirate and discard the supernatant using a P1000 pipette.
35. Resuspend cells in cell buffer using a P1000 pipette and place back on ice. If you started with more than 500,000 cells, resuspend in 300 μL of cell buffer without BSA. If you started with fewer than 500,000 cells, resuspend in 150 μL of cell buffer without BSA.
36. Pass cells through a 40 μM strainer into a 1.5 mL Eppendorf tube with a P1000. Keep samples on ice.

Note: These Eppendorf tubes should be properly labeled with key identifying information such as experiment number, pathogen and tissue name, date of collection, researcher initials, etc.

37. Add DMSO to samples as follows:
    a. If you started with more than 500,000 cells, add 5 μL of DMSO. If you started with fewer than 500,000 cells, add 2.5 μL of DMSO. Gently flick the tube 3 times to mix and wait 1 min with samples on ice.
    b. Repeat the previous step two more times to add a total of 15 μL of DMSO if you started with more than 500,000 cells, or 7.5 μL of DMSO if you started with fewer than 500,000 cells.
    c. Mix the final suspension by gently pipetting up and down five times with a P200 to ensure that the DMSO is well mixed. To avoid creating bubbles, set the P200 to 150 μL if you started with more than 500,000 cells, or 75 μL if you started with fewer than 500,000 cells.

Note: Do not vortex cells. At this step, we can split cells into aliquots in Eppendorf tubes before freezing if the cells will be run in multiple barcoding experiments. Place tubes containing cells into a Mr. Frosty (or similar device which enables cooling at around −1°C/min) and place into a −80°C freezer.

After fixation, cell counting was performed, and cell morphology was evaluated by microscopy (10X magnification) as shown in Figure 2. At 48 h post fixation, we again performed cell counting and took images to assess thawing effect on cells.

We loaded 8333 fixed footpad cells from each sample for three rounds of barcoding followed by lysis to isolate barcoded cDNA and prepare sub libraries. These sub libraries were sequenced and generated 30–60 thousand reads from each cell according to the standard Parse Biosciences scRNA-seq protocol www.parsebiosciences.com/products/evercode-whole-transcriptome and the detected cell clusters from mock-infected and MAYV-infected mice footpad cells are presented in Figure 3.

Note: We used Parse Biosciences kits, but users can choose other platforms depending on sample size, the required sequencing depth, and the budget.

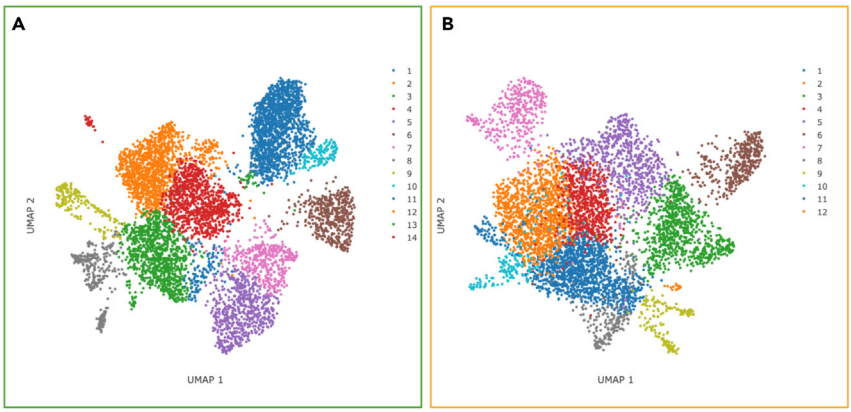

Figure 3. scRNA-seq from mouse footpad cells

(A) Uniform manifold approximation and projection (UMAP) of the identified cell clusters from mock infected animals footpad cells at 7dpi.

(B) UMAP plot of MAYV infected animals footpad cells at 7 dpi.

We have also performed flow cytometry from the isolated footpad cells to assess immune cell infiltration in MAYV-infected mice; data is presented in Figure 4.

Extracellular staining and fixation for flow cytometry

Timing: 2 h

38. Transfer up to 1 × 10^6 cells from each sample into a V bottom 96 well plate.
39. Pellet cells by centrifuging at 400 × *g* for 5 min at 4°C and discard supernatant. Again, wash cells with 200 μL PBS.
40. Dilute Zombie aqua dye at 1:400 in PBS. Resuspend cells in diluted 100 μL Zombie aqua solution.

Note: Don't use Tris buffer as a diluent and be sure that the PBS does not contain any other protein like BSA or FBS.

41. Incubate the cells at room temperature, in the dark, for 30 min.
42. Add 100 μL FACS buffer, pellet cells by centrifuging at 400 × *g* for 5 min at 4°C and discard supernatant.
43. Resuspend cells in 100 μL Fc antibody solution (1 μg per one million cells) and incubate for 15 min on ice. Keep one sample as an unstained control.
44. Prepare combined antibody solution for each sample. Here, we prepared two antibody cocktails: one for gating innate immune cells (CD45 0.125 μg, CD11b 0.125 μg, Ly6G 0.06 μg, Ly6C 0.06 μg) and other for gating adaptive immune cells (CD45 0.125 μg, CD3 0.25 μg, CD4 0.25 μg, CD8a 0.50 μg). We used UltraComp eBeads compensation beads as a single-color control for each antibody to achieve reliable and robust positive and negative populations for the fluorochromes listed in separate panels.
45. Add 100 μL FACS buffer containing antibodies to each sample, resuspend cells and incubate on ice for 30 min in dark.
46. Centrifuge at 400 × *g* for 5 min at 4°C and discard supernatant.
47. Add 200 μL FACS buffer, pellet cells by centrifuging at 400 × *g* for 5 min at 4°C and discard supernatant.
48. Again, wash cells with 200 μL FACS buffer.
49. Resuspend cells in 200 μL 4% formalin and incubate for 30 min at room temperature in dark.

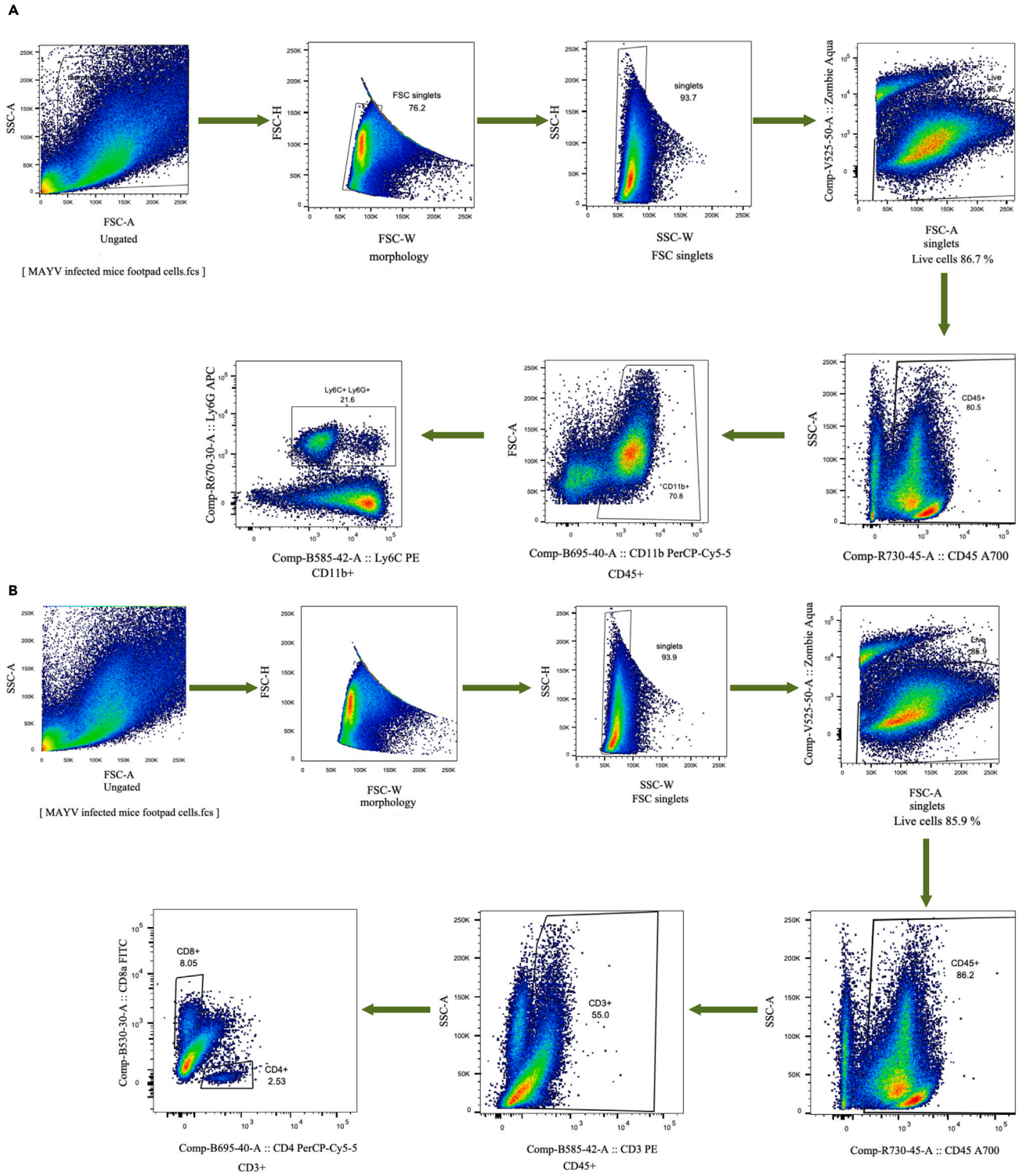

Figure 4. Flow cytometry gating strategy to identify innate and adaptive immune cells population in MAYV infected mice footpads (n = 6) at 7 dpi

(A) Innate immune cells ($CD45^+CD11b^+Ly6G^+Ly6C^+$) in MAYV infected animals footpads.

(B) T cytotoxic ($CD45^+CD3^+CD8a^+$) and T helper ($CD45^+CD3^+CD4^+$) lymphocytes in MAYV infected animals footpads.

50. Centrifuge at 400 × *g* for 5 min at 4°C and discard supernatant.
51. Wash cells twice with 200 μL FACS buffer.
52. Resuspend cells in 200 μL PBS, cover plate in aluminum foil and proceed to analyze cells using a flow cytometer equipped with the necessary lasers and detectors. We captured 100,000 cells/events for each sample.

EXPECTED OUTCOMES

The present single cell isolation protocol is optimized to isolate immune cells from mouse footpads (Figure 1). Following this protocol, we obtained approximately 200,000–300,000 and 200,000–400,000 cells per foot of non-infected and infected 6–8 week old mice, respectively. It is likely that cell yield will vary depending on the age and infection status of the animal (e.g., infecting virus, days post infection, footpad swelling, mouse strain, etc.). It is expected that more than 80% leukocytes will be viable as determined by manual cell counting with trypan blue and flow cytometry with Zombie aqua dye. This protocol allows for the isolation of single cells with high viability, which is required for scRNA-seq. This protocol can likely be adapted to isolate single cells from other experimental lab animals such as guinea pigs, hamsters, rats, rabbits, primates, etc. and human skin samples collected through punch biopsy by changing collagenase digestion time and concentration. This can also be useful to study conditions involving joint swelling such as rheumatoid arthritis and diabetes, as well as protozoal, bacterial, and other viral diseases. Overall, our data shows that mouse footpad immune cells isolated with this protocol can be used to investigate the cellular and molecular characteristics to define the cells pathophysiological properties and characterize their role during infections.

LIMITATIONS

Recently, scRNA-seq emerged as the state-of-the-art method for exploring the heterogeneity and complexity of individual cells at the transcriptional level.[6–9] We performed scRNA-seq from cells pooled from multiple mice. Obtaining sufficient cells from a single mouse may require further optimization. Second, we used CD45 positive selection to purify immune cells expressing CD45 markers. Our isolated single cell suspension from mouse footpads could also be used to isolate specific cell types such as neutrophils, macrophages, T lymphocytes subsets with magnetic bead based positive selection or fluorescence-activated cell sorting to characterize specific cell types more deeply. Similarly, a protocol without CD45 positive may be useful for researchers interested in studying non-hematopoietic cell types; it is possible that collecting the cells that do not bind to the CD45+ beads (e.g., epithelial, endothelial, and fibroblast cells, among others) would allow for analysis of these cells.

TROUBLESHOOTING

Problem 1

Cell yield and quality affected by using higher collagenase concentration.

Potential solution

We compared 2.5 mg/mL and 5 mg/mL collagenase concentration in digestion media and observed impact on cell yield and viability percentage. We observed that 5 mg/mL collagenase concentration yields a slightly higher number of cells; however, cell viability decreased, and more tissue debris was observed. It is possible that lower concentrations may produce higher viability if incubated for longer periods. In addition, RNase inhibitor (SUPERase•In) was added to increase RNA integrity for scRNA-seq. We added SUPERase·In during tissue dissociation, cell isolation and fixation steps.

Problem 2

Cell yield decrease if we filter more footpad suspension with single strainer.

Potential solution

We used 5 animals in each group (10 total footpads). We observed that if we digested all 10 footpads in a single tube and filtered through a single cell strainer, the overall cell yield decreased. The possible reason was that cell strainers were blocked with tissue debris. We observed improved cell yield when we digested 5 footpads in a single tube and used one filter for 5 or less footpads.

Problem 3

Single cell suspension contains tissue debris.

Potential solution

We observed that single cell suspension after digestion contained considerable tissue debris. We used M-P M to isolate immune cells and remove the debris.

Problem 4

There was a small amount of red blood cells (RBCs) observed after leukocyte isolation with M-P medium. We sought to remove all RBCs in our scRNA-seq samples. Therefore, we attempted 1× RBC lysis buffer treatment; however, when we fixed these cells, we found that there was a significant cell loss (>80%).

Potential solution

Lysis buffer treatment releases RNA which creates cell aggregation and block filters, and we were unable to get enough cell numbers as well as a single cell suspension. Therefore, we purified immune cells using the CD45 positive selection kit.

Problem 5

Perform manual cell counting at steps to determine an accurate cell number for each sample

Potential solution

An accurate cell count is necessary for the scRNA-seq workflow. We suggest performing manual cell counting before the start of cell fixation as we found that digital cell counters gave variable cell numbers from the same samples.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, James Weger: weger@vt.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code. The scRNA-seq data will be published in a future manuscript and data will be deposited in the SRA database.

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AUTHOR CONTRIBUTIONS

M.H. and J.W.-L. developed the protocol and wrote the manuscript. M.H. and P.R. performed experiments. M.M. performed flow cytometry and analyzed the raw flow cytometry data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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