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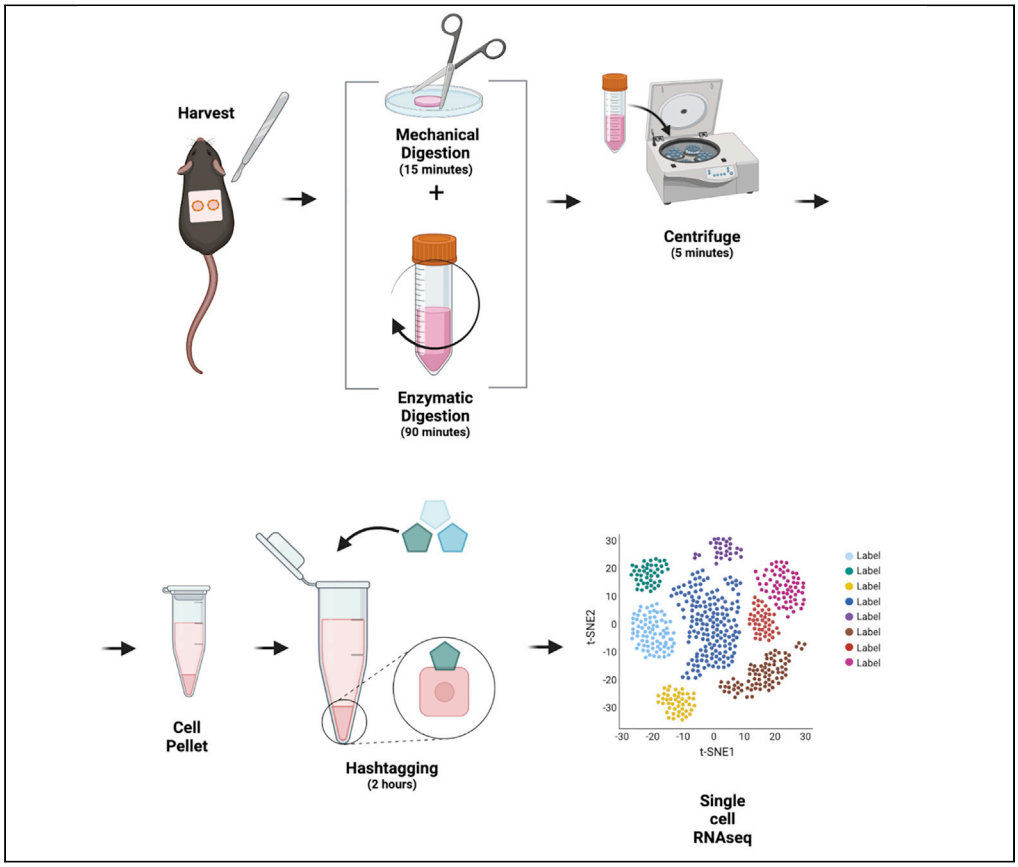
Protocol

Multiplexed evaluation of mouse wound tissue using oligonucleotide barcoding with single-cell RNA sequencing



Despite its rapidly increased availability for the study of complex tissue, single-cell RNA sequencing remains prohibitively expensive for large studies. Here, we present a protocol using oligonucleotide barcoding for the tagging and pooling of multiple samples from healing wounds, which are among the most challenging tissue types for this application. We describe steps to generate skin wounds in mice, followed by tissue harvest and oligonucleotide barcoding. This protocol is also applicable to other species including rats, pigs, and humans.

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

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Highlights

Oligonucleotide barcoding for the tagging and pooling of multiple scRNA-seq samples

Mouse wound healing using a splinted excisional model

Optimized tissue processing for cell viability throughout barcoding process

Januszyk et al., STAR Protocols 4, 101946
March 17, 2023
https://doi.org/10.1016/j.xpro.2022.101946

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Multiplexed evaluation of mouse wound tissue using oligonucleotide barcoding with single-cell RNA sequencing

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https://doi.org/10.1016/j.xpro.2022.101946

SUMMARY

Despite its rapidly increased availability for the study of complex tissue, single-cell RNA sequencing remains prohibitively expensive for large studies. Here, we present a protocol using oligonucleotide barcoding for the tagging and pooling of multiple samples from healing wounds, which are among the most challenging tissue types for this application. We describe steps to generate skin wounds in mice, followed by tissue harvest and oligonucleotide barcoding. This protocol is also applicable to other species including rats, pigs, and humans. For complete details on the use and execution of this protocol, please refer to Stoeckius et al. (2018),[1] Galiano et al. (2004),[2] and Mascharak et al. (2022).[3]

BEFORE YOU BEGIN

The protocol below describes the specific steps for using mouse dorsal wound tissue. However, we have also used this protocol for pig and human wound samples. The times presented below are listed for a cohort of ten mice and will scale accordingly.

Institutional permissions

All mice used for experiments in this protocol were housed at the Stanford University Comparative Medicine Pavilion under the care of the Department of Comparative Medicine in the Veterinary Service Center (VSC), in accordance with Stanford APLAC guidelines (APLAC-11048).

Dorsal skin wounding

Timing: 2 h

1. Create two splinted excisional wounds on the dorsum of each mouse, as described in detail in Galiano et al.[2]
   a. Site preparation.

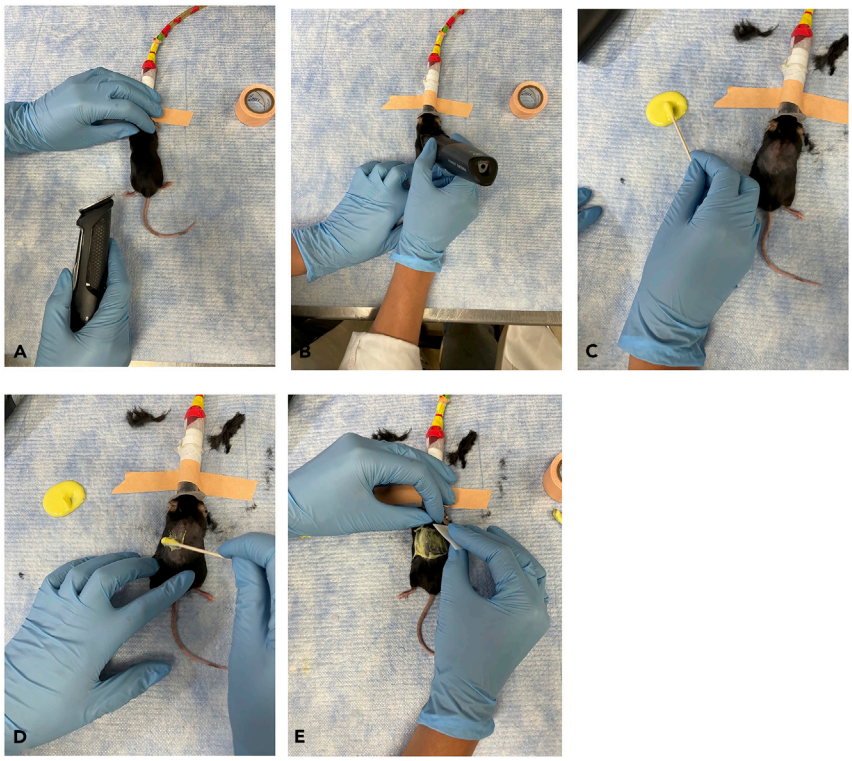

Figure 1. Site preparation I

(A–E) The mouse dorsum is first shaved from the neck to hindlimbs using a standard electric razor (A and B), after which the remaining fine hair is removed from skin using a depilatory cream (Nair) (C–E).

i. Anesthetize ten adult C57BL/6J mice (age 6–8 weeks; 5 male, 5 female) using 2% isoflurane.
ii. Shave each mouse dorsum using a standard electric razor (Figures 1A and 1B).
iii. Remove the remaining fine hair from skin using a depilatory cream (Nair) (Figures 1C–1E).
iv. Prep the dorsal skin with alternating betadine and alcohol wipes for 3 min (Figures 2A–2C). Then lay sterile surgical drapes over the surgical area to create a sterile field.

b. Wounding.
i. Prepare silicone rings using 6 and 8 mm punch biopsy tools to a standard sheet of silicone (Figures 3A–3G).
ii. Use a 6 mm punch biopsy tool to create two circular full-thickness tissue defects (extending through the panniculus carnosus), roughly 6 mm below the ears and 4 mm lateral to the midline on each side (Figures 4A–4D).
iii. Discard the excised tissue.

c. Ring placement.
i. Secure each ring around the wound perimeter with adhesive (Figures 5A–5C).
ii. Apply eight simple interrupted 6-0 nylon sutures (Ethicon) to each ring (Figures 5D and 5E).

d. Dressing application.
i. Cover both wounds with an adhesive dressing to keep the wounds sterile throughout the healing process (Figures 6A–6E).

Interval wound care

Timing: 1 h

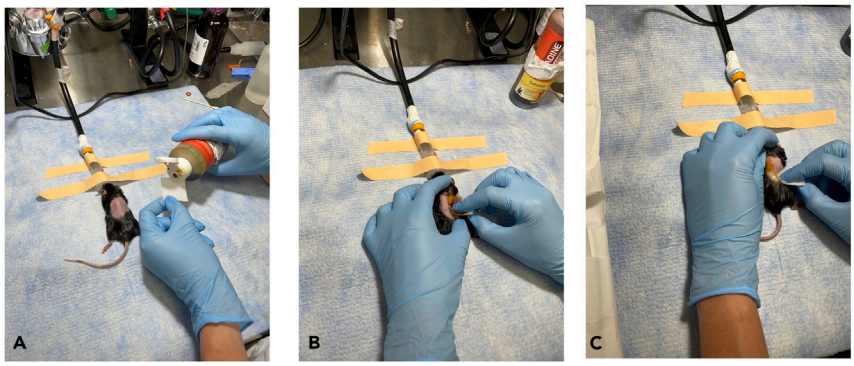

Figure 2. Site preparation II
(A–C) The dorsal skin is prepped with alternating betadine (A and B) and alcohol (C) wipes for 3 min.

2. Wound care and dressing changes.
   a. House all mice at the standard animal density (5 animals per cage at our institution).
   b. Ensure that mice undergo daily monitoring for pain or distress that may require analgesia or sacrifice.
   c. Remove dressings and clean wounds every 48 h in a sterile field under general anesthesia (2% isoflurane) (Figures 7A–7D).
   d. Replace silicone rings and apply new sutures as needed during dressing changes (typically every 1–2 weeks).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TotalSeq Series B anti-mouse hashtag antibodies 1–10 (1:50 dilution)	BioLegend	Cat# 155831, 155833, 155835, 155837, 155839, 155841, 155843, 155845, 155847, 155849; RRID: AB_2814067, AB_2814068, AB_2814069, AB_2814070, AB_2814071, AB_2814072, AB_2814073, AB_2814074, AB_2814075, AB_2814076
Anti-Mouse Monoclonal eFluor 450, CD45 (1:100 dilution)	Thermo Fisher Scientific	Cat# 48-0451-82; RRID: AB_1518806
Anti-Mouse Monoclonal eFluor 450, Ter-119 (1:100 dilution)	Thermo Fisher Scientific	Cat# 48-5921-82; RRID: AB_1518808
Anti-Mouse Monoclonal eFluor 450, CD31 (1:100 dilution)	BioLegend	Cat# 303114; RRID: AB_2114316
Anti-Mouse Monoclonal eFluor 450, Tie-2 (1:100 dilution)	Thermo Fisher Scientific	Cat# 13-5987-82; RRID: AB_466848
Anti-Mouse Monoclonal eFluor 450, CD326 (1:100 dilution)	Thermo Fisher Scientific	Cat# 48-5791-82; RRID: AB_10717090
Anti-Mouse Monoclonal eFluor 450, CD324 (1:100 dilution)	Thermo Fisher Scientific	Cat# 13-3249-82; RRID: AB_1659688
Goat Anti-Rabbit Alexa Fluor 488 (1:100 dilution)	Thermo Fisher Scientific	Cat# A32731; RRID: AB_2633280
Goat Anti-Rabbit Alexa Fluor 568 (1:100 dilution)	Thermo Fisher Scientific	Cat# A-11011; RRID: AB_143157
Goat Anti-Rabbit Alexa Fluor 647 (1:100 dilution)	Thermo Fisher Scientific	Cat# A-21245; RRID: AB_2535813
Rabbit Anti-CD45 (1:100 dilution)	Abcam	ab10558; RRID: AB_442810
Rabbit Anti-CD31 (1:100 dilution)	Abcam	ab281583
Rabbit Anti-EpCAM (1:100 dilution)	Abcam	ab221552
Chemicals, peptides, and recombinant proteins		
Fluoromount-G with DAPI	Thermo Fisher Scientific Cat	Cat# 00-4959-52
Permount	Fisher Chemicals	Cat# SP15

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Liberase DL Research Grade	Millipore Scientific	Cat# 5466202001
Biogenex Laboratories Power Block	Fisher Scientific	Cat# NC9495720
Trypsin antigen retrieval solution	Abcam	Cat# ab970
Ethanol	GoldShield	Cat# 64175
Xylene	Sigma	Cat# 534056
Triton-X 100	Sigma	Cat# X100
Phosphate buffered saline	Sigma	Cat# P5368
DAPI	BioLegend	Cat# 422801
Cell staining buffer	BioLegend	Cat# 420201
Critical commercial assays		
Agilent Bioanalyzer	Agilent	RRID: SCR_018043
Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1, 16 rxns	10X Genomics	Cat# PN-1000121
Experimental models: Organisms/strains		
Mouse: C57/BL/6J (age 6–8 weeks; 5 male, 5 female)	The Jackson Laboratory	Stock No. 000664
Other		
6-0 nylon sutures	Ethicon	Cat# 1698G
Red Silicone Rubber Sheet, 60A, No Adhesive Backing, High Temp Gasket Material	Exactly LLC via Amazon.com	Cat# B096MYWM4X
6 mm disposable biopsy punches, Integra™ Miltex®	VWR	Cat# 21909-144
8 mm disposable biopsy punches, Integra™ Miltex®	VWR	Cat# 21909-146
Tegaderm dressings	3M	Cat# 1624W
40 μm Flowmi Cell Strainer	Scienceware	Cat# H13680-0040

STEP-BY-STEP METHOD DETAILS

Part 1: Tissue harvest

Timing: 1 h

In this section, full-thickness wound tissue is excised and processed into a single cell suspension. Wounds may theoretically be harvested at any time post-surgery, but we have only tested this protocol at timepoints between 2 days and 6 months.

1. Tissue harvest and processing.
   a. Harvest each wound *en bloc* with the splinting ring in place (Figure 8A).
   b. Harvest the dorsal skin using dissecting scissors by separation below the panniculus carnosus to ensure the complete thickness of the skin is harvested (Figures 8B–8D).
   c. At this stage, up to one-third of each wound may be set aside and processed separately for histology.
   d. Isolate the remaining wound tissue from any underlying attached hypodermis with a scalpel.
   e. Rinse the resulting skin in cold PBS.
   f. To achieve a cell suspension:
      i. Finely mince the harvested skin using sharp scissors into sub-millimeter diameter pieces (Figures 8E–8H).
      ii. Minced tissue should be roughly 0.5 mm in diameter.
      iii. Enzymatically digest the minced skin using Liberase DL (0.5 mg/mL; 10 mL needed per 100 mg of tissue) for 1 h at 37°C (Figure 8I).
      iv. Filter the resulting suspension through a 40 mm nylon mesh (Figures 8J and 8K).

Optional: FACS isolation

Timing: 2 h

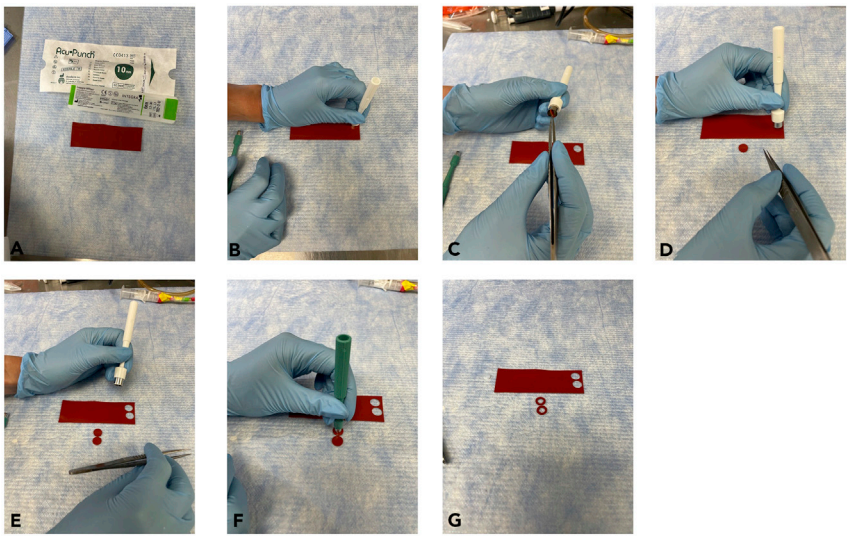

Figure 3. Creation of silicone rings

(A) The rings required for wound splinting are created from a standard sheet of silicone.

(B–G) An 8 mm punch biopsy tool is first used to create circular cutouts from this sheet (B–E), after which a 6 mm punch biopsy tool is used to carve out the center in order to generate the final silicone rings (F and G).

This optional step allows for the isolation of cell subsets prior to barcoding.

2. Optional: FACS isolation.
   a. Isolate cells from single cell suspensions using a pre-specified fluorescence-activated cell sorting (FACS) strategy.
   b. One approach to isolate fibroblasts uses a negative lineage gating strategy, wherein a lineage gate (Lin) for hematopoietic (CD45, Ter-119), endothelial (CD31, Tie2), and epithelial (CD326, CD324) cell markers are used as a negative gate to isolate fibroblasts (Lin-).[3]

Part 2: Oligonucleotide barcoding

Timing: 1.5 h

This step tags cells from each sample with unique oligonucleotide barcodes to permit their downstream identification.

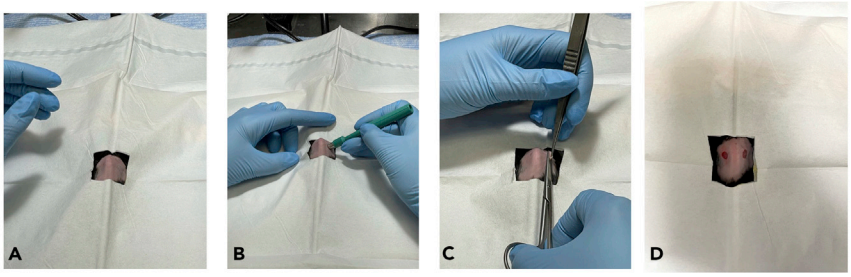

Figure 4. Wounding

(A) Sterile surgical drapes are laid over the surgical area to create a sterile field.

(B–D) A 6 mm punch biopsy tool is used to create two circular full-thickness tissue defects (extending through the panniculus carnosus), roughly 6 mm below the ears and 4 mm lateral to the midline on each side.

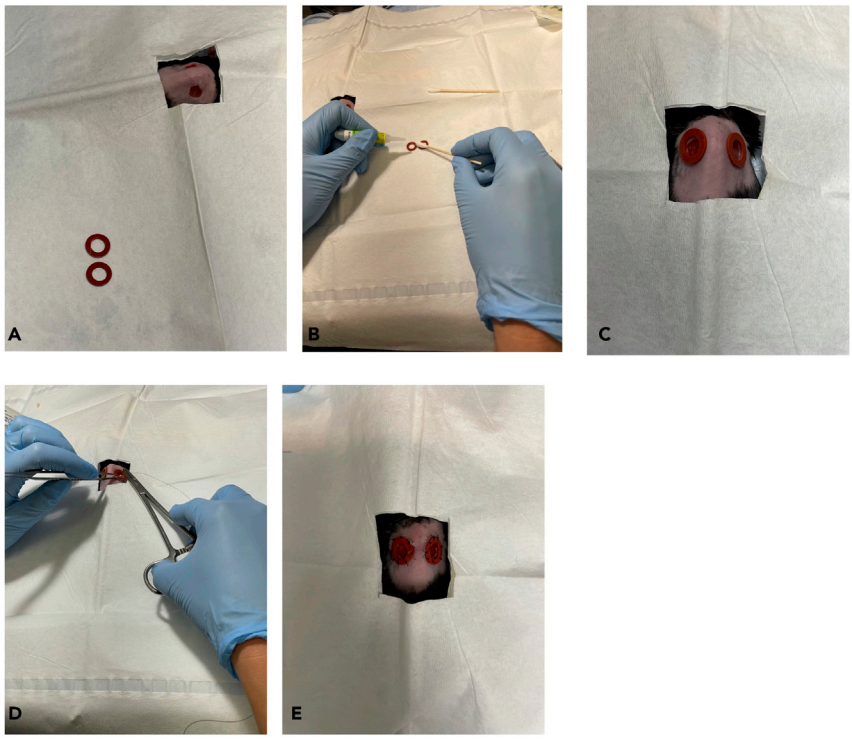

Figure 5. Ring placement

(A–E) Each ring is secured around the wound perimeter with adhesive (A–C) and eight simple interrupted 6-0 nylon sutures (Ethicon) per ring (D and E).

3. Hashtag oligonucleotide barcoding.
   a. Preparation of cell suspensions.
      i. Enzymatic digestion may cause a cleavage of tissue-specific epitopes, which may not be applicable to non-wound tissue specimens, resulting in reduced staining with TotalSeq antibodies.
      ii. Staining conditions and concentrations should be optimized separately for each tissue type.
   b. Assessment of cell viability.
      i. Perform cell counts using a hemocytometer to ensure accurate assessment of cell viability. An ideal cell viability is $\geq$ 95%.
   c. Dilute cells in an appropriate volume prior to staining.
      i. Dilute 1 million cells in 49.5 μL of Cell Staining Buffer (pre-made from BioLegend) in 1.5 mL Eppendorf tubes (Figures 9A and 9B).
      ii. If working with pig or human cells, instead dilute 1 million cells in 45 uL of Cell Staining Buffer.

   Note: Staining buffer should be stored at 2°C–8°C, for no longer than 6 months.

   d. Block cells.
      i. Add 5 μL of Human TruStain FcX Fc Blocking reagent or 0.5 μL of TruStain FcX PLUS (anti-mouse CD16/32) antibody.
      ii. The final blocking volume should be 50 μL.
      iii. Incubate for 10 min at 4°C.
      iv. While cells are incubating in Fc Block, proceed to next step.
   e. Prepare antibody pool using titrated amounts (up to 1 μg) of each TotalSeq hashtag antibodies.
      i. If the antibody cocktail volume is less than 50 μL, add Cell Staining Buffer up to 50 μL. Then centrifuge the antibody pool at 14,000 g at −8°C for 10 min before adding to the cell suspension prepared above.

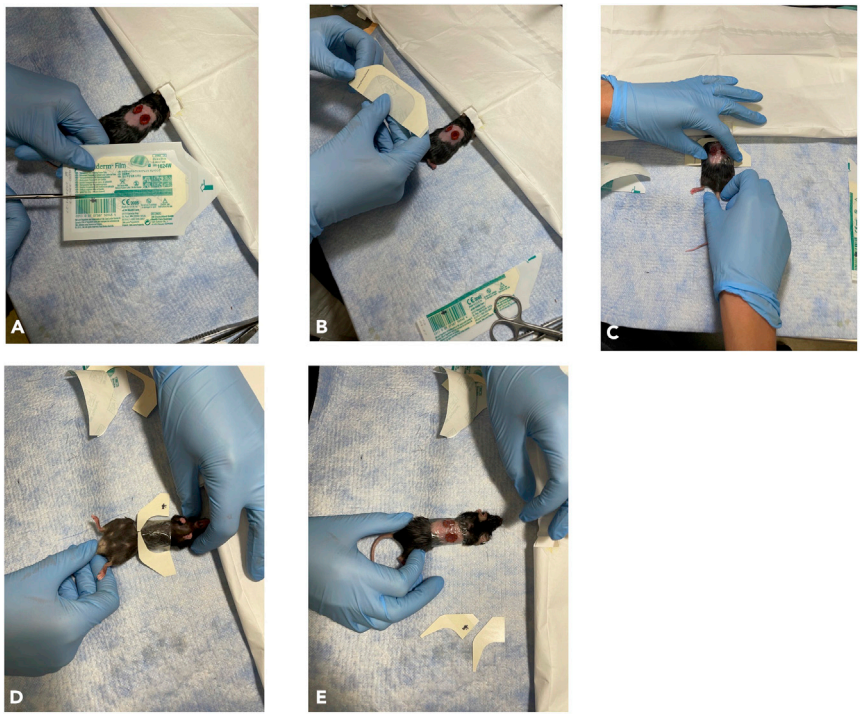

Figure 6. Wound dressing

(A–C) A small sheet of Tegaderm is cut in half lengthwise (A and B) and applied transversely to the wound region (C). (D and E) The remaining half of the Tegaderm is then applied to the mouse ventrum to create a circumferential coverage.

ii. Carefully pipette out the prepared antibody pool, avoiding the bottom of the tube, and add the 50 μL of TotalSeq antibody cocktail to the 50 μL blocked cell suspension (Figures 9C and 9D).

f. Incubate the stained suspension for 30 min at 4°C.

g. Wash the stained suspension (repeat 3 times).
   i. Add 0.5 mL of Cell Staining Buffer and spin at 4°C for 5 min at 500 g.
   ii. Carefully remove the supernatant using a pipette.

h. Following the last wash step.
   i. Resuspend the cells in PBS at a concentration of 500–800 cell/μL.
   ii. Slowly filter cells through a 40 μm Flowmi Cell Strainer (Figures 9E–9I).

i. Verify cell concentration and viability after filtration using a hemocytometer.

j. Dilute cells in PBS as necessary for appropriate input (10–20 uL) into the 10× Chromium chip for cell capture and subsequent library preparation and sequencing.
   i. Ideal loading volume is 10–20 uL per single well of the 10× Chromium controller.
   ii. Manufacturer recommendation for cell number is 10,000 cells, but well-clustered data may be obtained with as few as 1,000 cells per capture.
   iii. Our group has found that 10 samples (with unique HTO barcodes) is the ideal number for pooling per 10× well/capture, but we have had success using as few as 2 and as many as 12 pooled samples.

EXPECTED OUTCOMES

Expected number of cells from each wound in the pooled sample is 5,000 cells. If FACS sorting, expect 20%–30% loss. Lin- fraction represents 15%–35%, with Lin+ as 65%–85%. After subsequent library prep and sequencing, typically yields 4,000–8,000 cells per pooled sample.

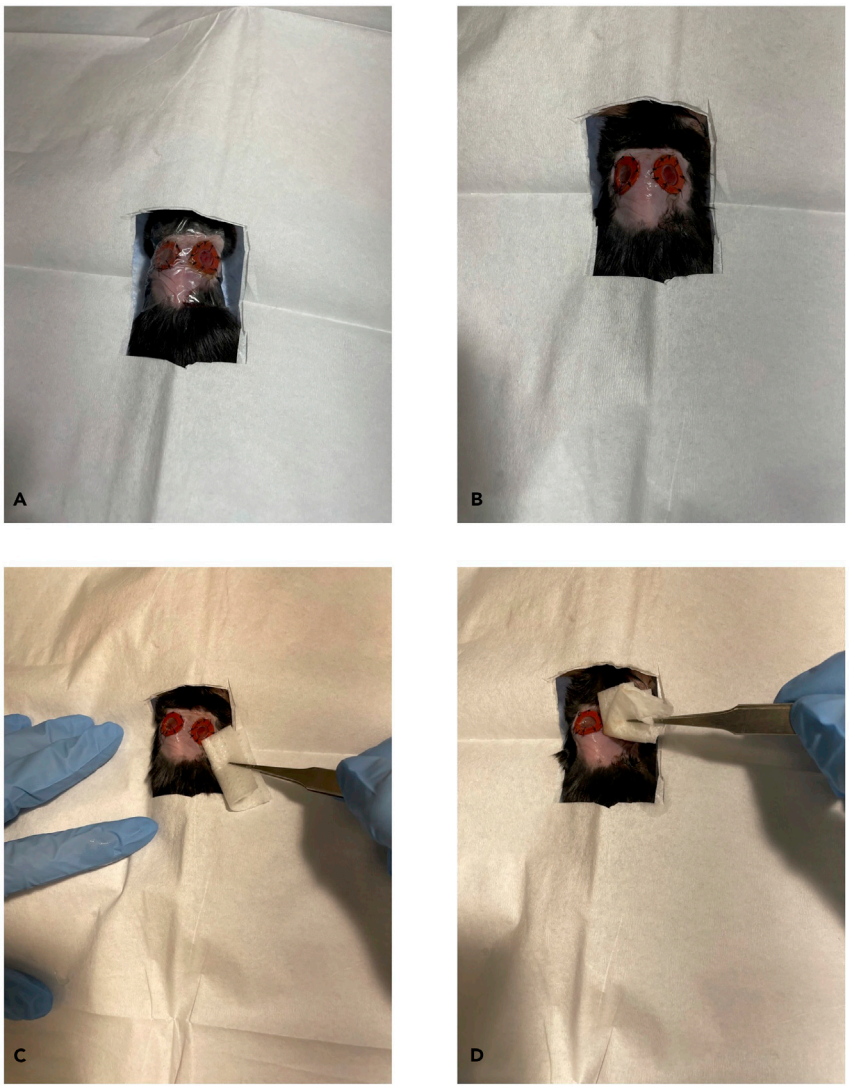

Figure 7. Dressing changes

(A–D) Dressings are removed in a sterile field under general anesthesia (2% isoflurane) (A and B), and wounds are gently cleaned with an alcohol swap using forceps (C and D).

LIMITATIONS

This approach is currently limited to a maximum of 12 hashtags per tube. It is also not compatible with nuclear extraction methods (e.g., for ATAC-seq), as it relies on ubiquitously expressed elements within the cell wall.

TROUBLESHOOTING

Problem 1

Step 3b: Presence of dead cells.

Potential solution

Optional FACS sorting step can be applied to remove dead cells. A less aggressive mincing process (step 1f) can also help to reduce the fraction of dead cells.

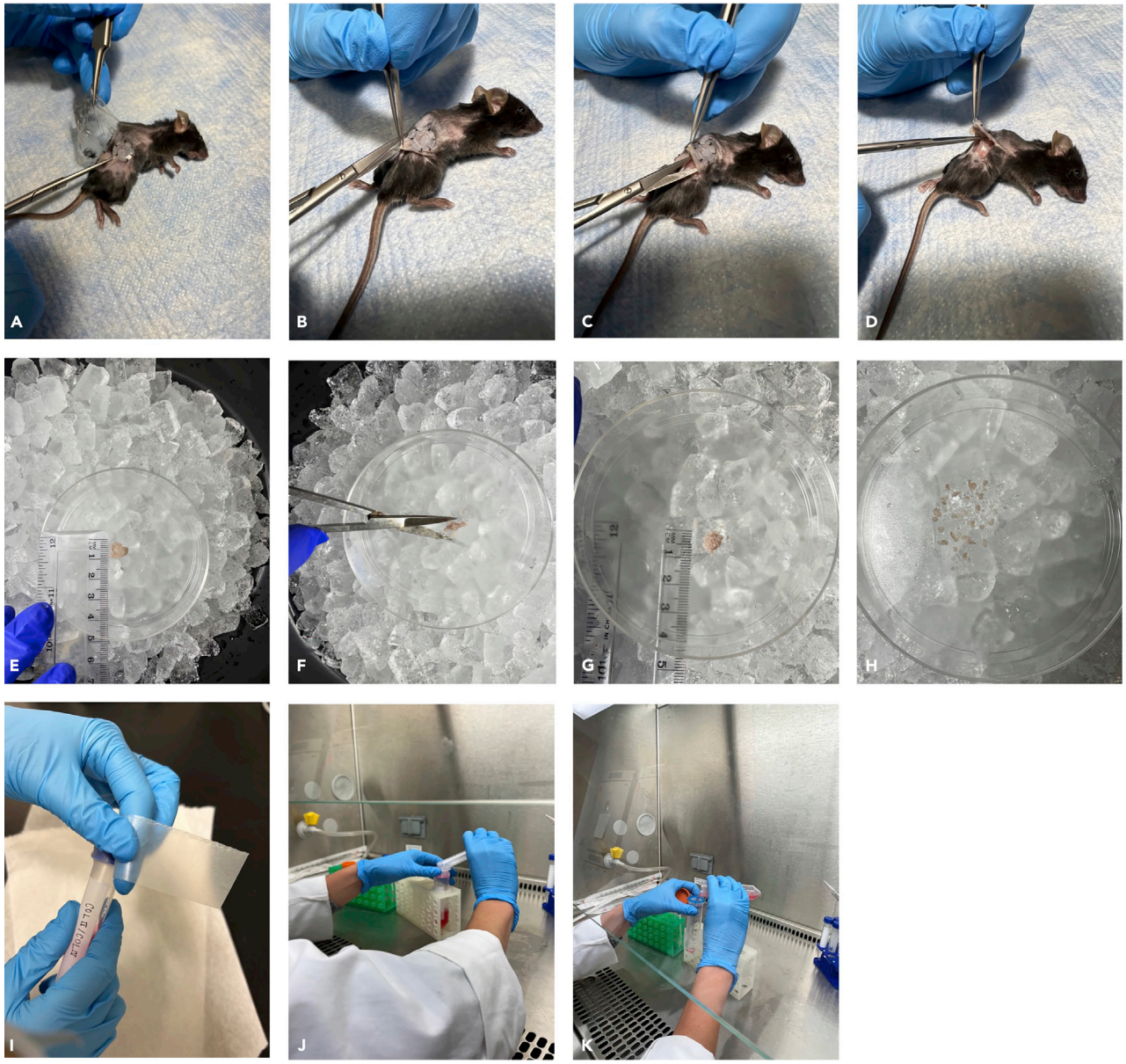

Figure 8. Wound tissue harvest

(A) The mouse is sacrificed. The adhesive dressing is removed prior to tissue harvest, but the silicone ring is left in place.

(B–D) The region of depilated dorsal skin is harvested using dissecting scissors by separation below the panniculus carnosus to ensure complete thickness.

(E–I) The skin is finely minced to sub-millimeter diameter pieces (E–H) and subjected to enzymatic digestion (I).

(J and K) The resulting suspension is then filtered through a 40 mm nylon mesh.

Problem 2

Step 3b: The cell yield is too low.

Potential solution

More finely mince the tissue to as small as 0.25 mm diameter fragments.

Problem 3

Step 3e: Hashtag signal too low.

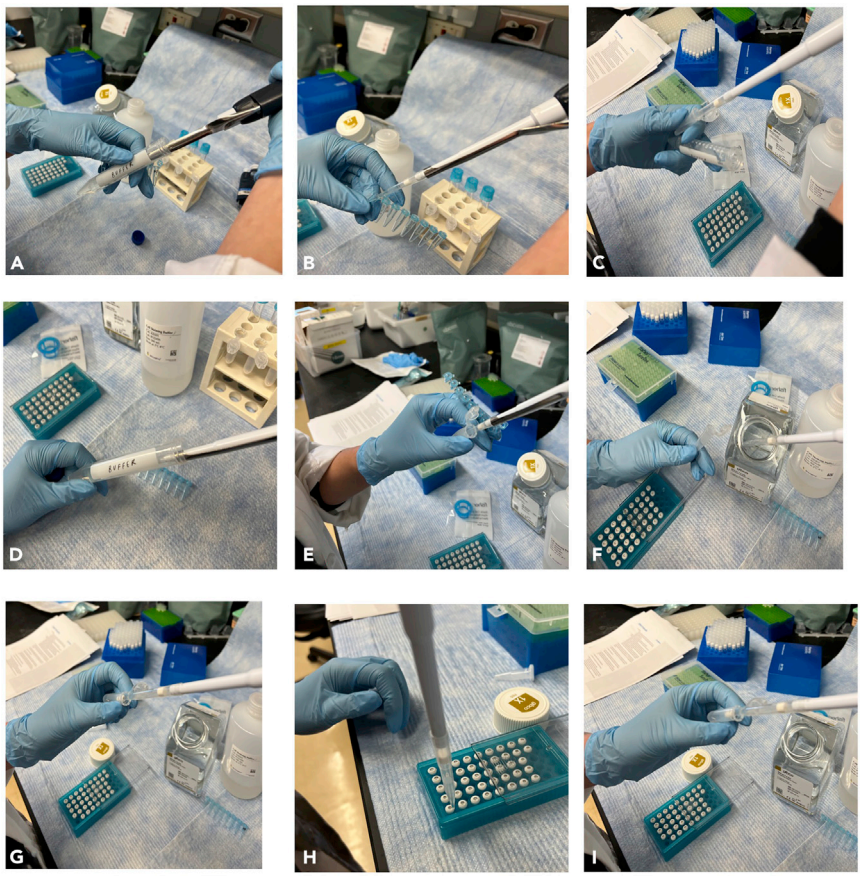

Figure 9. Single cell suspension preparation and hashtag oligonucleotide barcoding

(A and B) Prior to staining, cell suspensions are washed, spun down, decanted, diluted to an appropriate volume.

(C and D) Cells are then blocked by incubating with TruStain FcX and tagged using titrated amounts of each TotalSeq hashtag antibodies.

(E–I) Following incubation, the cells are filtered using a pipette tip cell strainer to remove any clumps of cells or debris.

Potential solution

Keep antibodies at 4°C to prevent loss of activity.

Problem 4

Step 3g: Excess, unbound antibodies are present in the skin sample.

Potential solution

Wash cells adequately after every antibody incubation step. Number of washes can be increased to as many as 3–5 at each step.

Problem 5

Step 3i: The cells are lysed or damaged.

Potential solution

- Optimize sample preparation to avoid cell lysis.
- Do not vortex or centrifuge cells at high speeds.
- Use fresh Cell Staining Buffers.

The authors should specify which buffers need to be fresh.

- Avoid storing the stained cells for longer than 30 min.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michael Longaker (longaker@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report any original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

We would like to acknowledge the Stanford Genomics Facility, Lokey Stem Cell FACS Core, and 10x Genomics for their assistance with experiments. We would also like to acknowledge Art and Elaine Taylor, the Rantz Foundation, and Warren and Judy Kaplan for their generous support of our research efforts. Funding sources include NIH 1F32CA239312-01A1 (D.S.F.), 1R01GM116892 (M.T.L.), 1R01GM136659 (M.T.L.), 5U01DK119094 (M.J., G.C.G.), 1R01DE027346 (D.C.W.), Sarnoff Cardiovascular Research Foundation (M.J.), Goldman Sachs Foundation (D.S.F., M.T.L.), the Child Health Research Institute at Stanford (D.C.W.), the Scleroderma Research Foundation (M.T.L.), the Damon Runyon Cancer Research Foundation (M.T.L.), the Wu Tsai Human Performance Alliance (M.T.L.), the Gunn/Olivier Fund, the California Institute for Regenerative Medicine, the Hagey Laboratory for Pediatric Regenerative Medicine, and the Steinhart/Reed Foundation. The graphical abstract for this work was created with BioRender.com.

AUTHOR CONTRIBUTIONS

M.J., G.C.G., D.C.W., and M.T.L. formulated the overarching research goals of this work. M.J., S.M., K.C., and D.H. designed the experiments. M.J., K.C., D.S.F., D.W., and J.C. validated the methodology. S.M., H.E.T., K.C., D.H., A.F.S., J.BL.P, N.E.L., A.C., and N.G. conducted the experiments. M.J., M.F.G., and M.T.L. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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