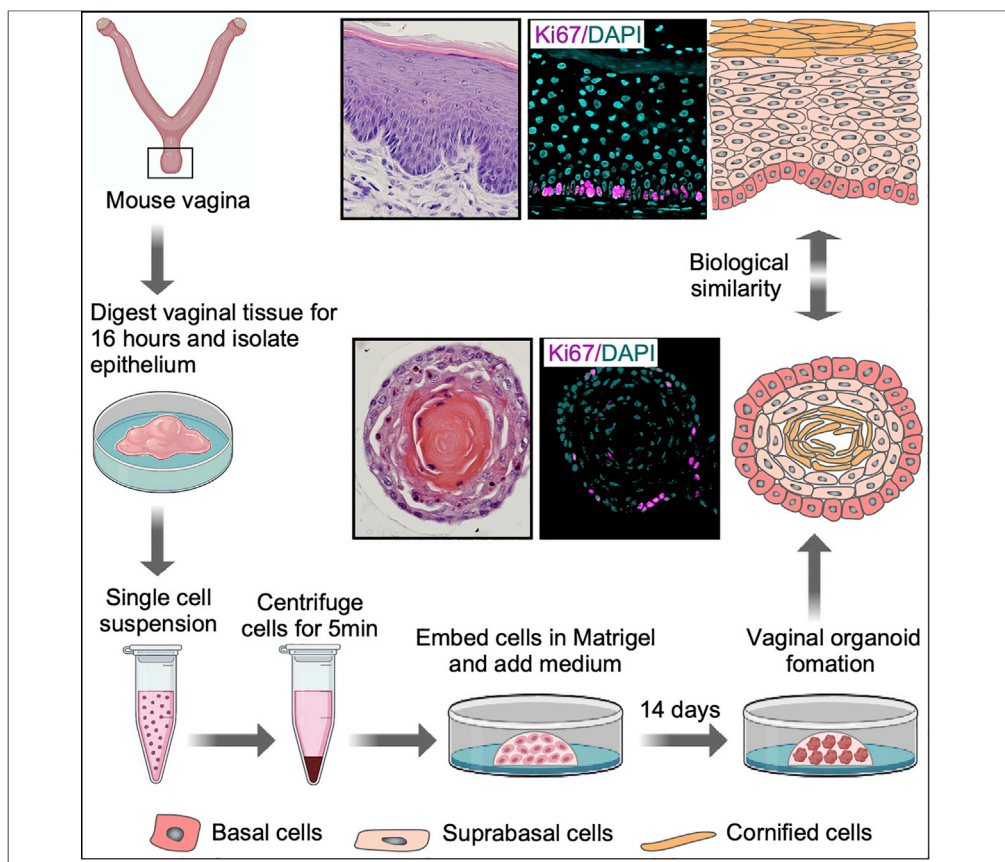


Protocol

Protocol for *In Vitro* Establishment and Long-Term Culture of Mouse Vaginal Organoids



Healthy vaginal epithelium is essential for normal reproductive functions and protects against infectious diseases. Here, we present a protocol for developing mouse vaginal organoids from single epithelial cells. These organoids recapitulate both functional and structural characteristics of vagina *in situ*. This model is a powerful tool for investigating how vaginal microbiome or chemicals in contraceptives and personal hygiene products interact with stem cells and alter the epithelial dynamics, which will lead to new insights into the pathogenesis of vaginal diseases.

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HIGHLIGHTS

A detailed methodology for generating and propagating vaginal organoids

Vaginal organoids functionally recapitulate *in vivo* vaginal epithelium

A procedure to maintain long-term cultures of vaginal organoids

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Protocol

Protocol for *In Vitro* Establishment and Long-Term Culture of Mouse Vaginal OrganoidsAyesha Ali,^{1,2,3,*} Shafiq M. Syed,^{1,2} and Pradeep S. Tanwar^{1,2,4,*}¹School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, NSW, Australia²Hunter Medical Research Institute, Newcastle, NSW, Australia³Technical Contact⁴Lead Contact*Correspondence: ayasha.ayasha@newcastle.edu.au (A.A.), pradeep.tanwar@newcastle.edu.au (P.S.T.)
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SUMMARY

Healthy vaginal epithelium is essential for normal reproductive functions and protects against infectious diseases. Here, we present a protocol for developing mouse vaginal organoids from single epithelial cells. These organoids recapitulate both functional and structural characteristics of vagina *in situ*. This model is a powerful tool for investigating how vaginal microbiome or chemicals in contraceptives and personal hygiene products interact with stem cells and alter the epithelial dynamics, which will lead to new insights into the pathogenesis of vaginal diseases.

For complete details on the use and execution of this protocol, please refer to Ali et al. (2020).

BEFORE YOU BEGIN

1. See the [Key Resources Table](#) for a complete list of materials.
2. All the procedures for the isolation of vaginal epithelial cells are carried out on ice. Therefore, before beginning, prepare ice-cold PBS and set centrifuges at 4°C. In order to minimize the chances of contamination during the harvesting of mouse vaginal tissue, work quickly at a steady pace and use sterile surgical instruments. We found that dipping surgical instruments in 70% ethanol overnight for 12 h or for a minimum of 3 h is sufficient.
3. All procedures following cell isolation are performed under standard aseptic conditions.
4. Vaginal organoids are cultured in a humidified incubator at 37°C with 5% CO₂.
5. Before beginning vaginal tissue harvest, prepare enzyme solution containing 1 mg/mL Pronase and 0.5 mg/mL DNase I in DMEM/F-12 supplemented with 1% Penicillin/Streptomycin.
6. Enzyme digestion takes place overnight for 12 h. Therefore, for culturing the epithelial cells on day 2 of the protocol, thaw BME at 4°C and pre-warm 24-well culture plates at 37°C, in advance.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
DMEM/F12 HAM	Sigma-Aldrich	Cat#D8437
DMEM/F-12 without HEPES	Gibco	Cat#11320-033
Fetal Bovine Serum	Bovogen	Cat#SFBS-F

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-glutamine	HyClone	Cat#SH30034.01
Penicillin-streptomycin	Thermo Fisher Scientific	Cat#15070-063
DPBS	Thermo Fisher Scientific	Cat#21600010
Pronase	Sigma-Aldrich	Cat#10165921001
DNase I	Sigma-Aldrich	Cat#10104159001
Trypan Blue	Sigma-Aldrich	Cat#T8154
Basement Membrane Matrix Extract (BME)	Trevigen	Cat#3433-010-01
Mouse EGF recombinant	Sigma-Aldrich	Cat#SRP3196
Y-27632 dihydrochloride	Tocris	Cat#1254
SB431542 TGF beta inhibitor	Seleckchem	Cat#S1067
Ultroser™ G serum substitute	PALL	Cat#15950-017
TrypLE express	Thermo Fisher Scientific (Gibco)	Cat#12604-021
Dimethylsulfoxide (DMSO)	Thermo Fisher Scientific (Ajax Finechem)	Cat#AJA2225
Paraformaldehyde, 16%	Proscitech	Cat#C004
HistoGel™	Thermo Scientific	Cat#HG-4000-012
Other		
Disposable Cryomolds 15 x 15 x 5 mm	Proscitech	Cat#RM475-2
Kimtech® Science™ Kimwipes™	Kimberly-Clark Professional®	Cat#34120
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory	Cat# JAX:000664, RRID:IMSR_JAX:000664

MATERIALS AND EQUIPMENT

Prepare following Media

Epithelial Cell Isolation (ECI) Medium

	Final Concentration	Amount
DMEM/F-12		440 mL
Fetal Bovine Serum	10% (v/v)	50 mL
L-glutamine	1% (v/v)	5 mL
Penicillin/Streptomycin	1% (v/v)	5 mL

Organoid Culture (OC) Medium

	Final Concentration	Amount
DMEM/F-12 without HEPES		48.4 mL
Ultroser™ G serum substitute (USG)	2% (v/v)	1 mL
Penicillin/Streptomycin	1% (v/v)	0.5 mL
Murine recombinant EGF	100 ng/mL	50 µL of 100 µg/mL stock
TGF-βR inhibitor (SB431542)	0.5 µM	5 µL of 50 mM stock
Rock inhibitor (Y-27632)	10 µM	50 µL of 10 mM stock

Freezing Medium for Cryopreservation of Organoids

	Final Concentration	Amount
DMEM/F-12		24.5 mL
Fetal Bovine Serum	40% (v/v)	20 mL
DMSO	10% (v/v)	5 mL
Penicillin/Streptomycin	1% (v/v)	0.5 mL

STEP-BY-STEP METHOD DETAILS

Isolation of Mouse Vaginal Epithelial Cells

⌚ Timing: 2 days

1. Sacrifice mice according to the protocol, approved by the Institutional Animal Care and Ethics Committee, and harvest the vaginal tissue.
2. Place the tissue into a sterile 10-cm culture dish containing 5 mL ice-cold DPBS. With the help of scissors and pointed forceps remove any fat and blood vessels (Figure 1A).
3. Transfer the tissue to another sterile 10-cm culture dish containing fresh ice-cold DPBS and rinse thoroughly. Using small scissors, slit open the vaginal tube exposing the epithelial lining for enzymatic digestion (Figures 1B and 1C).
4. Incubate the tissue in enzyme solution (1 mL/vagina) containing 1 mg/mL pronase and 0.5 mg/mL DNaseI for 16 h at 4°C with mild rocking/shaking.

Note: Use a 2 mL Eppendorf tube as it is important to have a wide bottom tube for efficient digestion.

5. The next day, following the digestion step, gently scrape off the epithelium from the underlying mesenchymal tissue using fine forceps (Figure 1D).
6. Transfer the isolated epithelium into a 15 mL Falcon tube containing 5 mL of ice cold ECI medium.
7. Disrupt the epithelial cell sheet by gently vortexing the tube for 1 min.

Alternatives: Pipette up and down approximately 50 times with a P1000 pipette to disrupt the epithelium.

8. Pellet down the epithelial cells by centrifuging the tube at 200 × g for 5 min at 4°C.
9. Aspirate and discard the supernatant and resuspend the pellet in fresh ECI medium.
10. Repeat steps 8 and 9 two more times.
11. Pass the cell suspension through a 40 μM cell strainer to remove any undigested tissue or cell clumps.
12. Collect the filtrate and pellet down the cells again by centrifuging the tube at 200 × g for 5 min at 4°C.
13. Aspirate and discard the supernatant. Resuspend the cell pellet in ECI medium.
14. Use hemocytometer to assess the cell number and cell viability. It is recommended not to proceed further with the protocol if the number is lower than 15,000–20,000 cells or the cell viability is lower than 80%–90%.
15. Before proceeding with plating wash the cells with organoid culture medium in order to remove any traces of FBS.

Culture of Mouse Vaginal Organoids

⌚ Timing: 2 weeks

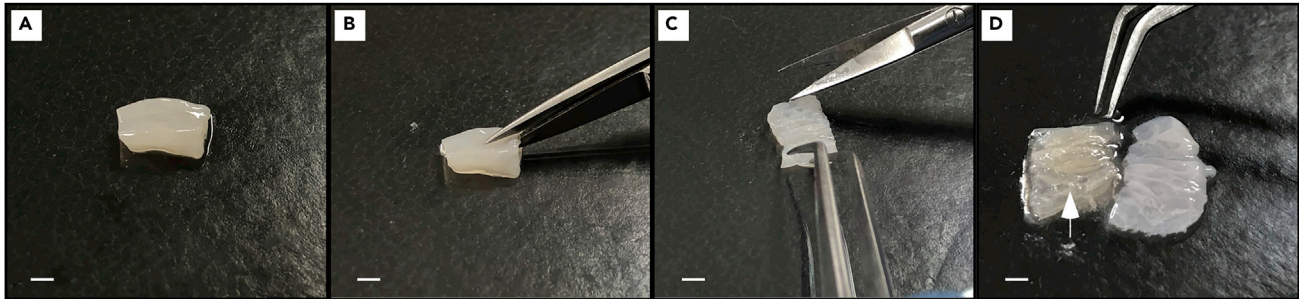


Figure 1. Overview of the Procedure for the Isolation of Mouse Vaginal Epithelium

(A) A representative image of a freshly harvested mouse vagina after removing fat and associated blood vessels.

(B) Cutting open the vagina.

(C) Slit open the vagina before proceeding with the enzymatic dissociation.

(D) Vaginal tissue post-enzymatic digestion. Arrow points to the separated vaginal epithelial sheet after enzymatic digestion.

Scale bar, 1 mm.

16. Pellet down the cell suspension by centrifuging the tube at $200 \times g$ for 5 min at 4°C from step 15 and resuspend in ice cold BME at a final concentration of 20,000 cells per 50 μL of BME.
17. Plate a 50 μL BME drop (containing 20,000 cells) in the center of a pre-warmed 24-well culture plate avoiding any bubbles.

△ CRITICAL: It is important that the cells are mixed with BME slowly and carefully so that no bubbles are introduced as it will affect the growth of organoids.

△ CRITICAL: BME should be handled on ice at all times and plating should be done as quickly as possible so that it does not solidify

18. Incubate the 24-well culture plate upside down in a CO_2 incubator (37°C , 5% CO_2) for 30 min to allow the BME to solidify.

△ CRITICAL: The plate should be placed upside down to prevent cell adherence to the plate bottom.

19. Once the BME is solidified, add 500 μL of pre-warmed organoid culture medium in each well, and return the plate to CO_2 incubator (37°C , 5% CO_2).
20. Replace the medium every 2 days. Organoid culture medium is supplemented with 10 μM Y-27632 for only the first 3 days of culture.
21. Check the organoid growth under a light microscope. After 4–6 days in culture, small organoids should appear (Figures 2A–2C).

Passaging of Mouse Vaginal Organoids

⌚ Timing: 10 days

22. Organoids can be passaged 10 days after seeding. Prepare all the reagents before proceeding with the experiment. Thaw BME on ice and pre-warm 24-well plates at 37°C .
23. To begin with, remove culture medium and add 1 mL of ice-cold DPBS to each well. Break the BME and dissociate the organoids by gentle pipetting up and down approximately 20 times using a P1000 pipette.
24. Transfer the organoid suspension into a 15 mL Falcon tube.
25. Centrifuge the tube at $200 \times g$ for 5 min and discard the supernatant.

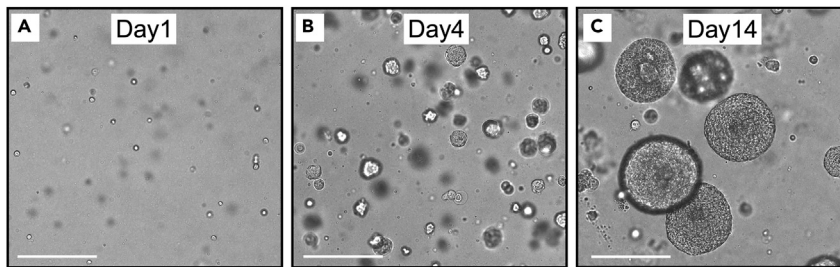


Figure 2. Timeline of Organoid Development from a Single Mouse Vaginal Epithelial Cell

(A) Day 1.

(B) Day 4.

(C) Day 14.

Scale bar, 250 μ m.

26. Incubate the pelleted organoids with 0.5 mL of TrypLE for 5 min at 37°C. Using a P1000 pipette, gently pipette the suspension up and down for 5–10 times to yield a single cell suspension.

△ CRITICAL: The organoid dissociation should be carried out gently to avoid cell death.

Note: Pipetting organoids after TrypLE treatment will result in a single cell suspension. However, some cell clumps might still remain which can either be removed by passing the suspension through a cell strainer or can be plated as such.

27. Add 10 mL of ECI medium to the cell suspension in order to stop the activity of TrypLE.
28. Pellet down the cells by centrifugation at 200 \times g for 5 min and discard the supernatant.
29. Resuspend the cells in organoid culture medium and centrifuge again.
30. Resuspend the pellet in 150 μ L of BME. Plate 50 μ L drops into the center of each well of a pre-warmed 24-well culture plate, thus splitting each organoid culture at a ratio of 1:3.
31. Place the 24-well plate upside down in a CO₂ incubator (37°C, 5% CO₂) for 30 min to allow the BME to solidify.
32. Once solidified, add 500 μ L of pre-warmed organoid culture medium into each well and return the plate into the incubator. Replace the medium every 2 days. Remember to supplement the organoid culture medium with 10 μ M Y-27632 for only the first 3 days of culture.

Cryopreservation of Mouse Vaginal Organoids

⌚ **Timing:** 30 min

33. After 14 days of culture, aspirate the medium and add 1 mL of ice-cold DPBS into each well of the 24-well culture plate to break the BME.
34. Once dissolved, transfer the organoid suspension into a 15 mL Falcon tube. Centrifuge the tube at 200 \times g for 5 min at 4°C.
35. Discard the supernatant and trypsinize the organoids by adding 0.5 mL of TrypLE to the pellet. Incubate for 5 min at 37°C.
36. Using P1000 pipette, gently pipette the organoids up and down for 15–20 times to yield a single cell suspension.
37. Add 10 mL of ECI medium to the suspension in order to stop the activity of TrypLE.
38. Centrifuge the tube at 200 \times g for 5 min at 4°C.
39. Resuspend the pellet in 1 mL of freezing medium containing 40% FBS and 10% DMSO with 1% Pen/Strep. Aliquot the suspension into cryovials.

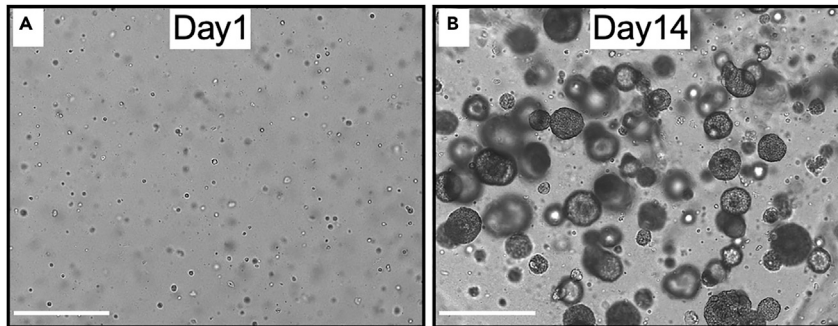


Figure 3. Vaginal Organoids Derived from Cells Stored in Liquid Nitrogen

(A) Day 1.

(B) Day 14.

Scale bar, 500 μ m.

Note: 1 mL of freezing medium is sufficient to freeze 1 well of a 24 well plate with approx. 1000 organoids.

40. Transfer the cryovials into a freezing container filled with 250 mL of isopropanol. Transfer the container to -80°C for 24 h, after which the cryovials need to be stored in liquid nitrogen for long-term storage.

△ CRITICAL: It is not recommended to leave and/or store cells at -80°C for more than 24 h.

Revival of Mouse Vaginal Organoids

⌚ **Timing:** 1 h

41. Retrieve and thaw the cryovial containing the frozen cells by briefly placing the cryovial in a 37°C water bath.

△ CRITICAL: It is recommended that the organoids are not completely thawed at 37°C but only about 50%. Rest of thawing should happen at 20°C – 22°C while preparing for next step of the protocol. Over-warming the medium may affect the growth efficiency of the organoids in culture, resulting in poor revival and more cell death.

42. When thawed, immediately transfer the contents of the cryovial into a 15 mL Falcon tube.
43. Slowly add 10 mL ECI medium to the Falcon tube containing the cell suspension.
44. Pellet down the cells by centrifugation at $200 \times g$ for 5 min at 4°C .
45. To remove the freezing medium completely, wash the cell pellet in ECI medium.
46. Centrifuge again at $200 \times g$ for 5 min at 4°C .
47. Discard the supernatant and wash the cells with vaginal organoid culture medium.
48. Plate the cells as described in steps 16–18.

Note: 1 vial of cells can be used to plate 3 wells of 24 well plate.

49. Once the BME is solidified, add 500 μ L of pre-warmed organoid culture medium in each well, and return the plate to the CO_2 incubator (37°C , 5% CO_2).
50. Replace the medium every 2 days. Organoid culture medium is supplemented with 10 μM Y-27632 for only the first 3 days of culture.
51. Check organoid growth under a light microscope (Figures 3A and 3B).

Processing of Vaginal Organoids for Immunofluorescence Staining

⌚ Timing: 1 day

52. After 14 days in culture, remove culture medium and add 1 mL of ice-cold DPBS to each well. Break the BME and harvest the organoids in a 1.5 mL Eppendorf tube. Proceed with their fixation, paraffin embedding and immunofluorescence staining as follows:

Note: All the organoids will not be released from BME at once. Cut the P200 pipette tip so that the barrel is large enough to suck up the organoids without breaking them and small enough to help break the BME and release the organoids.

53. Fix the organoids in 4% PFA in a 1.5 mL Eppendorf tube at 20°C–22°C for 30 min.
54. Allow the organoids to settle down at the bottom of the tube. Discard the PFA as supernatant and wash the organoids in PBS twice.

Note: During the washing steps, organoids should not be centrifuged as it will result in clumping. They should be allowed to settle under gravity for 1 min.

⏸ **Pause Point:** If not using immediately, organoids can be stored at 4°C for 7 days in PBS.

55. Before proceeding with the paraffin embedding, organoids are encapsulated in Histogel specimen processing gel. Warm the Histogel in a 65°C water bath for 1–2 h until it liquifies.

Note: Make sure that the Histogel is maintained at 65°C all the time, as it will solidify at lower temperature.

56. Coat the bottom of a cryomold (15 x 15 x 5 mm) with a layer of Histogel by adding 200 µL per cryomold and gently spreading it with a pipette tip. Place the cryomold on ice for 5–10 min to allow the Histogel to solidify.
57. Gently place the organoids into the Histogel coated cryomold using a cut P200 pipette tip. Use Kimwipes to soak up as much PBS as possible, and make sure that the organoids lie in the center of the cryomold.

Note: Before adding the second layer of Histogel onto the organoids, use Kimwipes to remove excess PBS otherwise it may result in the shrinkage of Histogel during processing

58. Gently add 200 µL of Histogel into the cryomold so that the organoids are sandwiched between the two layers of the Histogel.
59. Return the cryomold to ice for 20 min until the Histogel is completely solidified.
60. Gently remove the solidified Histogel containing the embedded organoids from the mold and transfer it into a tissue cassette for further processing.
61. Proceed with the standard paraffin embedding process by passing the cassettes through a gradient of 70%, 80% and 95% ethanol for 1 h each, followed by two changes of 100% ethanol for 1 h each and two changes of xylene for 1 h each. Finally, the cassettes are incubated in molten paraffin wax for 2 h, before the Histogel sandwiches, containing organoids are embedded into a paraffin block.
62. For immunofluorescence staining, sections are cut at 4 µm thickness using a microtome.

EXPECTED OUTCOMES

This protocol describes an efficient method for establishing organoid cultures from mouse vaginal epithelium. Within 3–4 days of plating, a single epithelial cell grows out into a small organoid. By

day 7 of culture, a differentiated suprabasal cell layer is formed in the center of the organoids. A fully differentiated vaginal organoid is formed after 14 days in culture. When cultured for a longer duration, organoid lumen increases in size and is filled with mucinous secretions. Immunofluorescence staining showed that the expression pattern of the markers for basal cells (CD271, TP63 and KRT5), differentiation (filaggrin) and proliferation (KI67) in these vaginal organoids is similar to *in vivo* vaginal tissue. Details of the immunostaining for different cell types and further characterization of these organoids can be found in our recent Cell Reports publication (Ali et al., 2020). Collectively, the vaginal organoids that we developed here recapitulate the functional and structural characteristics of the vagina *in situ*.

LIMITATIONS

One of the limitations of establishing and culturing vaginal organoids is using a serum-based medium. The addition of FBS in the organoid culture medium negatively affects the efficiency of organoid formation and growth. To avoid this, we recommend using serum supplements, such as Ultra-serum-G, instead of FBS.

TROUBLESHOOTING

Problem 1

No, few or small organoids appear.

Potential Solutions

- Extended duration of enzymatic digestion will decrease the viability of cells. Therefore, the digestion process should not exceed 16 h at 4°C.
- Inadequate growth factor activity in the culture medium is one of the main reasons for poor organoid development. Recombinant mouse EGF is a very critical component of the organoid culture medium. Failing to add it in the medium will result in no or fewer organoids. It is important that the medium is changed every 2 days to ensure that the growth factors in the culture medium are active. The OC medium can be stored for a month at 4°C without the growth factors. It is recommended to add growth factors just before changing the medium.
- Make sure all the growth factors are appropriately stored in smaller aliquots to avoid repeated freeze-thaw cycles.
- Splitting the cells with TrypLE results on 80%–90% cell viability however incubating the organoids for a longer time in TrypLE or harsh pipetting could reduce the cell viability.
- At the time of passaging and freezing down, avoid overdoing the pipetting to break the organoids as this could also affect the growth of organoids.

Problem 2

Stromal contamination in culture.

Potential Solutions

When isolating cells, it is recommended to do a differential attachment where cells are plated in a 10 cm cell culture plate and incubated in a CO₂ incubator (37°C, 5% CO₂) for 2–2.5 h. This will result in preferential attachment of stromal cells but not epithelial cells. After the incubation, collect medium in a 15 mL tube and centrifuge at 200 × *g* for 5 min, the pellet will contain epithelial cells with minimum stromal contamination.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Assoc Prof Pradeep Tanwar pradeep.tanwar@newcastle.edu.au.

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The published article includes all [datasets/code] generated or analyzed during this study.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.A., S.M.S., and P.S.T. designed the research. A.A. and S.M.S. performed the research. A.A. analyzed the data. A.A., S.M.S., and P.S.T. wrote the paper. P.S.T. supervised the study, provided financial support, editing, and final approval of the manuscript. All authors reviewed, approved, and commented on the manuscript.

DECLARATION OF INTERESTS

P.S.T. is an inventor on a patent application regarding vaginal organoid culture and its uses. All other authors have no competing interests to declare.

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