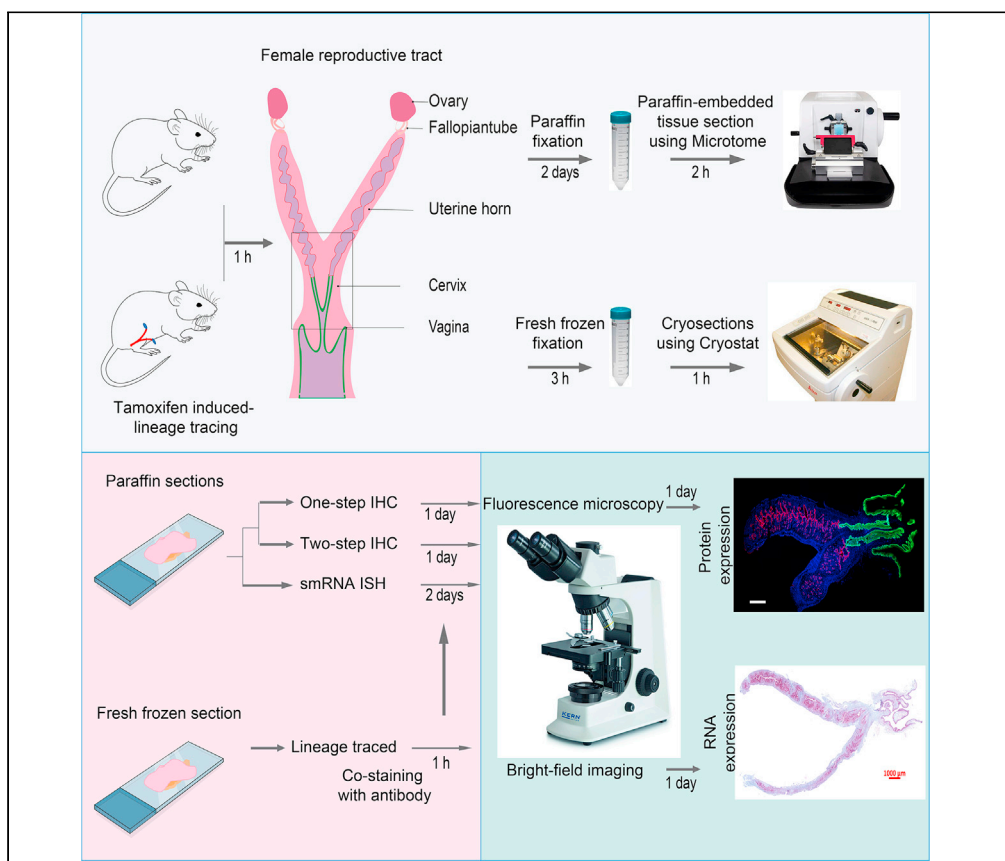


Protocol

Spatial analysis of organ-wide RNA, protein expression, and lineage tracing in the female mouse reproductive tract



Visualizing precise spatial patterns of an organ-wide gene and protein expression among diverse cell types can provide critical insights into the fundamental processes underlying normal tissue homeostasis and disease development. Here, we describe an optimized protocol for single-molecule RNA *in situ* hybridization (smRNA-ISH), immunohistochemistry, and cell lineage analysis of the female reproductive tract organs using commercially available smRNA-ISH probes, antibodies, and inducible Cre-mice. The high-resolution multispectral fluorescence imaging is performed using wide-field epifluorescence or confocal microscopy combined with a slide scanner.

Rajendra Kumar
Gurumurthy,
Naveen Kumar,
Cindrilla Chumduri

cindrilla.chumduri@
uni-wuerzburg.de

Highlights

Tissue preparation for fixation of female reproductive tract (FRT) organ

The optimized protocol for labeling of the protein and RNA in the FRT tissue

Spatial analysis of gene, protein expression, and lineage development in FRT

Gurumurthy et al., STAR
Protocols 2, 100969
December 17, 2021 © 2021
The Author(s).
<https://doi.org/10.1016/j.xpro.2021.100969>



Protocol

Spatial analysis of organ-wide RNA, protein expression, and lineage tracing in the female mouse reproductive tract

Rajendra Kumar Gurumurthy,¹ Naveen Kumar,² and Cindrilla Chumduri^{2,3,4,*}¹Department of Molecular Biology, Max Planck Institute for Infection Biology, 10117 Berlin, Germany²Chair of Microbiology, University of Würzburg, 97074 Würzburg, Germany³Technical contact⁴Lead contact*Correspondence: cindrilla.chumduri@uni-wuerzburg.de
<https://doi.org/10.1016/j.xpro.2021.100969>

SUMMARY

Visualizing precise spatial patterns of an organ-wide gene and protein expression among diverse cell types can provide critical insights into the fundamental processes underlying normal tissue homeostasis and disease development. Here, we describe an optimized protocol for single-molecule RNA *in situ* hybridization (smRNA-ISH), immunohistochemistry, and cell lineage analysis of the female reproductive tract organs using commercially available smRNA-ISH probes, antibodies, and inducible Cre-mice. The high-resolution multispectral fluorescence imaging is performed using wide-field epifluorescence or confocal microscopy combined with a slide scanner.

For complete details on the use and execution of this protocol, please refer to Chumduri et al. (2021).

BEFORE YOU BEGIN

All experiments involving animals were performed following regulations for animal research and approvals from national legal, institutional, and local authorities at Max Planck Institute for Infection Biology and the University of Würzburg.

Background

This protocol describes tissue preparation and different fixation methods of the female reproductive tract. Further, it provides step-by-step descriptions of immunohistochemistry, lineage tracing procedures and co-immunostaining of lineage traced fresh frozen tissue and the single-molecule RNA in-situ hybridization (smRNA-ISH) (Figure 1).

Prepare before beginning the experiment

⌚ Timing: 1 day

1. Prepare all the buffers and solutions according to the tables below.
2. Autoclave Eppendorf tubes and dissection sets, including forceps and scissors.
3. Prepare the dissection platform and a dissection microscope.
4. Keep the petri dish and scalpel/blades ready for performing the dissection.
5. Keep Falcon tubes containing 70% ethanol and sterile PBS ready for cleaning dissection tools.
6. Turn on the laminar flow at least 15 min before starting the work.
7. Set the water bath temperature to 37°C.



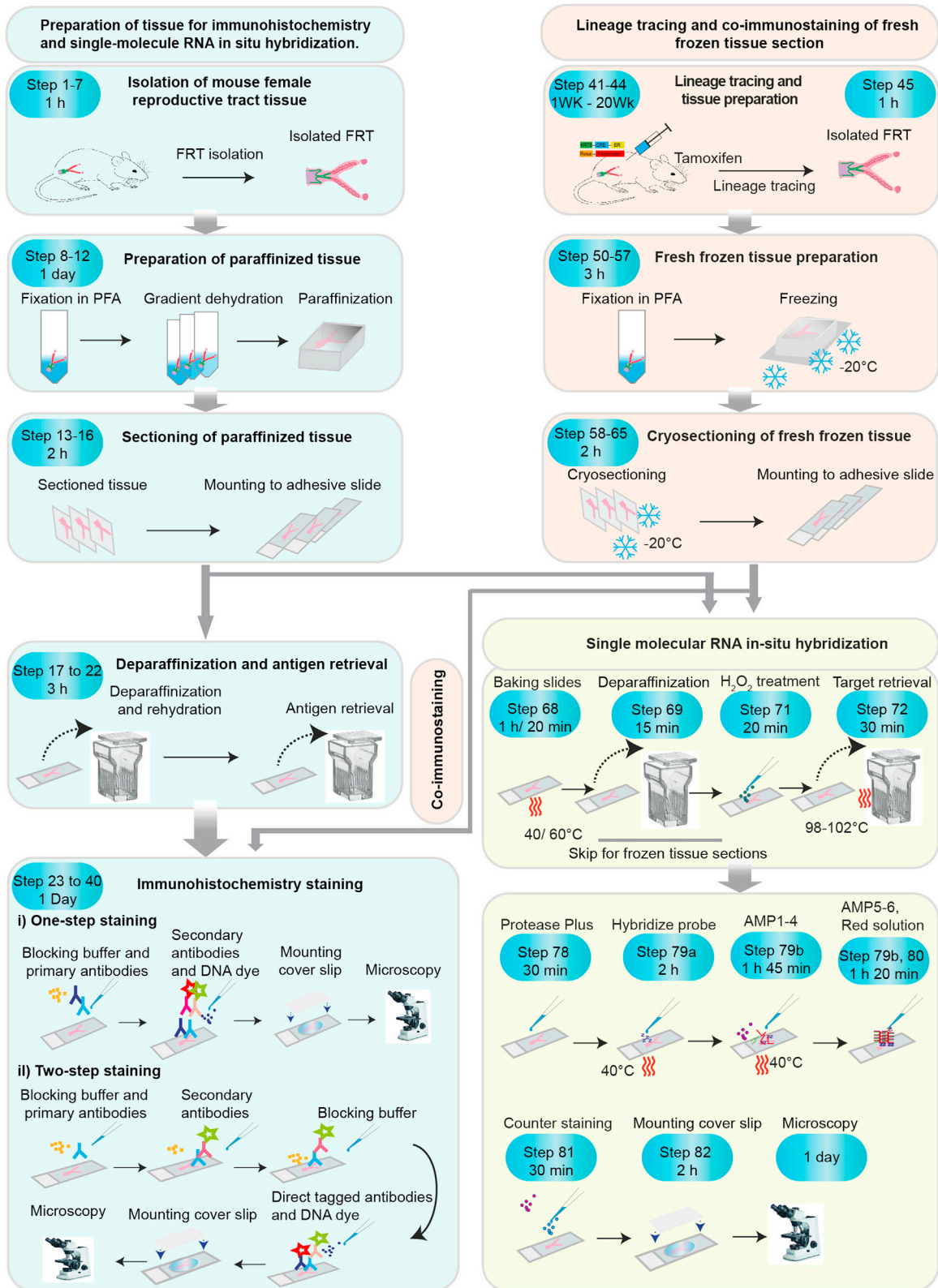


Figure 1. Flowchart representing different steps of the protocol and time needed to complete the steps

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-KRT5 (dilution 1:250)	Abcam	Cat#ab52635
Rabbit-anti-KRT8 (dilution 1:200)	Abcam	Cat#ab59400
Chemicals, peptides, and recombinant proteins		
DPBS	Gibco	Cat#14190-169
BSA	Carl Roth	Cat#80763
Paraformaldehyde	Sigma-Aldrich	Cat#4441244
FCS	Biochrom	Cat#S0115
Tween 20	Carl Roth	Cat#91927.2
TritonX-100	Carl Roth	Cat#3051.4
Glycerin	Carl Roth	Cat#4043.1
Mowiol	Sigma-Aldrich	Cat#81381
Tris-base	AppliChem	Cat#A1086.5000
Xylene	Carl Roth	Cat#9713.2
Ethanol	Merck	Cat#1.00983.2511
EcoMount	Biocare	Cat#EM897L
Tamoxifen	Sigma-Aldrich	Cat#T5648
Gill's Hematoxylin I	Sigma-Aldrich	Cat#GHS1128
TrypLE	Gibco	Cat#12605-028
Isopropanol	Merck	Cat#1.09634.2511
Paraffin	Carl Roth	Cat#6642.6
Target Retrieval solution	Dako	Cat#S1699
Draq5	Cell Signaling Technology	Cat#4085
Hoechst	Sigma-Aldrich	Cat#B2261
Isopentane	Carl Roth	Cat#3926.1
Autoclaved distilled water	N/A	N/A
Corn oil	Sigma-Aldrich	Cat#C8267
Ammonium Hydroxide	Merck	Cat#221228
Paraformaldehyde Solution, 4% in PBS	Thermo Fisher Scientific	Cat#AAJ19943K2
Mowiol® 4-88 Permanent Mountant Solution	Biotrend	Cat#MWL4-88-25
Tissue-Tek® optimum cutting temperature (OCT) medium	Sakura Finetek USA	Cat#4583
Critical commercial assays		
RNAscope® 2.5 HD Reagent Kit – RED (contains following sub kits)	ACD	Cat#322350
RNAscope® 2.5 HD Detection Kit (RED)	ACD	Cat#322360
RNAscope® 2.5 Pretreat Reagents-H202 and Protease Plus	ACD	Cat#322330
RNAscope® Target Retrieval	ACD	Cat#322000
RNAscope® Wash Buffer	ACD	Cat#310091
RNAscope® Positive Control Probe- Mm-Ppib	ACD	Cat#313911
RNAscope® Negative Control Probe – DapB	ACD	Cat#310043
RNAscope® Probe - Mm-Krt5	ACD	Cat#415041
RNAscope® Probe- Mm-Krt8	ACD	Cat#424521
Experimental models: Organisms/strains		
C57bl/6-Krt5-CreERT2;Rosa26-tdTomato	Previously generated (Chumduri et al., 2021)	N/A
C57bl/6 wild type	The Jackson Laboratory	https://www.jax.org
Software and algorithms		
Zen 3.2 (blue edition)	ZEISS	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Others		
0.22 µm filter	Merck Millipore	Cat#SLGP033RS
Coverslips	Marienfeld	Cat#0101242
Thermo Scientific™ SuperFrost Ultra Plus™ Adhesion Slides	Thermo Fisher Scientific	Cat#J3800AMNZ
Pipette tips, 10 µL	Sarstedt	Cat#3010
Pipette tips, 200 µL	Sarstedt	Cat#3030
Pipette tips, 1000 µL	Sarstedt	Cat#1181
1 mL syringe	BD	Cat#309628
Sterile scissors	B. Braun	Cat#BC050R
Bent forceps	Bochem Lab Supply	Cat#1130
100 mm Petri dish	TPP	Cat#P93150
15 mL Falcon tube	Sarstedt	Cat#62.554.100
50 mL Falcon tube	Sarstedt	Cat#62.547.004
Neubauer counting chamber	Marienfeld	Cat#0640210
Tissue cassette	Simport Scientific	Cat#M490-11
Tissue Sponges	Thermo Fisher Scientific	Cat#84-53
Pap pen	BIOZOL	Cat#CED-MU12
Microcentrifuge tubes 1.5 mL	Sarstedt	Cat#72.690.001
40 µm cell strainer	BD Falcon	Cat#352340
Microtome blade	Hartenstein	Cat#S35
26 G needle	VWR	Cat#612-0147
Immedge hydrophobic barrier pen.	Vector Laboratories	Cat#VECH-4000
Scalpel blade	Carl Roth	Cat#Ex75.1
Magnetic stirrer with a heating plate	IKA	Cat#0003810000
Fume hood	N/A	N/A
pH meter	Carl Roth	Cat#TA10.1
High-speed centrifuge	Eppendorf	Cat#5810 R
HybEZ™ Oven (110 or 220 VAC)	ACD	Cat#310010 or 310013
HybEZ™ Humidity Control Tray (with lid)	ACD	Cat#310012
HybEZ™ Slide Rack (20 slide capacity)	ACD	Cat#310014
Water bath	GFL	Cat#1002
Eppendorf centrifuge	Eppendorf	Cat#5417R
Fluorescence microscope	Olympus	Cat#CKX41
Bright-field microscope	Olympus	Cat#IX50
Drying oven	Thermo Fisher Scientific	Cat#11-475-153
Incubated Tabletop Orbital Shaker	Thermo Fisher Scientific	Cat#SHKE420HP
Rotary Shaker	VWR	Cat#444-1039
Tissue processor	Leica Biosystems	Cat#TP1020
Paraffin embedding station	Medite	Cat#Tes 99
Paraffin rotation microtome	Leica Biosystems	Cat#SM2010 R
Cryostat	Leica Biosystems	Cat#CM3050s
Biological safety cabinet	Thermo Fisher Scientific	Cat#10110910
Pipette, 0.5–10 µL	Eppendorf	Cat#3120000020
Pipette, 2–20 µL	Eppendorf	Cat#3120000038
Pipette, 10–100 µL	Eppendorf	Cat#3120000046
Pipette, 20–200 µL	Eppendorf	Cat#3120000054
Pipette, 100–1,000 µL	Eppendorf	Cat#3120000062
Axioscan Imager	Carl Zeiss Microscopy	ZEISS Axio Scan.Z1

MATERIALS AND EQUIPMENT

Refer to material safety data sheets of chemicals before reagent preparation and take protective measures whenever handling hazardous chemicals. Follow biosafety precautions when handling biological material.

3.7% Paraformaldehyde solution

Reagent	Final concentration	Amount
Distilled water	N/A	900 mL
PFA	3.7%	37 g
10× PBS	1×	100 mL
Total	N/A	1000 mL

- Dissolve the PFA in 100 mL of 10× PBS and 900 mL autoclaved water (prewarmed to 60°C).
- Place it on the magnetic stirrer with a hot plate set to 60°C inside the fume hood. Stir the solution with a magnetic bead.
- Add 1 mL 0.5 M NaOH to dissolve PFA.

⚠ **CRITICAL:** PFA is highly toxic; avoid contact with skin and eyes. Use suitable protective gloves and a mask.

⏸ **Pause point:** After cooling to 25°C, adjust the pH to 7.4, store at 4°C for a few weeks or at –20°C for up to 1 year.

Note: Alternatively, the PFA solution can also be purchased from commercial vendors (See [key resources table](#)).

Immunohistochemistry (IHC) blocking solution

Reagent	Final concentration	Amount
DPBS	N/A	9.79 mL
BSA	1%	100 mg
Fetal calf serum (FCS)	2%	200 µL
Tween- 20	0.01%	10 µL
Total	N/A	10 mL

Note: Filter by passing through 0.22 µm filter and store at –20°C.

Permeabilization buffer

Reagent	Final concentration	Amount
Immunohistochemistry (IHC) blocking solution	N/A	10 mL
Triton-X 100	0.2%	20 µL
Total	N/A	10 mL

Note: Filter by passing through 0.22 µm filter and store at –20°C.

Mowiol

Reagent	Final concentration	Amount
Milli Q water	N/A	30 mL
Glycerin	35%	35 g
Mowiol	12%	12 g
0.2M Tris HCl (pH 8.5)		60 mL
Total	N/A	100 mL

- Dissolve Mowiol and glycerin in water by slowly stirring without forming air bubbles for 2 h at 25°C.
- Add 0.2 M Tris/HCl (pH 8.5), incubate for 1 h at 50°C, then stir for 14 h at 50°C.
- Centrifuge at 500 g for 15 min to remove any undissolved Mowiol.
- Aliquot the clear solution, and store at 4°C for short term and at –20°C for long-term.

Note: Alternatively, the Mowiol mounting medium can be purchased from commercial vendors (see [key resources table](#)).

1 × target retrieval solution for smRNA-ISH		
Reagent	Final concentration	Amount
10× target retrieval reagent	1×	70 mL
Milli Q water	N/A	630 mL
Total	N/A	700 mL

⌚ **Timing:** 10–15 min

Note: Prepare freshly at step 70.

⚠ **CRITICAL:** Use baked glass beaker and autoclaved distilled water.

- Place the beaker with RNA scope 1× target retrieval reagents on the hot plate.
- Cover the beaker with aluminum foil and turn the hot plate on high.
- Check the temperature of the solution with the thermometer.
- Once the solution reaches to mild boil (98°C–102°C), turn the hot plate to a lower setting to maintain the temperature before use.

⚠ **CRITICAL:** Do not boil the 1× target retrieval reagent more than 15 min before use to avoid excessive evaporation.

1 × wash buffer		
Reagent	Final concentration	Amount
50× wash buffer	1×	60 mL
Milli Q water	N/A	2.94 L
Total	N/A	3 L

Note: Warm RNA scope 50× wash buffer up to 40°C for 10–20 min before preparation. 1× wash buffer can be stored at 25°C for up to one month.

Hematoxylin staining solution		
Reagent	Final concentration	Amount
Gill's Hematoxylin 1	50%	100 mL
Milli Q water	N/A	100 mL
Total	N/A	200 mL

Note: Solution can be reused for 1 week.

Ammonia water (Bluing reagent) (0.02%, w/v)		
Reagent	Final concentration	Amount
1N Ammonium Hydroxide	0.02%	1.43 mL
Milli Q water	N/A	248.57 mL
Total	N/A	250 mL

Tamoxifen stock		
Reagent	Final concentration	Amount
Tamoxifen	20 mg/mL	20 mg
Ethanol	N/A	1 mL
Total	N/A	1 mL

- Prepare the tamoxifen solution for injection at a concentration of 0.25 mg/g bodyweight of the mouse.
- Calculate the amount of tamoxifen stock required for the injection by measuring the bodyweight of the mouse.
- Dissolve the required amount of tamoxifen in corn oil by shaking for 14 h at 37°C and 120 rpm on a rotary shaker, and store at 4°C.

△ **CRITICAL:** Tamoxifen is light-sensitive; avoid exposure of the solution to direct light.

- Aliquot tamoxifen stock and store at –80°C.

Note: Do not reuse after thawing.

STEP-BY-STEP METHOD DETAILS

Isolation of female mouse reproductive tract tissue

⌚ Timing: 1 h

1. Euthanize the experimental mice according to the ethically approved technique.
2. Fix the mice to the dissection platform with the ventral side facing up and pin the legs with wall needles.
3. Sterilize the entire surface of the mouse with 70% ethanol.
4. Lift the skin of the abdomen and abdominal muscles with a pair of forceps.
 - a. Make a small cut giving an incision extending down to the genital opening.
 - b. Give transverse incisions along the length of the limbs.
 - c. Pin down the separated skin and the muscular folding to the dissection platform (Figure 2A).
5. Carefully remove the entire female mouse reproductive tract (FRT) from the abdominal cavity (Figure 2B).
6. Place the FRT tissue in a Petri dish and clear off any adipose and connective tissue surrounding the FRT.
7. Transfer the tissue to a Falcon tube and wash tissue with PBS by inverting the tube several times.

Preparation of paraffinized tissue

⌚ Timing: 1 day

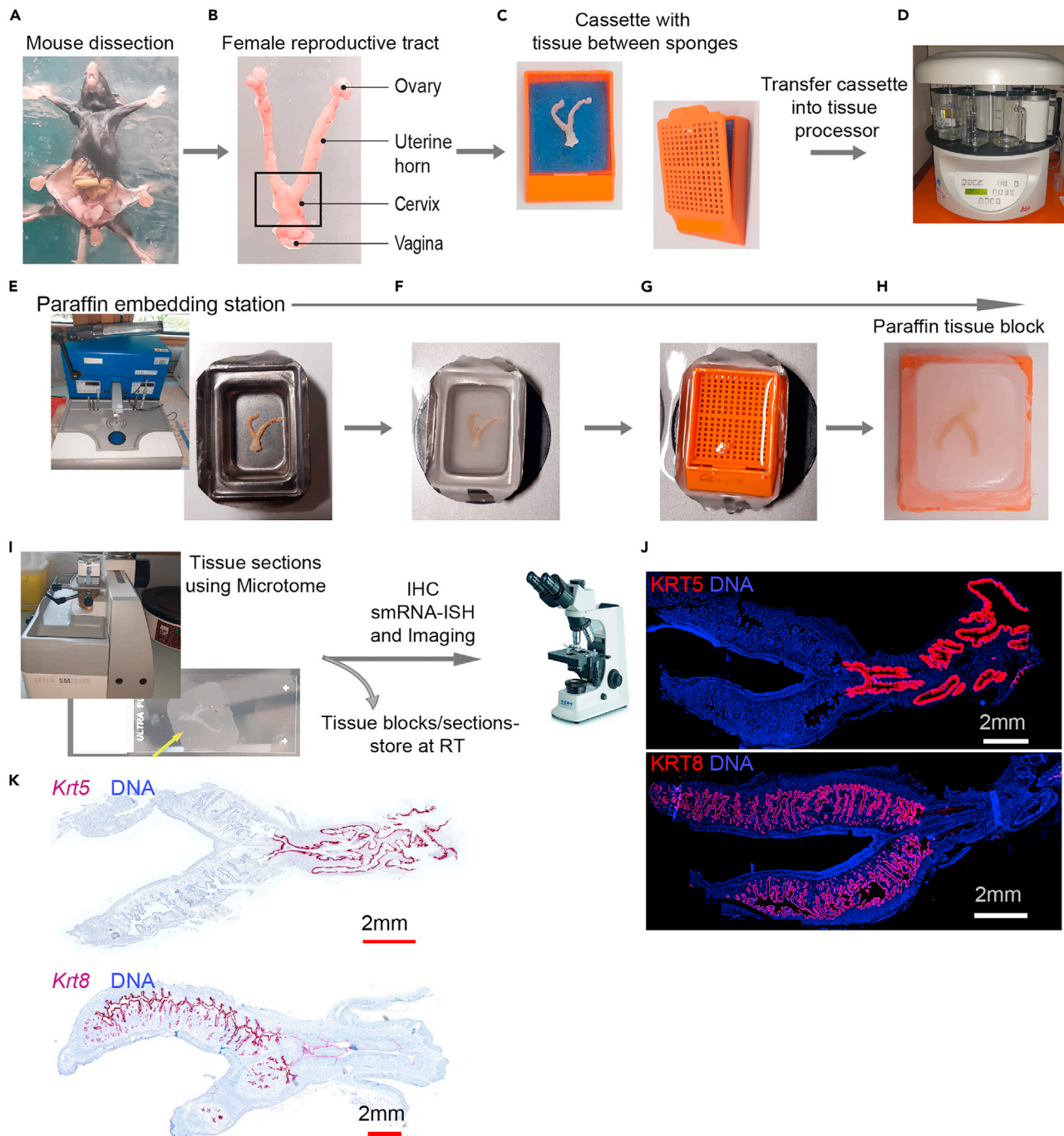


Figure 2. Isolation of FRT and preparation for IHC and smRNA-ISH

(A) Euthanized mice dissected.

(B) The isolated FRT.

(C) FRT tissue placed on a sponge inside the embedding cassette (left) and a closed cassette containing FRT tissue sandwiched between sponges (right).

(D) Tissue processor.

(E) The embedding station (left) and FRT tissue placed inside a paraffin embedding mold (right).

(F) FRT tissue placed inside an embedding mold with molten paraffin.

(G) Tissue embedding cassette placed in the embedding mold with FRT tissue.

(H) Paraffin-embedded FRT tissue block.

Figure 2. Continued

(I) Microtome for sectioning of paraffin blocks and a slide containing a 5 μm section of the FRT tissue.

(J) Whole tissue images of FRT tissue stained with antibody against cytokeratin 5 (KRT5) expressed exclusively in the stratified epithelia of ectocervix and vagina (upper panel) and cytokeratin 8 (KRT8) expressed exclusively in the columnar epithelium of the upper FRT, including endocervix and uterus (lower panel). Nuclei are shown in blue. (K) Whole FRT tissue smRNA-ISH images of *Krt5* (upper panel) and *Krt8* (lower panel). Nuclei are shown in blue. Images in J and K are acquired with AxioScan imager.

8. Transfer the entire tissue to a 50 mL Falcon tube containing 3.7% PFA prepared in sterile 1 \times PBS and keep for 14 h at 25°C (Figures 1 and 2B).
9. Discard the PFA, wash tissue thrice with 10 mL sterile PBS.

△ CRITICAL: Tissue used for IHC can be stored in PBS at 4°C for 1 week until the next step. The tissue used for smRNA-ISH should be processed immediately for the next step.

△ CRITICAL: PFA is highly toxic. Use protective gloves and a mask.

10. Place the tissue between the sponges inside the embedding cassette. Label the cassette with a pencil (Figure 2C) (troubleshooting 1).
11. Place the cassette inside the processing chamber of the tissue processor (Figure 2D).
 - a. Immerse the tissue in each reagent for 1 h with agitation (Series of regents to include: 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol X 2, 100% isopropanol X 2, 100% Xylene and Molten paraffin).

△ CRITICAL: Xylene causes systemic toxicity by ingestion or inhalation. Use protective gloves and a mask.

12. Embed dehydrated and paraffinized tissue using a paraffin embedding machine (Figures 2E–2H).

△ CRITICAL: Place the entire FRT horizontally and ensure the whole tissue is in the same plane before adding the paraffin.

- a. Cool the paraffin block on the cooling pad.
- b. After cooling, remove the paraffin-embedded tissue with cassette from the metal jar using a scalpel blade.
- c. The paraffinized tissue blocks can be stored for years at 25°C.

Sectioning of paraffinized tissue for immunohistochemistry and single molecular RNA *in situ* hybridization

⌚ Timing: ~2 h

13. Keep the paraffinized tissue block on ice for at least 30 min (troubleshooting 2).
14. Cut 5–7 μm thick sections for the IHC and 10 μm sections for the smRNA-ISH using a microtome (Figure 1).

△ CRITICAL: Sharp blade: contact may result in injury.

15. Place the sections on the water bath with the temperature set to 40°C for at least 5–10 min (Figure 2I).

△ CRITICAL: For smRNA-ISH, clean water bath with autoclaved distilled water and fill with fresh autoclaved distilled water each time (troubleshooting 3).

16. Using a glass slide, fish out tissue sections onto the middle of the slide.

△ **CRITICAL:** Use “SuperFrost Plus” adhesion slide.

- a. Place the slide on top of a heating plate at 37°C until sections dry completely.
- b. For IHC, the section can be stored at 25°C for years.
- c. For smRNA-ISH, prepare fresh sections ([troubleshooting 4](#)).

Deparaffinization and antigen retrieval of tissue section for immunohistochemistry

⌚ **Timing:** ~ 3 h

17. Pour 250 mL of DAKO target retrieval solution into a glass jar and place it in a water bath. Set the temperature to 95°C ([Figure 1](#)).
18. Keep the slides with tissue sections in an oven for 1 h at 60°C.
19. Immerse slides through a series of reagents as follows inside the fume hood:

	Reagent name	Time
1	100% Xylene	10 min
2	100% Xylene	10 min
3	100% ethanol	5 min
4	95% ethanol	5 min
5	70% ethanol	5 min
6	50% ethanol	5 min
7	Milli Q water	5 min
8	Milli Q water	5 min

- a. Move the slides up and down at least 5 to 10 times, every 2–3 min in each step.

△ **CRITICAL:** Xylene causes systemic toxicity by ingestion or inhalation. Use protective gloves and a mask.

20. Immerse slides in target retrieval solution, close the lid, and incubate for 30 min.

△ **CRITICAL:** Make sure water bath temperature has reached 95°C.

21. After boiling, keep the target retrieval solution with slides for 20 min at 25°C.
22. Remove the slides and wash 5 times with tap water by dipping up and down 3 to 4 times per wash.

One-step immunohistochemistry staining

⌚ **Timing:** 1 day

Note: All the primary antibodies targeting different proteins and raised in different species without a directly coupled fluorochrome can be incubated together in the one-step IHC. Then followed by respective fluorochrome tagged secondary antibodies (steps 23–36). In the two-step IHC protocol, all the primary antibodies not coupled to fluorochrome are first incubated with the tissue, followed by their respective fluorochrome coupled secondary antibodies. Next, the primary antibody that targets a different protein and is coupled directly to a fluorochrome is incubated together with DNA staining dye (steps 37–40) ([Figure 1](#)).

△ **CRITICAL:** The two-step IHC protocol greatly reduces the non-specific staining of tagged primary antibodies.

23. Remove slides and carefully wipe the slides with tissue paper to remove the moisture without disturbing the tissue section.
24. Using a Pap pen or Immedge hydrophobic barrier pen, draw a circle around the tissue section.

△ **CRITICAL:** Make sure not to touch the tissue section with the Pap pen.

25. Add 50–100 μL of PBS on top of the tissue section to prevent it from drying out.

Note: Volume of the PBS depends on the size of the tissue section.

26. Remove PBS and add 50–100 μL of IHC blocking solution onto the tissue section.
27. Incubate for 60 min at 25°C in a moisture chamber (to prevent drying).
28. Remove IHC blocking solution and add 50–100 μL of primary antibodies (without a directly coupled fluorochrome) diluted in IHC blocking solution, incubate for 14 h at 4°C in a moisture chamber.

△ **CRITICAL:** Make sure the tissue section is completely covered with antibody solution.

29. Wash the tissue section 5 times with PBS for 2–5 min at 25°C each time.
30. Add 50–100 μL of fluorochrome-linked secondary antibodies diluted in IHC blocking solution and your choice of DNA dye (Eg: Draq5 (1:1000), Hoechst (1:2000)).

△ **CRITICAL:** From this step onwards, work in low light.

31. Incubate for 60 min at 25°C in a moisture chamber in the dark.
32. Wash the tissue section 5 times with PBS for 2–5 min at 25°C each time.
33. Wash the tissue section once with distilled water.
34. Remove the excess water with tissue paper.

△ **CRITICAL:** Do not touch the tissue region and do not let the tissue section dry.

35. Add 3 to 4 drops of Mowiol on top of the tissue section, place a coverslip (24 × 60 mm) above, gently squeeze out excess Mowiol using the backside of bent forceps.

△ **CRITICAL:** Do not press hard as this may break the coverslip or disrupt the tissue. Make sure that no air bubbles are trapped near the tissue section.

36. Keep the slides for 14 h at 25°C in a dark place ([troubleshooting 5](#)).

▣ **Pause point:** Store at 4°C in the dark until fluorescence microscopy ([troubleshooting 6](#)).

Two-step immunohistochemistry staining

37. For staining, which includes both antibodies with unconjugated and conjugated fluorochromes, follow protocol steps 23–32.
38. After the wash in step 32, add 50–100 μL of IHC blocking solution onto the tissue section, incubate for 60 min at 25°C in a moisture chamber in the dark.
39. Remove IHC blocking solution and add directly tagged primary antibody diluted in IHC blocking solution and your choice of DNA dye (e.g., Draq5 (1:1000), Hoechst (1:2000)).
40. Follow steps 31–36.

Lineage tracing and tissue preparation

Here, we described the protocol for the lineage tracing of squamous epithelial marker KRT5 by utilizing mice expressing Krt5-CreERT2; Rosa26-tdTomato to visualize directly the endogenous tdTomato fluorescence in the FRT. Further, the protocol describes co-immunostaining of the lineage traced tissue to visualize additional proteins of interest. For lineage tracing, use four-week-old female mice housed in a BSL 2 level animal laboratory and provided with sterile drinking water and chow.

Note: Timing varies for lineage tracing depending on the experimental requirements.

For lineage traced tissue preparation

⌚ Timing: 1 h

41. Prepare fresh tamoxifen solution in corn oil before starting the experiment (see [materials and equipment](#) section).
42. Inject 50 μ L of tamoxifen solution per mouse (0.25 mg/g body weight) intraperitoneally.
43. Repeat the injection 2 times on consecutive days.

⚠ CRITICAL: Wipe the abdominal surface with 70% ethanol before injection and observe the health of the mice daily.

44. As per the requirement, euthanize mice (between 1 week to 20 weeks post-tamoxifen injection) according to standard ethically approved procedure.
45. Isolate FRT following steps 1–7.
46. Proceed for sectioning (steps 50–57, steps 58–62) and detect lineage traced cells directly (steps 47–49) or by co-immunohistochemistry according to the procedure below steps 58–65.
47. Dilute choice of DNA Dye in permeabilization buffer (Draq5 (1:1000), Hoechst (1:2000)), add 100 μ L per tissue section, incubate for 30 min at 25°C.
48. Follow steps 31–36.
49. Visualize the lineage traced cells by standard microscopic methods ([troubleshooting 7](#)) ([Figure 3J](#)).

Fresh frozen tissue preparation for immunohistochemistry

⌚ Timing: ~3 h

This protocol is applicable for the lineage traced fresh frozen mouse tissue.

50. Isolate FRT following steps 1–7.
51. Place the tissue in 10 mL of 2% PFA solution in a 50 mL Falcon tube, incubate for 1 h at 25°C in a dark place.

⚠ CRITICAL: PFA is highly toxic; avoid contact with skin and eyes. Use suitable protective gloves and a mask.

52. Remove the PFA and wash tissue 3 times with 1 \times PBS and immediately proceed for freezing the tissue.
53. Keep ready the freezing apparatus as follows inside a fume hood ([Figures 3A–3C](#)).
 - a. Fill a small icebox 1/4th with dry ice and add an equal volume of isopropanol ([Figures 3A and 3B](#)).
 - b. Place a small plastic box filled with 5 mL of isopentane on top of isopropanol, incubate for 15 min to cool down ([Figure 3C](#)).

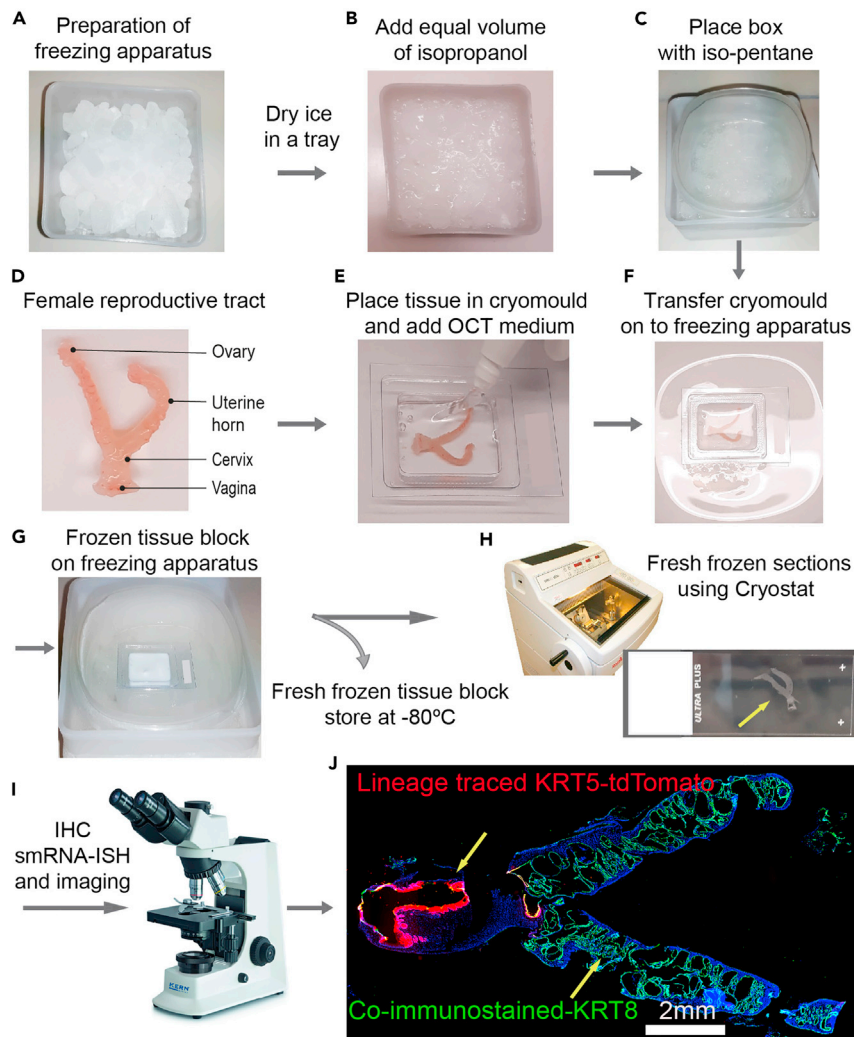


Figure 3. Preparation of fresh frozen tissues for analyzing lineage tracing and co-immunostaining

(A–C) Prepare a freezing apparatus as shown.

(D) FRT tissue isolated freshly and fixed.

(E) FRT tissue placed in a cryomould overlaid with OCT medium.

(F) Cryomould containing FRT tissue and OCT medium placed on the isopentane containing tray over the dry ice and isopropanol mixture for freezing.

(G) Fresh frozen tissue block in the cryomould ready to be stored or sectioned.

(H) Cryostat for sectioning fresh frozen tissues (left) and FRT tissue section placed on a slide.

(I) IHC or smRNA-ISH staining followed by imaging.

(J) FRT tissue showing the lineage traced stratified epithelia using a KRT5-tdTomato shown in red and the co-staining of the section with an antibody against KRT8 specific to columnar epithelium shown in green. Image in J is obtained with an AxioScan imager.

54. Remove tissue from the PBS and dry excess liquid using a tissue pad (Figure 3D) (troubleshooting 8).

55. Spread a small amount of OCT medium inside the plastic cryomould.

56. Place the tissue in the cryomould, add OCT medium until the tissue is completely covered (Figure 3E).

57. Place the cryomould on top of pre-cooled isopentane in the freezing apparatus and incubate until OCT medium has hardened (Figures 3F and 3G) (troubleshooting 9).

▮▮ **Pause point:** Immediately store frozen tissue at -80°C for long-term storage.

Cryosectioning and immunohistochemistry staining of fresh frozen tissue

⌚ Timing: ~2 h

58. Cut 5–7 μm thick tissue sections in the cryotome. Place the tissue sections on the “SuperFrost Plus” adhesion slides (Figure 3H).

⚠ **CRITICAL:** Always use SuperFrost Plus Slides.

59. Air-dry the slides at a dark place and proceed immediately for smRNA-ISH procedure described in steps 66–82 or step 60 for IHC.

⏸ **Pause point:** Can store for up to one month at 4°C before proceeding to step 60.

60. Before starting IHC, keep the slides inside an oven for 20 min at 40°C.

61. Wash slides 5 times with 1 \times PBS for 3 min at 25°C each time.

62. Using a Pap pen circle around the tissue section.

63. Add 50–100 μL of PBS on top of the tissue section to prevent it from drying out.

64. Remove PBS and add 50–100 μL of permeabilization solution for 1 h at 25°C.

65. Proceed for either the one-step IHC procedure described above in the protocol steps 23–36 or the two-step IHC procedure described above in steps 37–40.

⚠ **CRITICAL:** Use permeabilization buffer instead of IHC blocking solution to prepare primary or secondary antibody dilutions.

Single molecular RNA *in situ* hybridization

⌚ Timing: 3 days

For smRNA-ISH, the RNAscope protocol from the ACDBio (https://acdbio.com/sites/default/files/322360-USM%20RNAscope%202.5%20HD%20RED%20Pt2_11052015.pdf) was adapted with modification for the FRT (Figure 1). Following protocol works for both fresh-frozen and paraffinized tissue sections.

⚠ **CRITICAL:** Autoclave equipments used for the smRNA-ISH protocol. Use sterile filter tips. Work in a place dedicated to RNA work.

66. Isolate FRT following steps 1–7.

67. Process intact FRT for paraffinization and sectioning (steps 8–16) or fresh frozen tissue preparation and sectioning (steps 51–59).

DAY-1

Follow the same procedure outlined below for both the paraffin and fresh frozen sections.

68. Bake slides in a dry oven for 1 h at 60°C for the paraffin section and 20 min at 40°C for the fresh frozen sections.

69. In a fume hood:

⚠ **CRITICAL:** This step is only for the deparaffinized tissue section. For the Fresh frozen section, skip this step

a. Incubate the slides 2 times in Xylene for 5 min at 25°C each time.

- b. Incubate the slides 2 times in 100% Ethanol for 1 min at 25°C each time.
- c. Air-dry the slides for at least 5 min at 25°C or until completely dry.

△ **CRITICAL:** Xylene causes systemic toxicity by ingestion or inhalation. Use protective gloves and a mask.

70. Freshly prepare 1× RNA scope Target Retrieval reagent (refer to [materials and equipment](#) section).
71. Apply RNAscope Hydrogen Peroxide.
 - a. Incubate slides with RNAscope Hydrogen Peroxide for 10 min at 25°C.

△ **CRITICAL:** Make sure that the Hydrogen Peroxide solution covers all the tissue regions.

- b. Keep the slide on the slide rack and immerse in the beaker containing distilled water.
- c. Wash slides 2 times in distilled water for 5 min at 25°C each time.

72. Apply RNAscope Target Retrieval Reagents.
 - a. Very slowly submerge the slide rack containing the slides into boiling RNAscope 1× target retrieval reagent solution.
 - b. Cover the beaker with aluminum foil and boil the slides for 20 min.

△ **CRITICAL:** Do not boil less or more than 20 min. This step is optimized for the FRT to remove crosslinks, preserve tissue morphology, and efficient target RNA accessibility.

- c. Wash slides 2 times in distilled water for 5 min at 25°C each time.
- d. Wash slides once with fresh 100% EtOH.
- e. Dry the slides completely at 25°C.

73. Create a barrier.
 - a. Draw a barrier 2 times around each section with the Immedge hydrophobic barrier pen.

△ **CRITICAL:** Do not let the barrier touch the tissue section.

- b. Dry slides for 14 h to use the following day.

DAY-2

74. Equilibrate equipment.
 - a. Turn on the HybEZ oven and set the temperature to 40°C.
 - b. Place a humidifying paper in the humidity control tray and wet thoroughly with distilled water.

△ **CRITICAL:** Warm the tray for 30 min at 40°C before use; keep the tray in the oven when not in use. Do not allow slides to dry in between the incubation and washing steps.

75. Keep 1× wash buffer ready (can be prepared on day 1 (refer to [materials and equipment](#) section)).
76. Prepare counterstaining reagents, hematoxylin staining solution, and Ammonia water (refer to [materials and equipment](#) section).
77. Equilibrate reagents.

△ **CRITICAL:** Remove AMP 1 to 6 reagents from the refrigerator and place them at 25°C.

△ **CRITICAL:** Ensure HybEZ oven and prepared humidity control tray are at 40°C.

78. Apply RNAscope Protease Plus.
- Place dried slides on the HybEZ slide rack.
 - Add RNA scope protease plus to cover each tissue section entirely.
 - Insert tray with slides back into the oven.
 - Incubate at 40°C for 25 min.

△ **CRITICAL:** The duration of 25 min in this step is optimized for the FRT to remove crosslinks, preserve tissue morphology, and efficient target RNA accessibility. Do not change the timings of the incubation period to avoid insufficient or over digestion ([troubleshooting 10](#)).

- Wash slides by immersing them 5 times in distilled water.

79. Hybridization and signal Amplification.
- Hybridize probe.

△ **CRITICAL:** Before each use, warm the target and control probes for at least 10 min at 40°C in a water bath or incubator. Swirl gently to mix.

- Add 3 drops of the appropriate probe to cover each tissue section entirely.
 - Cover the HybEZ humidity control tray with a lid and insert it into the oven for 2 h at 40°C.
 - Wash slides two times in 1 × wash buffer for 2 min at 25°C each time
- Hybridize AMP.

△ **CRITICAL:** During the washing step, always keep the tray inside the oven to maintain the temperature at 40°C.

- Add ~2–3 drops of AMP1 to cover each section entirely.
- Close the tray and insert it into the oven for 30 min at 40°C.
- Wash slides two times in 1 × wash buffer for 2 min at 25°C each time.
- Add ~2–3 drops of AMP2 to cover each section entirely.
- Close the tray and insert it into the oven for 15 min at 40°C.
- Wash slides two times in 1 × wash buffer for 2 min at 25°C each time.
- Add ~2–3 drops of AMP3 to cover each section entirely.
- Close the tray and insert it into the oven for 30 min at 40°C.
- Wash slides two times in 1 × wash buffer for 2 min at 25°C each time.
- Add ~2–3 drops of AMP4 to cover each section entirely.
- Close the tray and insert it into the oven for 15 min at 40°C.
- Wash slides two times in 1 × wash buffer for 2 min at 25°C each time.

△ **CRITICAL:** Do not insert the tray into the HybEZ oven for the rest of the procedure.

- Add ~2–3 drops of AMP5 to cover each section entirely.
- Incubate for 45 min at 25°C.
- Wash slides two times in 1 × wash buffer for 2 min at 25°C each time.
- Add ~2–3 drops of AMP6 to cover each section entirely.
- Incubate for 15 min at 25°C.
- Wash slides two times in 1 × wash buffer for 2 min at 25°C each time.

80. Signal detection.

△ **CRITICAL:** Use the Fast RED-B solution within 5 min. Do not expose to direct sunlight or UV light.

- a. Prepare sufficient RED working solution per section by using a 1:60 ratio of Fast RED-B to Fast RED-A (2 μ L of Red B to 120 μ L of Red A)
 - b. Pipette \sim 100 μ L RED solution onto each tissue section.
 - c. Incubate for 10 min at 25°C.
 - d. Wash twice with Milli Q water
81. Counterstain the slides.
- a. Immerse slides in 50% Hematoxylin solution for 1 min at 25°C ([troubleshooting 11](#)).
 - b. Wash 1 s with Milli Q water.
 - c. Wash 5 times with tap water for 3 min each time (sections appear purple).
 - d. Replace tap water in the staining dish with 0.02% ammonia water, incubate for 1 min (Section color turns to blue).
 - e. Replace ammonia water with Milli Q water, wash slides 3 to 5 times
82. Mounting the samples.
- a. Dry the slides at 60°C inside the dry oven for at least 15 min.
 - b. Briefly dip the slide into fresh, pure xylene and immediately place 1–2 drops of Eco mount on the slide before the xylene dries.
 - c. Place a coverslip over the tissue section, gently press with the help of the backside of bent forceps to squeeze out excess eco mount, avoid the formation of air bubbles.
 - d. Air dry slides for at least 1 h and store at 25°C until microscopy.

△ CRITICAL: Xylene causes systemic toxicity by ingestion or inhalation. Use protective gloves and a mask.

EXPECTED OUTCOMES

This protocol provides a procedure for IHC to visualize the protein of interest from both paraffinized and fresh frozen sections of the entire FRT ([Figures 2J and 3J](#)) ([Chumduri et al., 2021](#)). This allows the utilization of antibodies that only work either with paraffinized or fresh frozen tissue samples. Lineage traced cells from the FRT can be visualized for the fluorescent protein used for tracing from fresh frozen tissue even after long-term storage. In addition, these tissues can also be stained with other antibodies to co-visualize multiple proteins of interest ([Figure 3J](#)). Further, the protocol also provides detailed steps for visualizing the spatial distribution of RNA of the gene of interest from paraffinized and fresh frozen tissue samples of the FRT ([Figure 2K](#)). This protocol could be implemented for the human tissue samples preserved similarly as described above. Thus, this protocol provides a complementary approach for validating single-cell RNA sequencing results and the possibility of analyzing different populations of cells and their spatial distribution with the native tissue context to study tissue dynamics in health and during pathogenesis.

LIMITATIONS

The IHC protocol describes the visual characterization of epithelial cells. However, to analyze different cell types, such as the immune cells, tissue preparation steps should be modified. The protocols described for smRNA-ISH using chromogenic probes can be applied to detect single or two genes simultaneously. However, multiplexing of up to four genes simultaneously would be possible using the RNAscope Multiplex Fluorescent Assay ([Wang et al., 2015](#)) by further optimizing the protocol. Additionally, this protocol can be adapted for the co-detection of RNA and protein simultaneously ([Dikshit et al., 2020](#)).

TROUBLESHOOTING

Problem 1

The tissue section from ectocervix to endocervix is not in one plane.

Potential solution

After PFA treatment, the tissue becomes stiff and slightly changes its original shape. To maintain the tissue in one horizontal plane, place the tissue between two sponges in the plastic cassette during dehydration and paraffinization in the tissue processor. If the tissue is still out of the plane, try to press the tissue with the help of hot forceps into one plane during paraffinization of tissue inside the metal mold while placing it on top of the cooling platform for solidification.

Problem 2

Tissue sections break during sectioning.

Potential solution

Use a fresh microtome blade to avoid breaking of tissue sections. Keep paraffin block on ice for at least 30 min before sectioning. Use the cooling option if available in the microtome.

Problem 3

Very few signals in the positive control.

Potential solution

The possible reason is that RNA is degraded during tissue processing. Maintain RNase free working area during tissue isolation, deparaffinization, sectioning, and development of signal. Use autoclaved water in the water bath. Make sure microtome blades and the water bath are free from RNase. Always try to use a freshly cut tissue section for the smRNA-ISH.

Problem 4

The tissue section is detached from the slide after the antigen retrieval step.

Potential solution

The tissue section has not adhered firmly to the slide. Use SuperFrost Plus slides to avoid detaching of tissue. Dry the slide at 37°C immediately after fishing out the tissue section from the water bath until the water completely evaporates.

Problem 5

Mowiol is dried out inside the coverslip

Potential solution

Not enough Mowiol was added to the slide. Make sure to add enough Mowiol onto the slide so that it oozes out from the edges of the coverslip while gently pressing with the backside of bent forceps.

Problem 6

No antibody signal was found.

Potential solution

Check whether antibody works (primary and secondary) with positive control and negative control samples. Check the dilution of antibodies recommended by the manufacturer. Make sure the right combination of secondary antibodies is used against animal species' source of primary antibodies. Check the settings in the microscope to make sure that it is set appropriately to the fluorochrome used.

Problem 7

No lineage traced cells were observed.

Potential solution

Cross-check the amount of tamoxifen in the diluted solution according to the weight of the mice. Do not reuse the tamoxifen solution the second time after thawing.

Problem 8

The tissue section is not intact.

Potential solution

The tissue is not appropriately embedded in the OCT medium. Remove any excess PBS from the tissue using dry tissue paper before adding an OCT medium.

Problem 9

The tissue section lost its cellular morphology

Potential solution

The tissue section is not frozen quickly. Make sure iso-pentane is adequately cooled by keeping in dry ice isopropanol mixture. Ensure enough dry ice is present at the bottom of isopropanol while freezing tissue in the OCT medium. Do not thaw frozen tissue blocks more than -10°C .

Problem 10

Tissue digestion compromised and no signal.

Potential solution

Protease plus treatment timing is optimized for the FRT tissue. Do not change the incubation period of protease plus, which may cause under digestion or over digestion that leads to low or no signal detection.

Problem 11

The red signal is not visible over the dark blue color.

Potential solution

A higher concentration of hematoxylin or extended incubation time may result in the dark blue color cells, which preclude the appearance of red-colored mRNA dots. Use 50% diluted hematoxylin with an incubation period of 1 min–2 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, Dr. Cindrilla Chumduri (cindrilla.chumduri@uni-wuerzburg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

C.C. is funded by the University of Wuerzburg and DFG (GRK 2157). N.K. is funded by DFG-DAAD and DFG (GRK2157).

AUTHOR CONTRIBUTIONS

C.C. and R.K.G. developed protocols, C.C., R.K.G., and N.K. carried out experiments. C.C. made the illustrations and graphical abstract, R.K.G., N.K., and C.C. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Chumduri, C., Gurumurthy, R.K., Berger, H., Dietrich, O., Kumar, N., Koster, S., Brinkmann, V., Hoffmann, K., Drabkina, M., Arampatzi, P., et al. (2021). Opposing Wnt signals regulate cervical squamocolumnar homeostasis and emergence of metaplasia. *Nat. Cell Biol.* 23, 184–197.

Dikshit, A., Zong, H., Anderson, C., Zhang, B., and Ma, X.J. (2020). Simultaneous visualization of RNA and protein expression in tissue using a combined RNAscope in situ hybridization and immunofluorescence protocol. *Methods Mol. Biol.* 2148, 301–312.

Wang, H.S.N., Wang, L.-C., Wu, X., Bui, S., Chang, K.-J., Nielsen, A., Vo, H.-T., Luo, Y., and Ma, X.-J. (2015). Multiplex fluorescent RNA in situ hybridization via RNAscope. In *In Situ Hybridization Methods*, G. Hauptmann, ed. (Humana Press), pp. 405–414.