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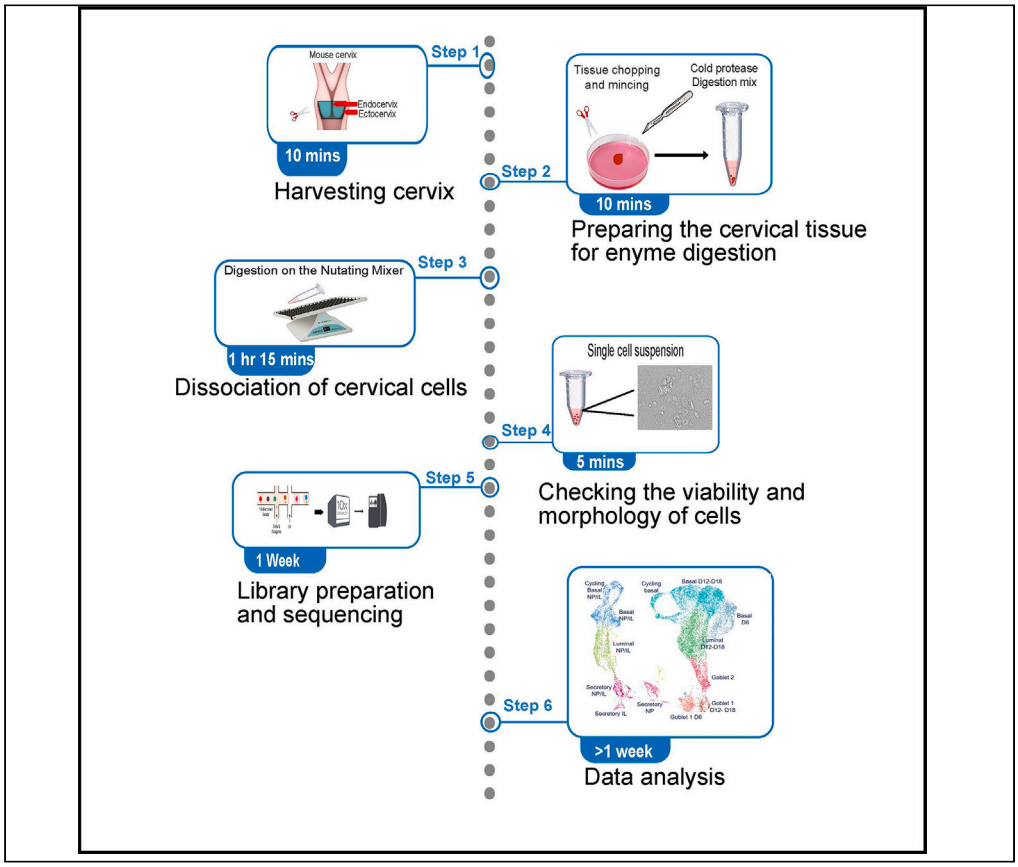
Protocol to dissociate epithelia from non-pregnant and pregnant mouse cervical tissue for single-cell RNA-sequencing



A challenge in studying cervical epithelial cell biology at the single-cell level is that differentiated subtypes, in particular mucus-secreting goblet cells, are sensitive to disassociating enzymes making isolation of all epithelial subpopulations difficult. Here we present a protocol to dissociate epithelia from non-pregnant and pregnant mouse cervical tissue for single-cell RNA-sequencing. We describe steps for harvesting cervices, preparing cervical tissue, dissociation of cervical cells, and viability checks. We then detail library preparation, sequencing, and procedure for data analysis.

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

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Highlights

Detailed steps for collection of cervices from non-pregnant and pregnant mice

Obtain sufficient viable single cells for single-cell RNA-sequencing libraries

Techniques to dissociate and enrich diverse epithelial subtypes from mouse cervix

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Protocol to dissociate epithelia from non-pregnant and pregnant mouse cervical tissue for single-cell RNA-sequencing

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SUMMARY

A challenge in studying cervical epithelial cell biology at the single-cell level is that differentiated subtypes, in particular mucus-secreting goblet cells, are sensitive to disassociating enzymes making isolation of all epithelial subpopulations difficult. Here we present a protocol to dissociate epithelia from non-pregnant and pregnant mouse cervical tissue for single-cell RNA-sequencing. We describe steps for harvesting cervices, preparing cervical tissue, dissociation of cervical cells, and viability checks. We then detail library preparation, sequencing, and procedure for data analysis.

For complete details on the use and execution of this protocol, please refer to Cooley et al. (2023).[1]

BEFORE YOU BEGIN

Here we outline a protocol using the cervix to enrich for viable epithelial cells from nonpregnant and pregnant C57BL/6 mice (8–12 weeks old). Before attempting this protocol ensure permission for animal experiments has been granted by a designated Institutional Animal Care and Use Committee and follow all safety precautions, as well as proper disposal of biological and chemical materials.

1. Plan to have enough cycling mice to collect cervical tissue from desired stage of the nonpregnant estrus cycle and timed pregnant mice at desired gestational time points on the day of cell isolation. A minimum of 8 females per non-pregnant cycle and 2 pregnant females per gestation time point is required to have at least 50,000 single cells for library preparation.
2. Ensure adequate supply of stock solutions as described in the materials and equipment.
3. Perform all preparation step for tissue samples and stock solutions in the biosafety cabinet.
4. Equilibrate the centrifuge (that holds 1.5 mL and 5 mL tube) to 4°C.
5. Make fresh digestion buffer and place it on ice as described in the materials and equipment section.
6. All dissecting tools should be sterile.
7. Perform cervical dissection in the surgery area under a dissecting microscope.
8. Dissociation should be performed in a biosafety cabinet on ice to reduce contamination and ensure cell viability.
9. Set up a clean working station in the tissue culture biosafety hood for tissue dissociation including sterile Lobind 1.5 mL tubes, Lobind tips, trypan blue, 40 μm cell strainer, scissors, and razor blades.

10. Use pipettes with barrier tips and Eppendorf tubes that are free from DNases and RNases.
11. Properly dispose biological and chemical materials.

⚠ CRITICAL: Always work under sterile condition and keep the sample on ice.

Institutional permissions (if applicable)

All laboratory mice were maintained under standard husbandry and housing conditions approved by the UT Southwestern Medical Centre Institutional Animal Care and Use Committee (IACUC). All animals were handled in accordance with NIH and Assessment and Accreditation of Laboratory Animal care (AAALAC) guidelines for laboratory animals. The animals had free access to food and water and were exposed to a 12-h light/12-h dark cycle. Timed matings were carried out to obtain cervical tissue from time points in pregnancy.

Setup timed mating

Timing: 0–19 days

12. Place one adult male (age 8–12 weeks) into a clean cage (prepare as many as needed, making sure males are single housed).
13. On the day of mating, add four females (age 8–12 weeks) to each breeding cage between 8:30–9:30 am.
14. Check for the presence of a vaginal plug after 6–7 h and record the vaginal plug as day 0.
15. Keep plugged females separate from the males.
16. On gestational day 6, 12, 15, 18 and in labor, collect the cervix, and digest as described in the step-by-step protocol. To collect cervix from laboring mice, females are monitored beginning at 12 am on day 19 for delivery of 2–3 pups at which time cervical tissue is collected.

⚠ CRITICAL: Pool cervical tissue from gestational time points to get enough cells for single-cell RNA sequencing library preparation

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's phosphate-buffered saline (PBS)	Sigma-Aldrich	Cat No: D8537
Cold protease	Sigma-Aldrich	Cat No: P5380
DNase I	Millipore Sigma	Cat No: 11284932001
EDTA 0.5M	Sigma-Aldrich	Cat No: SKU-20-158
RBC lysis buffer	BioLegend	Cat No: 420301
Trypan blue	STEMCELL Technologies	Cat No: 07050
Experimental models: Organisms/strains		
C57BL6/129 Mouse strain (Adult females between 3- 6 months old were used)		Taconic B6129F1 | Taconic Biosciences
Other		
DNA LoBind tubes 1.5 mL	Eppendorf	Cat No: 022431021
DNA LoBind tubes 1.5 mL	Eppendorf	Cat No: 0030122348
Cell strainer 40 μm	Fisher Scientific	Cat No: 08-771-1
Microscopic slides	Fisher scientific	Cat No: 12-550-15
Coverslips	Corning	Cat No: 2980-225
5 mL tubes	Eppendorf	Cat No: 0030119401
GyroMini Nutating Mixer	Labnet	Cat No: S0500

MATERIALS AND EQUIPMENT

Preparation of enzyme stock solutions

Timing: 15 min

- 100 mg/mL cold protease.
  - Dissolve 100 mg of cold protease in 1 mL of PBS.
  - Make aliquots of 100 μL and store at −20°C for up to 6 months.
- 15 mg/mL DNase I.
  - Dissolve 15 mg of DNase I in 1 mL of PBS.
  - Filter-sterilize with 0.22 μm syringe filter.

Make aliquots of 50 μL and store at −80°C for 1 year.

⚠ CRITICAL: Do not vortex while dissolving as DNase I is sensitive to physical perturbation.

Prepare the digestion solution using the stock solution prepared

Reagent	Final concentration	Amount
Cold protease	20 mg	200 μL
Ice cold PBS	-	1698 mL
DNase I	1 mg	100 μL
0.5 M EDTA	-	2 μL
Total	N/A	2. 0 mL

⚠ CRITICAL: Make fresh on the day of the experiment. Mix and place on ice until use.

STEP-BY-STEP METHOD DETAILS

Check the female estrus cycle phase by examining vaginal cells

Timing: 30 min

1. Place the mice on the surface of a metal cage rack. Hold the tail base securely elevating the rear end with one hand. The mouse will have her front paws grasping the cage.
2. With the other hand hold the pipette tip with 20 μL of 1× PBS and flush the vagina gently by pushing the end of the pipette tip into the opening of the vaginal canal. Flush the fluid into the vagina and back up into the pipette two to three times using the same tip.
3. Collect the final flush on a glass slide (25 × 75 × 1.0 mm) and place a cover slip (thickness 1 $^{1}/_{2}$, 22 × 50 mm).
4. Examine the slide under a bright field microscope (for example, Leica DMi8) using a 10× or 20× objective.

Collect cervical tissue from females at the desired cycle phase. As shown in the examples, the estrus stage is characterized by the presence of cornified epithelial cells in the vaginal lavage. Diestrus phase is dominated by leukocytes (Figures 1A and 1B).

⚠ CRITICAL: It is important to check the estrus cycle using vaginal cytology just before collecting cervical tissue.

Harvest cervix from non-pregnant and pregnant mice

Timing: 15 min

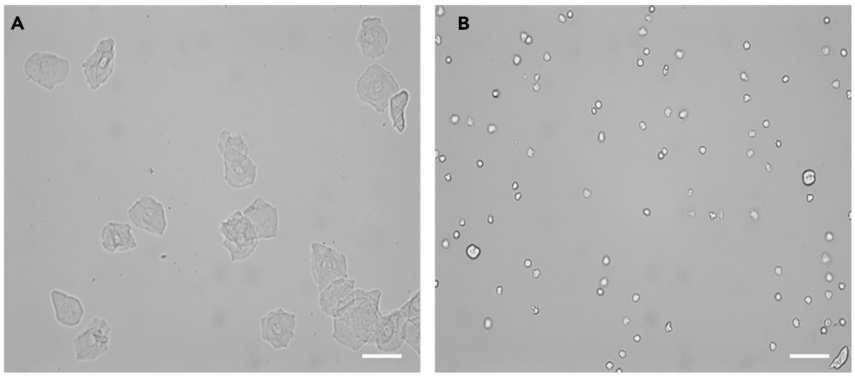

Figure 1. Mouse estrus cycle using vaginal cytology
(A) Estrus stage.
(B) Diestrus stage. 20× objective and Scale bar 50 μm.

5. Sacrifice the mouse using method approved by the local Institutional Animal Care and Use Committee (IACUC) (e.g., cervical dislocation and/or ketamine injection).
6. Lay the mouse back side up on an absorbent pad and generously spray the abdomen and other exposed side of the mouse with 70% ethanol to reduce the risk of contamination.
7. Using clean forceps and scissors, lift the skin and make an incision at the midline cutting through the viscera and the peritoneum, into the abdominal cavity.
8. Expose the abdominal cavity by lifting the skin up and away from you. Push the intestines aside to visualize the uterus, cervix and vagina. Remove the mesometrial fat and other connective tissues.
9. Cut the upper end of the uterine horns, hold both horns with the forceps and pull upward. Simultaneously using the scissors cut the vagina with intact cervix.
10. Place the dissected uterine horn, cervix and vagina on a new absorbent pad and remove the vagina and the uterine horns under the dissecting microscope (Figure 2).
11. Transfer the cervix to a 30 mm Petri dish containing cold PBS on ice.
12. Weigh the tissue followed by washing the tissue with enough PBS twice to get rid of hair and blood. The weight of mouse cervix obtained varies between 10–30 mg per cervix depending on non-pregnant stage and pregnant gestational age.
13. Split or pool the cervices in 5 mL Eppendorf tubes such as each tube has no more than 30 mg of tissue.

⚠ CRITICAL: Do not digest more than 30 mg of tissue in a single tube.

Enzymatic dissociation of cervix from non-pregnant and pregnant mice

Timing: 2 h

Note: All subsequent steps must be carried out under sterile condition on ice.

14. Working inside a hood under sterile conditions, transfer the cervix to a new Petri dish with 20–30 μL of digestion solution on ice.
15. Start chopping the cervix into tiny pieces (less than 0.5 mm^3) using sterile scissors followed by finely mincing with razor blades.

Note: Chopping with scissors followed by mincing with razor blade is a critical step to get representation of different cell types.

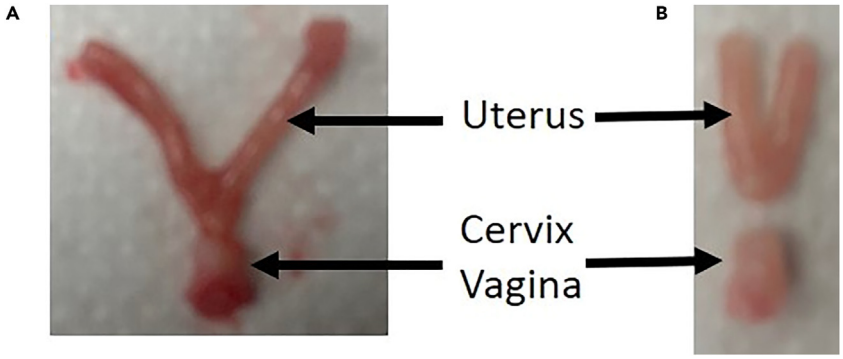

Figure 2. Mouse female reproductive tract
Mouse female reproductive tract (A) uterus, cervix and vagina.
(B) Vagina and cervix separated from the uterus.

16. Transfer minced tissue to a 5 mL Eppendorf containing 2 mL of digestion solution depending on the weight of the cervix and incubate for 1 h 15 min at 4°C with mild shaking/rocking in the nutating mixer.

Note: Use 2 mL digestion solution for 20–30 mg tissue.

17. Centrifuge the 5-mL tube at 1200 rpm for 5 min at 4°C. Discard the supernatant.
18. Resuspend the pellet in 1 mL of ice-cold PBS by gently pipetting up and down a few times.
19. Gently pass the cell suspension (1 mL) 3–5 times through a 21-gauge blunt-end needle using a 1 mL syringe to break the cell chunks (Methods video S1).
20. Transfer the entire cell suspension through a 40 μm filter into a 1.5 mL Lobind tube to remove any cell clumps.

Note: Prewet the 40 μm filter using 1 mL PBS.

21. Centrifuge the 1.5 mL tube at 1200 rpm for 5 min 4°C. Discard the supernatant.
22. Resuspend the pellet in 1 mL of ice-cold PBS by gently pipetting up and down few times.

Optional: Perform red blood cell lysis if necessary (if you see red blood cells on the bottom of the tube)by resuspending the cells in 300 μL of RBC lysis buffer and incubating for 5 minutes on ice. Centrifuge the 1.5 mL tube at 1200 rpm for 5 mins. Discard the supernatant. A white pellet should be visible at the bottom of the tube. Resuspend the pellet with 1 mL of ice-cold PBS (to remove lysis buffer) by gently pipetting up and down few times.

23. Repeat step 21 and resuspend the pellet in 1 mL ice cold 10× Chromium buffer containing PBS and 0.04% BSA to reduce cell loss and clumping. Keep cells/samples on ice and proceed immediately to determine cell counts and viability.

Optional: Perform red blood cell lysis if necessary (if you see red blood cells on the bottom of the tube)by resuspending the cells in 300 μL of RBC lysis buffer and incubating for 5 minutes on ice. Centrifuge the 1.5 mL tube at 1200 rpm for 5 mins. Discard the supernatant. A white pellet should be visible at the bottom of the tube. Resuspend the pellet with 1 mL of ice-cold PBS (to remove lysis buffer) by gently pipetting up and down few times.

Cell counting, viability, and morphology

Timing: 15 mins

24. On a small piece of Parafilm or in a 1.5 mL Eppendorf tube mix 5 μL of cell suspension with 5 μL of Trypan blue.
25. Load 10 μL onto the Neubauer hemocytometer and count the cells under the microscope to check the cell density and viability.

Optional: Count cells twice and take the average cell count.

26. Add 5 μL of cell suspension to a clean slide and place a cover slip to check the morphology.
27. Adjust the concentration to 1000 cells/ μL for optimal density in the 10× Genomics Chromium system and reconfirm the cell count again by repeating steps 24 and 257.
28. Load the cells onto the 10× Genomic Chromium Controller chip and reverse transcription of single-cell GEMs to get barcoded cDNA. Using the Thermal cycler (Applied Biosystems) the cDNA was amplified. 10× Chromium controller chip was used to partition single cells into nanoliter-scale Gel-Bead-In-Emulsions (GEMs) and reverse transcription performed using Single-cell 3′ Reagent Kit v2 and library construction for Illumina sequencing. (https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v2-chemistry).
29. The cDNA Libraries were quantified using the Qubit 3.0 Fluorometer and the quality was checked using a 2100 Bioanalyzer with a High Sensitivity DNA kit (Agilent). Sequencing was performed in paired-end mode with S1-100 cycles kit using a Novaseq 6000 sequencer (Illumina). Approximately 400 million reads were allocated per sample with at least 70,000 reads per cell.

⚠ CRITICAL: Minimize the amount of time between cell counting and library preparation.

EXPECTED OUTCOMES

This protocol allows for successful isolation and enrichment of mouse cervical epithelial cells from both nonpregnant and pregnant mice. This protocol can be adapted for epithelial cells in other tissues as demonstrated in kidney.[2,3] Successful digestion/dissociation should yield a viability of 90% or greater for single-cell RNA-sequencing. The cell number is dependent upon a number of factors including tissue size and time point in pregnancy. Critical adherence to temperature, incubation time, tissue handling, and processing time are essential to achieve yield viability above 90%. In this protocol, tissues were processed within 15 min of tissue collection and cell yield may vary with longer processing time. Collectively, the cold-active protease digestion method enriches for viable, healthy secretory and non-secretory epithelial cells and reduces the degree of transcriptional changes associated with stress.[1]

LIMITATIONS

The quality of single cell transcriptomic data is dependent on isolation of representative cell types within a tissue and on utilization of dispersion methods that limit transcriptional changes due to cell stress. This protocol describes methods to efficiently isolate the diverse subtypes of epithelial cells that populate the mouse cervix in nonpregnancy and pregnancy. A limitation of this method is that other cervical cells, in particular fibroblasts and smooth muscle cells are not well represented using this method of cell dispersion. The reduced number of SMCs could be due to their size which could exceed the capacity of single cell microfluidic channel, as reported in other studies.[4,5] We speculate that the abundant extracellular matrix of the cervix was not readily digested using the cold protease dispersion method to yield a sufficient number of single cell fibroblasts for single-cell RNA-sequencing. Other studies have reported the use of traditional collagenase enzymatic digestion methods to generate cervical single-cell RNA-sequencing libraries that include stromal cells.[6,7] We anticipate that protocols for protease dispersion of viable cervical stroma subtypes will be distinct from optimized methods for isolation of cervical epithelia.

TROUBLESHOOTING

Problem 1

Cell suspension cloudy after dissociation (Steps 14 to 23).

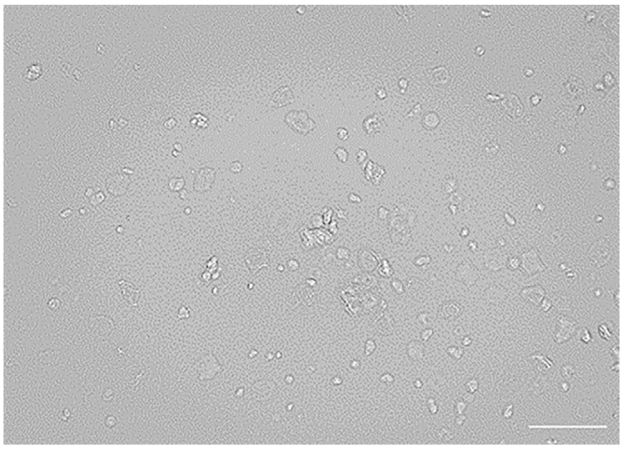

Figure 3. Brightfield image showing the different morphology and size of cervical cells after cold protease digestion
10× objective. Scale bar 100 μm.

Potential solution

- Make the digestion solution fresh just prior to use.
- Ensure DNase I and EDTA has been added to digestion solution.
- Adding ice cold PBS to cold protease is critical to prevent cell clumping and cloudiness during and after dissociation.
- Make sure always the samples are on ice and centrifuge samples at 4°C.

Problem 2

Reduced cell viability and cell count (Steps 24–27).

Potential solution

- Optimize the volume of digestion solution required for weight of the tissue.
- Optimize the incubation time for cell digestion/dissociation.
- Consider using plastic made of polypropylene, which minimizes cell binding to the side of tubes such as Lobind tubes and pipette tips.
- Cell count could be reduced due to accidental aspiration of the pellet. After centrifugation, carefully remove the supernatant.
- Increase the starting material by increasing the tissue number, but to achieve good cell count and viability perform the dissociation using no more than 20–30 mg of tissue per tube in 2 mL volume of digestion solution.
- Before loading the cells to 10× Chromium platform, samples from similar stage or gestation age can be pooled.

Problem 3

Few epithelial subtypes (Step 26).

Potential solution

- It is critical to use scissors to chop the tissue and then a sharp blade to finely mince the tissue.
- Ensure that you have different cell types with different morphology and size at the final stage using the microscope (Figure 3).

Another option to assess subtype diversity is to perform immunostaining or flow analysis using epithelial subtype antibodies on digested cells to confirm the subtypes.

Problem 4

High mitochondrial gene expression during sequencing.

Potential solution

- High mitochondrial gene expression can be due to cell death/apoptosis.
- Work quickly to process tissue and keep it on ice during processing.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mala Mahendroo, (Mala.mahendroo@utsouthwestern.edu).

Materials availability

This study did not generate new unique data or codes.

Data and code availability

This study did not generate data or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102631.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.P.M. developed the protocol, performed all the experiments, wrote the manuscript, and generated figures. G.H. and M.M. supervised the study, provided financial support, and edited and provided final approval of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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