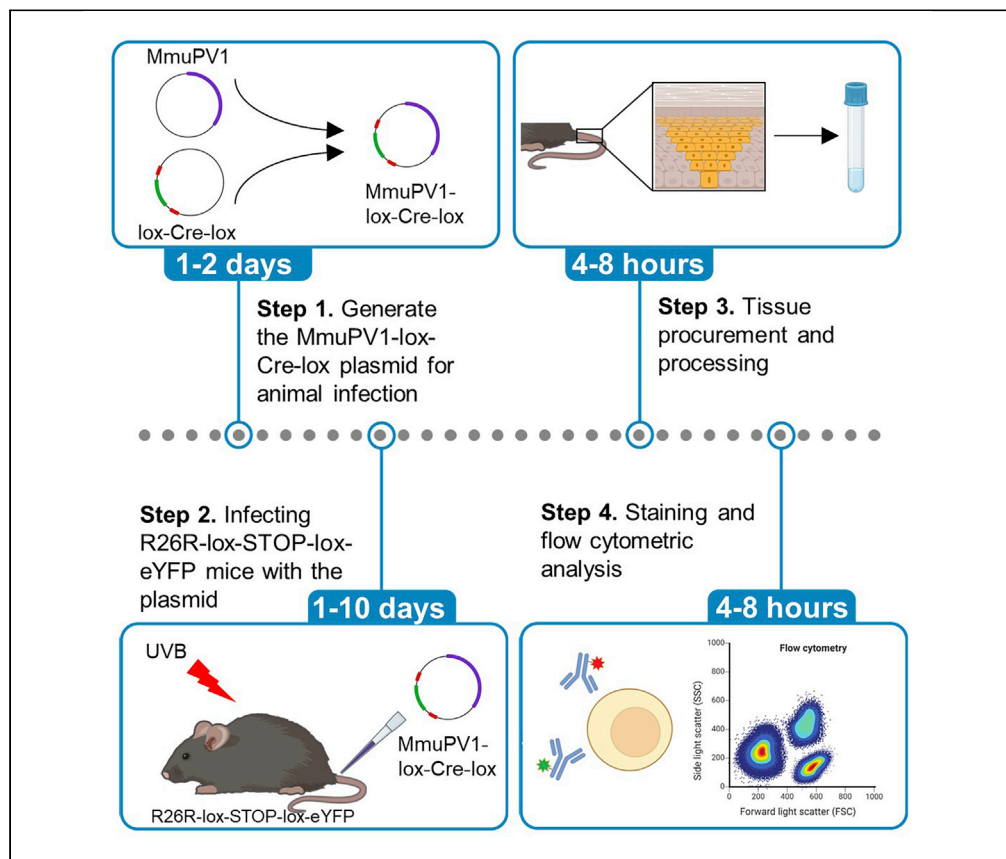


Protocol

Protocol for *in vivo* lineage tracing of the mouse-papillomavirus-type 1-infected cells in mice



Here, we present a protocol to create an *in vivo* lineage-tracing mouse model for mouse-papillomavirus-type 1 (MmuPV1)-infected cells. We describe the steps to generate and deliver the MmuPV1 lox-Cre-lox plasmid for the infection of mice, followed by skin tissue extraction and processing. We then detail how to use flow cytometry to trace, quantify, and analyze MmuPV1-harboring cells and their progeny. This model is suitable to investigate the early effects of papillomavirus on the target cells.

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

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Highlights
An *in vivo* lineage-tracing model for MmuPV1-infected cells in mice

Steps to generate and deliver the plasmid for animal infection

Trace, quantify, and analyze MmuPV1-harboring cells and their progeny by flow cytometry

Suitable tool for answering a variety of outstanding questions in papillomavirus biology

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Protocol

Protocol for *in vivo* lineage tracing of the mouse-papillomavirus-type 1-infected cells in miceVural Yilmaz^{1,2} and Katerina Strati^{1,3,*}¹Department of Biological Sciences, University of Cyprus, Nicosia 2109, Cyprus²Technical contact: vyilma01@ucy.ac.cy³Lead contact*Correspondence: strati@ucy.ac.cy<https://doi.org/10.1016/j.xpro.2022.101994>

SUMMARY

Here, we present a protocol to create an *in vivo* lineage-tracing mouse model for mouse-papillomavirus-type 1 (MmuPV1)-infected cells. We describe the steps to generate and deliver the MmuPV1 lox-Cre-lox plasmid for the infection of mice, followed by skin tissue extraction and processing. We then detail how to use flow cytometry to trace, quantify, and analyze MmuPV1-harboring cells and their progeny. This model is suitable to investigate the early effects of papillomavirus on the target cells.

For complete details on the use and execution of this protocol, please refer to Yilmaz et al. (2022).¹

BEFORE YOU BEGIN

This protocol allows lineage tracing of cells which have received a mouse specific papillomavirus genome (MmuPV1) *in vivo* via flow cytometry and assess their phenotypical changes such as proliferation dynamics and levels of MHC-I expression on the cell surface during the acute phase of the infection. Here, we specifically describe first, how to combine lox-Cre-lox plasmid (which was created in-house) with MmuPV1 genome to create MmuPV1-lox-Cre-lox plasmid which we deliver to the tail skin of reporter R26R-lox-STOP-lox-eYFP mice. Expression of self-deleting Cre from the plasmid results in recombination of the loxP sites and excision of the sequence flanked by the loxP sites. Therefore, introduction of the plasmid in mouse cells leads to the production of Cre recombinase and loss of the Cre sequence and its promoter from the plasmid and, more importantly, recircularization of the MmuPV1 genome, and the loss of the stop codon upstream of the yellow fluorescence protein (YFP) gene. A similar strategy has been used in the past to deliver human papillomavirus genomes in organotypic raft cultures.² This allows observation and tracing of the cells that initially harbored the plasmid containing the viral genome. Since YFP expression is genetically activated, it is conserved in the progeny of the initial cells that have taken up the plasmid. Secondly, we describe here how we perform the plasmid delivery to the R26R-lox-STOP-lox-eYFP mice; Thirdly, how we collect and process the tissue samples and finally we describe here how we perform the flow cytometric analysis to detect the YFP expressing MmuPV1-harboring cells and assess their various characteristics such as Ki-67 and MHC-I expression.

Institutional permissions

The R26R-lox-STOP-lox-eYFP mice are currently in use in the lab and are housed in the animal facility of the department of Biological Sciences at the University of Cyprus. The facility is licensed by the Veterinary Services (Republic of Cyprus Ministry of Agriculture and Natural Resources), the government body in charge of approving and overseeing laboratory animal work in Cyprus (license number CY.EXP.105). The Veterinary Services are also in charge of approving proposed experimental



protocols and overseeing their implementation according to local and European regulations. Mouse work currently underway in the lab is covered under license number CY/EXP/PR. L1/2019 which is valid until 2024.

Plasmid extraction from bacteria cultures

⌚ Timing: 1 day

1. Culture bacteria containing the lox-Cre-lox, puCMmuPV1 or CAG-Cre plasmids in 200 mL of standard LB medium in the presence of Ampicillin (100 µg/mL) overnight (16–18 h) at 37°C and shaking at 180 rpm in a shaker incubator. Then transfer the culture in to 50 mL tubes.
2. Spin down cultures at 4,700 rpm (2,420 rcf) for 7 min at room temperature (20°C–22°C).
3. Remove supernatant and resuspend bacteria pellet in P1 buffer (2.5 mL for every 50 mL culture) of the QIAGEN Plasmid Midi Kit. Collect them all to one 50 mL tube.
4. Add 2.5 mL of P2 buffer for every 50 mL culture.
5. Add 3.5 mL N3 buffer for every 50 mL culture.
6. Spin down samples at 4,700 rpm (2,420 rcf) for 6 min.
7. Transfer supernatant to a clean 50 mL tube.
8. Add equal volume 1:1 chloroform:isopropanol solution under a chemical fume hood.
9. Spin down at 4,700 rpm (2,420 rcf) for 6 min.
10. Transfer the top phase to a clean 50 mL tube.
11. Add equal volume of isopropanol and mix well under a chemical fume hood.
12. Spin down at 4,700 rpm (2,420 rcf) for 6 min.
13. Remove the supernatant and wash pellet with 70% ethanol (Break up the pellet with a blue tip and transfer in to an Eppendorf tube).
14. Spin down the samples at 13,000 rpm (15,871 rcf) for 1 min at room temperature (20°C–22°C), remove the supernatant and air-dry pellet (can take between 1 to 24 h at room temperature of 20°C–22°C).
15. Resuspend in 50 µL of autoclaved water (50–300 µL depending on the size of the pellet).
16. Keep in –20°C until further processing.

Autoclave mouse equipment and consumables

⌚ Timing: 2–4 h

17. Autoclave mouse cages containing mouse bedding prior to mouse reallocation for the experiment or use a sterile single-use mouse cages designed for short-term housing of mice.
18. Autoclave sufficient mouse food and water to be used for the duration of the experiment.
19. Autoclave the surgical tools to be used for the tissue procurement and processing.

⚠ **CRITICAL:** It's important to sterilize and keep the mice equipment and consumables clean for the duration of the experiment since mice go through UV irradiation and are susceptible to other potential pathogens which can halt the experiment itself or change the outcome.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Ki-67 (PerCP/Cy5.5) (1:100 dilution)	Biolegend	Cat#652423, RRID: AB_2629530
Anti-MHC-I (H-2Kb) (Brilliant violet 421) (1:100 dilution)	Biolegend	Cat#116525, RRID: AB_2876430

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Tail skin of R26R-lox-STOP-lox-eYFP mice	This study	N/A
Chemicals, peptides, and recombinant proteins		
0.25% trypsin containing EDTA solution	Sigma-Aldrich	Cat#T4049-500ML
BD FACS™ Lysing Solution	BD Biosciences	Cat#349202
Tween-20	Sigma-Aldrich	Cat#P1379
1X PBS (phosphate buffered saline) solution	Invitrogen	Cat#70011-044
CountBright™ Absolute Counting Beads	Invitrogen	Cat#C36950
Proline™ Universal Calibration Beads	BIO-RAD	Cat#1451086
Plasmid Midi Kit	QIAGEN	Cat#12143
NucleoSpin Gel and PCR Clean-up	MACHEREY-NAGEL	Cat#740609.250
T4-DNA ligase	NEB	Cat#M0202M
T4-DNA ligase reaction buffer	NEB	Cat#B0202S
DEPC-treated water	Invitrogen	Cat#AM9915G
Ammonium acetate, Puriss. p.a., ACS Reagent	Honeywell Fluka	Cat#32301-500G
Experimental models: Organisms/strains		
Laboratory mice (<i>Mus musculus</i>): R26R-lox-STOP-lox-eYFP 6–9 weeks old Females	Jackson Laboratory http://jaxmice.jax.org/strain/006148.html	B6.129X1-Gt (ROSA)26Sortm1(EYFP)Cos/J, RRID: IMSR_JAX:006148
Recombinant DNA		
Lox-Cre-Lox plasmid	This paper	N/A
puC19-MmuPV1 plasmid	Gift from Paul F. Lambert, Joh et al. ³	N/A
pCAG-cre control plasmid	Matsuda et al. ⁴	Addgene plasmid # 13775
Software and algorithms		
FlowJo v10.6.2 for FACS data analysis	FlowJo, LLC	https://www.flowjo.com/ , RRID: SCR_008520
GraphPad Prism v.8.0	La Jolla, CA	RRID: SCR_002798
Other		
Flow cytometer	Bio-Rad, S3e Cell Sorter	Cat#12007058, RRID: SCR_019710
handheld homogenizer: POLYTRON PT 1200 E	KINEMATICA	Cat#11010026/100-120V
NanoDrop™ 2000c Spectrophotometer	Thermo Fisher Scientific	Cat#ND2000CLAPTOP RRID: SCR_020309

MATERIALS AND EQUIPMENT

To generate a plasmid with a self-deleting Cre sequence and the full MmuPV1 genome, we combined sequences from four plasmids: puC19-MmuPV1 (gift from Paul F. Lambert), pCAG-cre (a gift from Connie Cepko (Addgene plasmid # 13775; <http://n2t.net/addgene:13775>; RRID: Addgene_13775)), small_pMA-T, and large_pMA-T (synthesized by Invitrogen). To construct the final plasmid, we first PCR amplified the sequence in large_pMA-T that contains the lox and part of the E6 sequence using primers to introduce PstI and HindIII restriction sites in the 5' and 3' ends of the sequence, respectively. Using PstI and HindIII, we cloned lox-E6 in the pCAG-Cre plasmid. In this new plasmid, we subcloned the L1-lox sequence from small_pMA-T plasmid using blunt end ligation (L1-Lox sequence was isolated using SacI and KpnI restriction enzymes, the Cag-cre plasmid was digested with Sall and T4 DNA polymerase was used to generate blunt ends). We refer to this plasmid as lox-Cre-lox which was sequenced to confirm the correct insertion of L1-lox-Cre-lox-E6 sequence. The protocol here starts at this point forward and does not include the above-mentioned preparations in details for the generation of the lox-Cre-lox plasmid.

Fixation/permeabilization solution for FACS staining (For 10 samples)

Reagent	Final concentration	Amount
BD Lysis Buffer	25% (v/v)	2.5 mL
Tween-20	0.1% (v/v)	12.5 μL

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Continued

Reagent	Final concentration	Amount
ddH ₂ O	N/A	10 mL
Total	N/A	12.5 mL

Store at room temperature (20°C–22°C) up to 8 h.

Ligation reaction mix

Reagent	Final concentration	Amount
T4-DNA ligase	2 m U/mL	8 μL
T4-DNA ligase buffer	10% (v/v)	500 μL
DNA to be ligated	10 μg/mL	30–50 μg
DEPC-H ₂ O	N/A	Up to 5 mL
Total	N/A	5 mL

Incubate at 16°C overnight (16–18 h).

Ammonium acetate recipe

Reagent	Final concentration	Amount
Ammonium acetate	7.5 M	23.12 g
ddH ₂ O	N/A	40 mL
Total	7.5 M	50 mL

Store at 4°C up to 1 week.

For the ammonium acetate solution, first add 40 mL of ddH₂O to the 23.12 g of ammonium acetate to dissolve the reagent completely and then replenish ddH₂O up to the final volume of 50 mL.

Flow Cytometer: The flow cytometer machine used in this study is Bio-Rad S3e Cell Sorter with a laser set up of 405/488/561 nm and filter set up of 447/60, 525/30, 586/25, 600LP.

Alternatives: Any other flow cytometers and fluorochromes could be used as long as suitable laser channels and detectors were appropriate for the corresponding fluorochromes and the necessary compensation controls were performed.

STEP-BY-STEP METHOD DETAILS

Part 1: Generating the plasmid for animal infection

⌚ Timing: 2 days

This part describes the generation of the plasmid for animal infection by removing the ampicillin bacterial selection cassette from lox-Cre-lox and puC19-MmuPV1 plasmids using XhoI and XbaI. The two fragments (lox-cre-lox and MmuPV1) are then gel purified and ligated overnight (16–18 h) at 16°C (ligation was done using a ratio of 1:2.5, MmuPV1 to lox-cre-lox).

1. XhoI and XbaI digestion of lox-cre-lox and puC19-MmuPV1 plasmids.
 - a. Set up a large-scale double digestion in Takara buffer M with the XhoI and XbaI restriction enzymes as per manufacturer's instructions: https://www.takarabio.com/us/products/molecular_biology_tools/restriction_enzymes/double_digestion_buffers.
 - b. Check that the digestion is completed by running a small amount on a 1% agarose gel.

- c. After the validation run a large agarose gel to separate the products and the cut bands as follows:
 - i. MmuPV1: isolate the 6 kb band.
 - ii. lox-Cre-lox: isolate the 4 kb band.
 - d. Purify DNA from the gel of these cut bands by using the NucleoSpin Gel and PCR Clean-up, Mini kit for gel extraction and PCR clean up and follow the manufacturer's instructions: <https://www.mn-net.com/media/pdf/02/1a/74/Instruction-NucleoSpin-Gel-and-PCR-Clean-up.pdf>.
 - e. Quantify DNA concentration via Nanodrop and confirm the size by running a 1% agarose gel with 1 kb DNA ladder in which, the mass of DNA in each of the bands is provided (assuming a 0.5 µg load) for approximating the mass of DNA samples of similar size.
2. Ligation of the two plasmids.
- a. Bring the digested DNA (30–50 µg total) up to a 5 mL volume with DEPC water and 1x T4-DNA ligase buffer (500 µL) in a 50 mL conical tube.
 - b. Add 8 µL of high concentration (2 m U/mL) T4-DNA ligase (NEB).
 - c. Incubate the ligation mix at 16°C overnight (16–18 h).
 - d. Precipitate the ligated DNA by adding 2.5 mL of 7.5 M ammonium acetate and 12.5 mL of 95% Ethanol (4°C) into ligation mix and vortex well.
 - e. Incubate at 4°C overnight (16–18 h).
 - f. Isolate the ligated DNA by centrifuging the mix at 4°C for 10 min at 10,000 rcf.
 - g. Decant the supernatant carefully and wash the pellet with 1 mL of 70% Ethanol (transfer the genome in an Eppendorf tube) and centrifuge the sample in room temperature (20°C–22°C) for 10 min at 5,000 rcf.
 - h. Carefully decant the supernatant and repeat the washing steps 2–3 times.
 - i. Decant the final supernatant and let the pellet air-dry for 10 min. Dilute the ligated DNA in DEPC treated water.
 - j. Quantify DNA concentration on nanodrop and confirm the size by running a 1% agarose gel with 1 kb DNA ladder with known mass.
 - k. Use the online calculator at <http://nebiocalculator.neb.com/#!/dsdnaamt> to calculate the generated DNA copy number from the known DNA length and DNA mass.

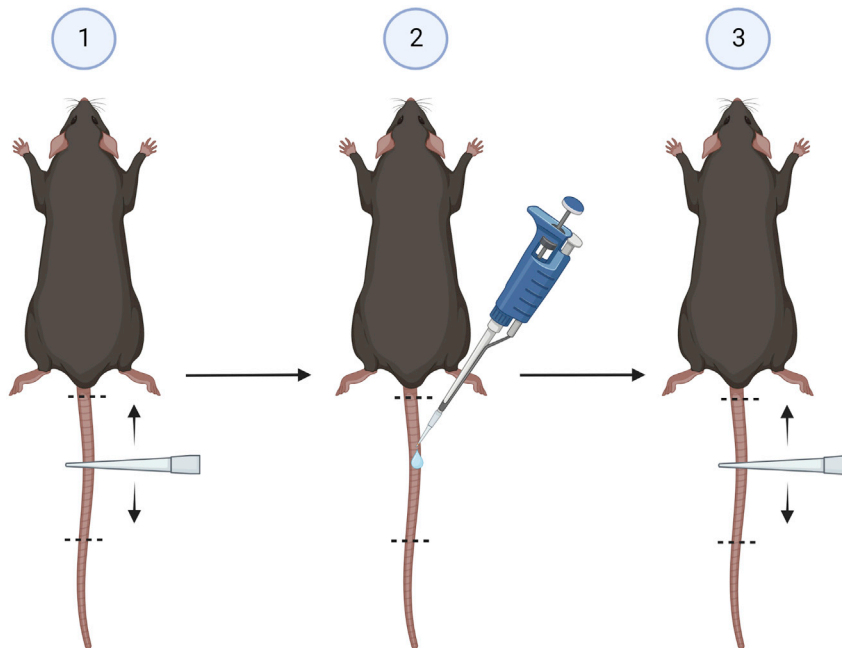
⚠ **CRITICAL:** After the calculations, prepare several aliquots with low volume to avoid freezing and thawing the plasmid repeatedly. Keep the aliquots in –80°C indefinitely, until the plasmid delivery day.

Part 2: Plasmid delivery to the R26R-lox-STOP-lox-eYFP mice

⌚ **Timing:** 4–6 h, depending on the number of mice

This part describes the steps to shave the back skin of 6–9 weeks old female R26R-lox-STOP-lox-eYFP mice and apply a single dose of 1,000 mJ/cm² UV-B irradiation for transient immunosuppression. Following the UV-B radiation, the mice are given at the same day with 2×10^9 DNA copies of the generated MmuPV1-lox-Cre-lox plasmid or the commercially available CAG-Cre plasmid (control plasmid) in a 10 µL of PBS solution applied at the base of the tail skin by a superficial dermabrasion with a pipette tip to expose the tail basal layer (Scheme 1). Perform all the steps below in a BSL2 biological safety cabinet.

3. Shave the back skin of the mice.
 - a. One by one, hold the mouse by its tail and lift it up and use a common electric hair trimmer to shave most of the back skin of the mouse starting from the base of the tail.
4. UV-B irradiation of the mice.
 - a. After the mice are shaved, put them all into a glass container without a lid and place the container with the mice inside the UV machine.



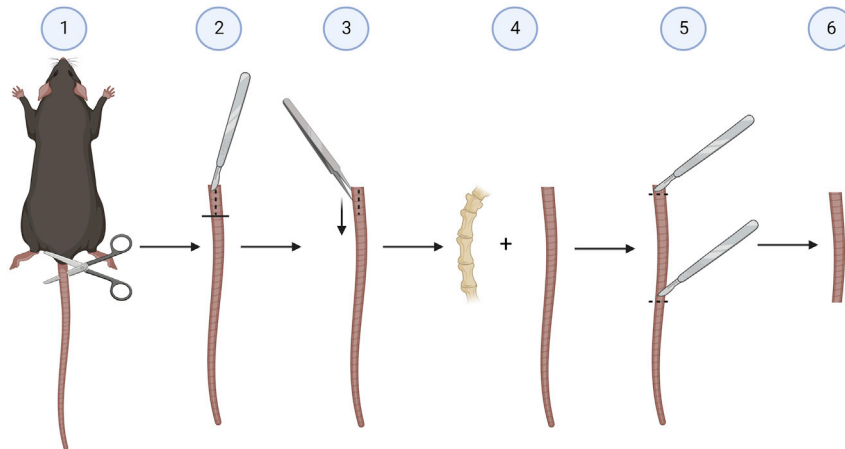
Scheme 1. Dermabrasion and plasmid delivery

(1) Place the mouse into a mouse restrainer and hold out the tail of the mouse. For dermabrasion, use a 20–200 μ L pipette tip, rubbing it back and forth with the side of the pipette tip on the surface of the base of the tail skin approximately 20–30 times while keeping the tail stretched by hand. (2) Apply the plasmid by pipetting 10 μ L of plasmid containing PBS solution on the top of the area of the dermabrasion (to the epidermis layer). (3) continue rubbing with the side of the pipette tip very slowly for another 10–20 times and wait until the solution is completely absorbed by the skin or dry out before continuing to the next mouse.

- b. Program the machine to apply UV-B irradiation in a dose dependent manner set to 1,000 mj/cm^2 . This generally takes 2–5 min per cage of mice.
5. Superficial dermabrasion of the tail skin and delivery of the plasmid, 5–7 min per mouse (Scheme 1).
 - a. One by one, place the mouse into a mouse restrainer which exposes and gives easy access to handle the tail of the mouse.
 - b. Dermabrasion is performed by using a 20–200 μ L pipette tip, rubbing it back and forth with the side of the pipette tip on the surface of the base of the tail skin approximately 20–30 times while keeping the tail stretched and strait by hand.
 - c. Then, apply the plasmid by pipetting 2×10^9 DNA copies of MmuPV1-lox-Cre-lox or CAG-Cre plasmid in a 10 μ L of PBS solution on the top of the area of the dermabrasion (to the epidermis layer).
 - d. Continue rubbing with the side of the pipette tip very slowly for another 10–20 times.
 - e. Wait until the solution is completely absorbed by the skin or dry out before continuing to the next mouse. By this method, the plasmid should have access to the basal cell layer of the epidermis via the minor cracks established by the mild dermabrasion.

Optional: One can also shave mice one day and perform the UV-B irradiation and infection the next day.

Note: Tail skin was selected for plasmid delivery for practical reasons such as the ability to track area where plasmid was delivered and fewer hairs which place barriers on cell dissociation for flow cytometric analysis. If the instructions are followed precisely, all mice should harbor the delivered plasmid.



Scheme 2. Extracting mouse tail skin for further processing

(1) Extract the mouse tail skin by cutting the tail from the body completely by the base with a scissor. (2) Make a small incision at the base end of the tail with a small surgical blade. (3) Grab the skin from here and strip the whole tail skin from the tail bone in one firm motion with using the tweezers. (4) Now, we have the tail skin completely peeled off from the tail bone. (5) Cut out the part of the tail skin where the plasmid was applied and discard the rest of the tail skin. (6) Continue with this skin sample for enzymatic digestion and separation of epidermis from dermis.

⚠ **CRITICAL:** Dermabrasion of the tail skin with a pipette tip should be done very gently as to only expose the tail skin basal layer, which does not require much force. Further, after applying the plasmid in PBS solution, wait 1–2 min until the solution is dried out. Also, perform the delivery of the control plasmid or mock infections first and then the delivery of the MmuPV1-lox-Cre-lox plasmid to avoid cross contamination between mice groups.

Part 3: Tissue procurement and processing

⌚ **Timing:** 1 day

This part describes the steps to sacrifice mice and extract tail skins, followed by the incubation of the tail samples in a 0.25% trypsin containing EDTA solution (Sigma-Aldrich) overnight (16–18 h) at 4°C. The next day, epidermis should be separated from dermis by using tweezers, the tissue should be cut into small pieces and mechanically dissociated using a handheld homogenizer (POLYTRON PT 1200 E). Perform all the steps below in a BSL2 biological safety cabinet.

6. Sacrifice mice at the desired time points (0, 5, 10, 30 days post-delivery).
 - a. Euthanize mice by using first a CO₂ chamber and then cervical dislocation.
7. Extract tail skin (Scheme 2).
 - a. Extract the tail skin by cutting the tail from the body completely by the base.
 - b. Make a small incision at the base end of the tail and grab the skin from here and strip the whole tail skin from the tail bone in one firm motion with using the tweezers.
 - c. Cut out the part of the tail skin where the plasmid was applied in a rectangular shape that is 3–4 cm and discard the rest of the tail skin.
8. Separating dermis from epidermis.
 - a. Place the rectangular tail skin samples into a 6-well plates that contains 4 mL of 0.25% trypsin containing EDTA solution.
 - b. Incubate overnight (16–18 h) at 4°C for enzymatic digestion.
 - c. After the incubation (the next day), use tweezers to separate epidermis from dermis by grabbing the tail skin sample with the tweezers and gently press down. The epidermis should easily come off from the dermis due to the trypsinization.

- d. Next, cut the separated epidermis to even smaller pieces with surgical blades to make it easier to create single cell suspension from the epidermis.
- e. Put the small pieces in to a 15 mL Falcon tube with the 4 mL of trypsin containing medium from the well and mechanically dissociate the cells by using a handheld homogenizer such as POLYTRON PT 1200 E.

Optional: One can also perform the trypsinization at step 8b for 2 h at 37°C instead of overnight at 4°C and continue with separating epidermis and preparing the single cell suspension for FACS staining.

Note: One does not have to use a handheld homogenizer at step 8e to dissociate the cells. This can also be done by pushing the tissue pieces through a cell strainer by using the piston/plunger from a sterile 3 cc syringe. Use the flat end of the plunger to mince the sample by crushing the sample on the strainer in gentle circular motions.

△ CRITICAL: It's very important to clean the homogenizer vigorously before every sample by dipping the tip of the homogenizer in to a 70% ethanol and turn it on and repeat this in clean PBS solution as well just before using it for the next skin sample. Also, process the skin of the control or mock infection group first and then the MmuPV1-lox-Cre-lox infected samples. This will further prevent cross contamination between samples.

Part 4: Antibody staining and flow cytometry

⌚ **Timing:** 4–6 h, depending on sample number and acquisition speed

This part describes the steps to wash the resulting single cell suspensions from previous step with PBS and incubate the cells with the mentioned fluorescently conjugated antibodies for 30 min at 4°C. After surface marker staining, cells should be fixed and permeabilized for 10 min with 2 × BD lysing solution (BD Biosciences) + 0.1% Tween, washed, and then stained in PBS for intracellular Ki-67 staining. We then perform multiparametric flow cytometric analysis using a Bio-Rad S3e Cell Sorter flow cytometer and analyze the data using FlowJo software (FlowJo, LLC). The population of live cells is detected depending on their size and complexity (FSC-SSC gate) and later the doublets are excluded (FSC-Height/FSC-Area gate) before the analyses of the markers.

9. Wash and stain cells.
 - a. Transfer the samples into 5 mL FACS tubes and fill up the tubes with PBS.
 - b. Centrifuge samples for 5 min at 300 rcf and 4°C and decant supernatant.
 - c. In the meantime, prepare the solution for MHC-I staining as follows: For 10 samples, calculate for 12 for the volume of 100 μL for each sample. This is 1,188 μL of PBS and 12 μL of the BV421 – MHC-I (H2Kb) antibody in a 1:100 ratio.
 - d. Resuspend cells with 100 μL of the staining solution, vortex well and incubate cells 30 min on ice or at 4°C.
10. Fixation and permeabilization for Ki-67 staining.
 - a. Prepare the fixation/permeabilization solution as follows: For 10 samples (calculate for 12) for the volume of 1 mL for each sample. This is 2.5 mL of BD Lysis Buffer (BD Biosciences), 10 mL of ddH₂O and 12.5 μL of Tween 20 (Sigma-Aldrich).
 - b. Add 1 mL of fixation/permeabilization solution to each sample without washing and incubate 10 min at room temperature (20°C–22°C) in the dark.
 - c. Wash the cells as mentioned at steps 9a and 9b.
 - d. In the meantime, prepare the solution for Ki-67 staining as follows: For 10 samples, calculate for 12 for the volume of 100 μL for each sample. This is 1,188 μL of PBS and 12 μL of PerCP/Cy5.5 – Ki67 antibody in a 1:100 ratio.

- e. Resuspend cells with 100 μ L of the staining solution, vortex well and incubate cells 30 min on ice or at 4°C.
 - f. Wash the cells as mentioned at steps 9a and 9b.
 - g. Resuspend the cells with 300–500 μ L of PBS and add 5000 of CountBright™ Absolute Counting Beads (Invitrogen) to each sample to be able to calculate absolute numbers.
11. Acquisition at Bio-Rad S3e Cell Sorter flow cytometer.
- a. Open ProSort software and start the Bio-Rad S3e Cell Sorter.
 - b. After 30 min of start-up which warms-up lasers, run QC with Proline™ Universal Calibration Beads.
 - c. Create a new worksheet and create plots resembling the basic gating strategy to be able to monitor cell populations while recording.
 - d. Pass the samples through a cell strainer cap to ensure single cell suspension. The volume of buffer can be adjusted to change the event rate.
 - e. Use and record an unstained control with the same cells to adjust SSC and FSC gains for cell population to be on scale.
 - f. Define acquisition settings such as events to record, the speed of acquisition and stopping volume or timing. Adjust stopping criteria to high thresholds to make sure that the whole sample is acquired.
 - g. Acquire samples and export fcs files to be analyzed on FlowJo.

Optional: The counting beads enable the calculation of absolute numbers when we analyze the results. This step provides better comparison between samples but remains as an optional step. If there is spillover of fluorescence signal between channels while acquiring, perform single stained controls and acquire with the machine to calculate the compensation for fluorescence signals. One can also use a Live/Dead dye during the FACS staining to better distinguish the live cells from the dead ones during the flow cytometric analysis.

Note: To dissolve the Tween-20 completely in the fixation/permeabilization solution, leave the pipette tip that was used for adding Tween-20 inside the tube of the solution and leave it on the shaker for 10–20 min while shaking gently.

△ CRITICAL: Keep everything on ice while working and use ice cold PBS. Also, for incubation steps and storage, samples and staining mixes should be placed in the dark, as the fluorophores are sensitive to light exposure.

▮▮ Pause point: Samples can be acquired directly or stored at 4°C overnight (16–18 h) to be measured the next day.

EXPECTED OUTCOMES

A representative analysis of expected results is provided in [Figures 1](#) and [2](#) where we identify the cell populations based on their size and granularity using the forward scatter (FSC) and sideward scatter (SSC) signal, followed by exclusion of doublets and dead cells. Then, the cells harboring the plasmids are identified by their YFP signal at the different time points of the infection tested here and further analyzed for their MHC-I and Ki-67 expression at those time points ([Figures 1](#) and [2](#)).

By following this protocol, one should expect to find the plasmid harboring cells via their YFP expression on flow cytometer and that both numbers and percentages of these YFP + cells are much higher in MmuPV1 samples at day 5 post-delivery (p.d.), which decreased overtime as seen at day 10 and day 30 p.d. Whereas, pCAG-Cre samples only start to have increased YFP+ cells around day 10 p.d. Coinciding with the increased number of YFP+ cells, one should observe that MmuPV1-harboring cells exhibit significantly increased levels of Ki-67 around day 5 p.d. compare to other time points and CAG-Cre control plasmid, indicating a higher proliferation rate around day 5 p.d. due to the presence of MmuPV1.

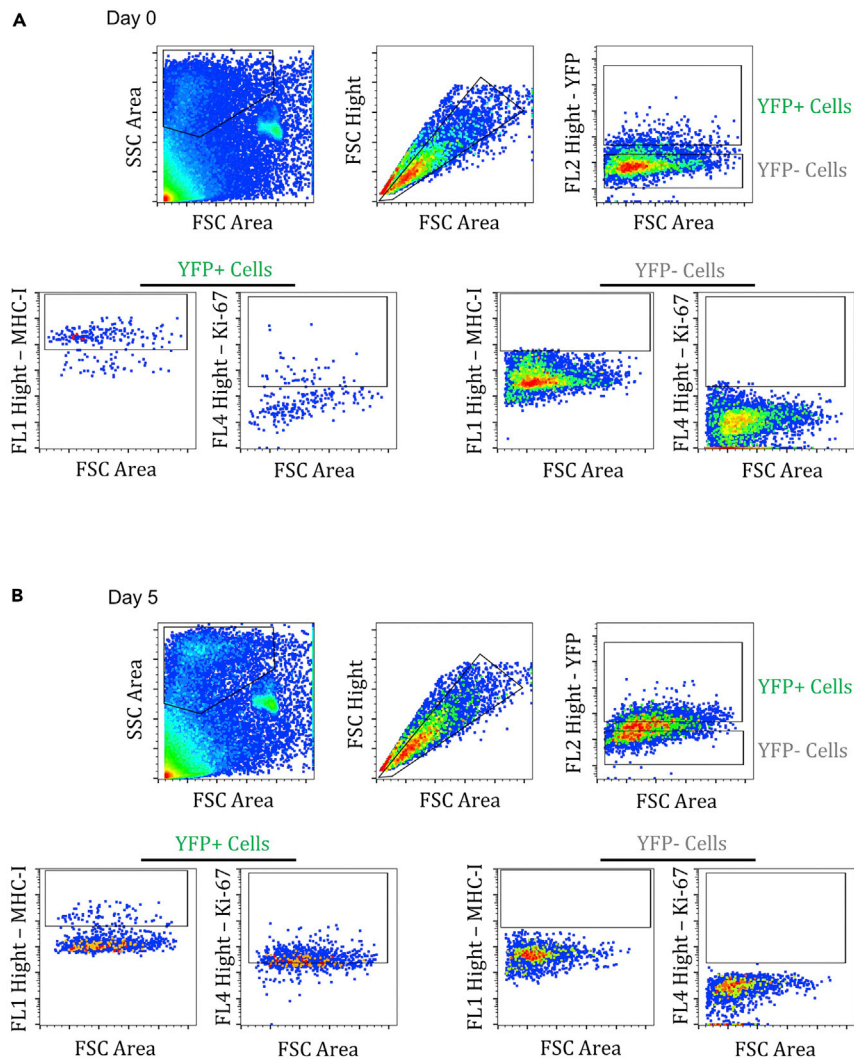


Figure 1. Gating strategy to detect and assess the MmuPV1-*lox-Cre-lox* or CAG-Cre control plasmid harboring cells (A and B) The population of the interest of cells from day 0 (A) or day 5 (B) of MmuPV1-*lox-Cre-lox* harboring skin samples was detected depending on their size and complexity, omitting the dead cells (FSC-SSC gate) and later excluding the doublets (FSC-Height/FSC-Area gate). Then, the plasmid bearing cells were detected by their YFP expression and grouped as YFP+ and YFP- cells. These populations then were assessed for their levels of MHC-I and Ki-67 expressions.

Furthermore, one should observe significantly lower expression of MHC-I surface receptors on the YFP+ cells of MmuPV1-treated tissues at day 5 p.d. which indicate a possible immune evasion strategy by the MmuPV1. We speculate that the virus is causing a decreased expression of MHC-I molecules on the cell surface to reduce the antigen exposure to the immune cells but further work is required to verify this. YFP+ cells in control treated tissues exhibit increased MHC-I expression at this time point, presumable due to YFP neo-antigen expression. These effects wanes at later time points.

As a control for our lineage-tracing model, we use CAG-Cre plasmid which drives the expression of a self-deleting Cre recombinase, just as in the MmuPV1-*lox-Cre-lox* plasmid, but it does not encode any MmuPV1 genes. In this control group, we expect to see the YFP+ cells which harbor the plasmid in the tail skin of R26R-*lox-STOP-lox-eYFP* mice but without the phenotypic changes imposed on the cells due to the presence of the MmuPV1 gene expression. This is indeed what we observe in the [Figure 2](#).

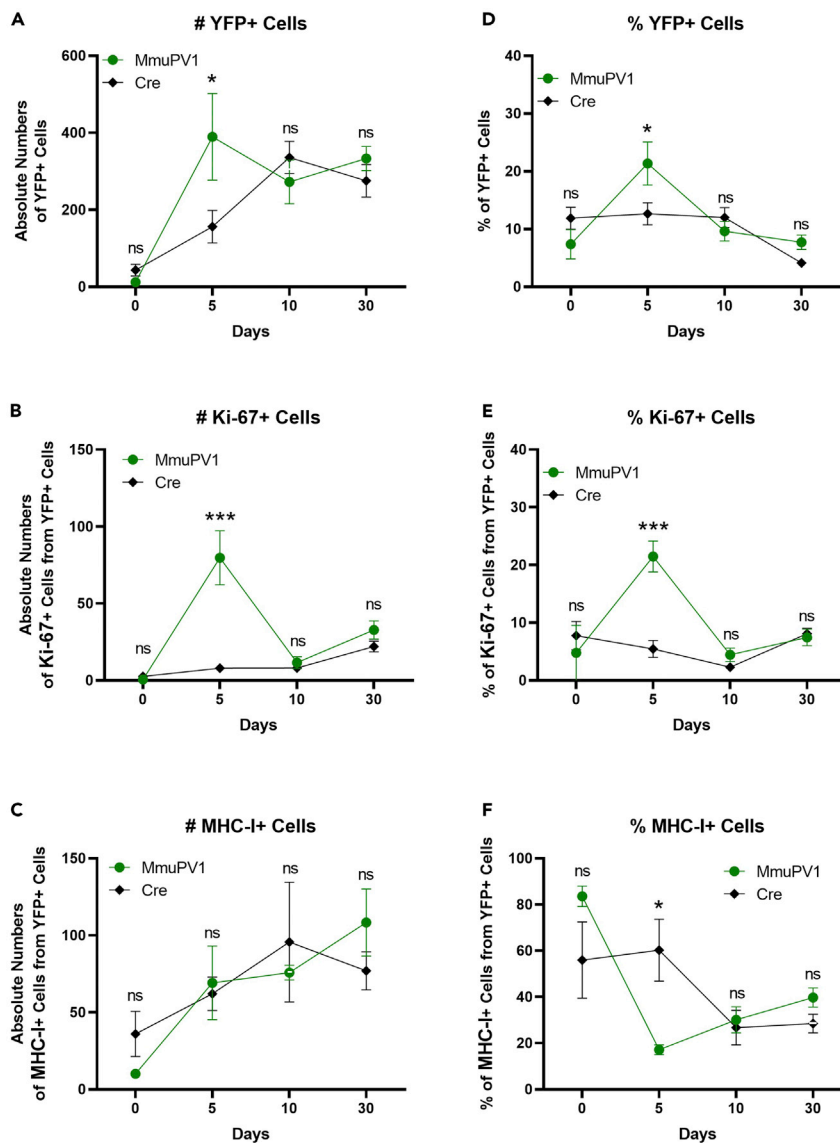


Figure 2. Assessing the characteristics of the plasmid-harboring cells during the acute phase of the infection

Cells with MmuPV1-lox-Cre-lox plasmid have increased proliferation rate and decreased MHC-I expression on the cell surface.

(A) Absolute numbers of YFP+ cells.

(B) Absolute numbers of Ki-67+ cells out of only YFP+ cells.

(C) Absolute numbers of MHC-I+ cells out of only YFP+ cells.

(D) Percentages of YFP+ cells.

(E) Percentages of Ki-67+ cells out of only YFP+ cells.

(F) Percentages of MHC-I+ cells out of only YFP+ cells. Mock infection with PBS (red square) was only performed for day 0 time point. Data representative of two independent experiments with $n = 3$ mice per group. The results are presented as means \pm SEM. not significant (ns), * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by nonparametric 2-way ANOVA test.

QUANTIFICATION AND STATISTICAL ANALYSIS

The raw data is obtained and recorded by flow cytometer where we have numbers and percentages of the YFP+ cells, MHC-I levels and Ki-67 levels of the YFP+ cells as shown in [Tables 1](#) and [2](#) below. We also obtain and record the same parameters for the YFP- cells (not included here). The following

Table 1. Raw data of absolute numbers measured by FACS

Sample	YFP+ cells	YFP+ Ki67+ cells	YFP+ MHC-I+ cells
Cre 1 d0	13.28903654	1.661129568	6.644518272
Cre 2 d0	55.29789511	3.567606136	48.16268284
Cre 3 d0	61.17834818	2.659928182	53.19856364
MmuPV1 1 d0	16.36393389	0	14.7275405
MmuPV1 2 d0	7.782101167	0	5.836575875
MmuPV1 3 d0	11.42857143	1.632653061	9.795918367
Cre 1 d5	167.8004535	4.535147392	80.70866142
Cre 2 d5	77.38095238	5.952380952	42.73504274
Cre 3 d5	223.0359521	13.31557923	62.58381761
MmuPV1 1 d5	592.519685	98.42519685	78.60922147
MmuPV1 2 d5	203.962704	44.67754468	23.80952381
MmuPV1 3 d5	371.032633	96.11086276	104.8601864
Cre 1 d10	416.2826421	13.82488479	173.5791091
Cre 2 d10	276.9979412	5.614823133	54.27662362
Cre 3 d10	313.7506003	4.802305106	59.22842965
MmuPV1 1 d10	304.028376	6.3339245	77.2738789
MmuPV1 2 d10	161.8929016	9.339975093	66.93648817
MmuPV1 3 d10	351.6369305	19.05421791	83.14567816
Cre 1 d30	278.8311399	17.84519295	99.26388579
Cre 2 d30	346.9322197	28.91101831	74.95449192
Cre 3 d30	200.4251442	19.23271586	56.68589938
MmuPV 1 1 d30	379.0030359	43.09078445	151.7970816
MmuPV 1 2 d30	273.6520855	22.38046796	88.50457782
MmuPV 1 3 d30	347.107438	33.05785124	84.7107438

tables show examples of obtained raw data, analysis and the calculations of absolute numbers by the use of counting beads (CountBright™ Absolute Counting Beads) added into the samples before the acquisition with FACS. This calculation is done by dividing 5000 with the recorded number of beads in the particular sample and multiplying the resulting number with the number of YFP+ cells, MHC-I+ cells from the YFP+ cell population or Ki-67+ cells from the YFP+ cell population, again recorded for that same particular sample. The resulting amount is the normalized absolute number from the given sample (example in [Table 3](#)).

Nonparametric 2-way ANOVA test was performed and statistical significance was considered at $p < 0.05$. The results are presented as mean \pm SEM. Not significant (ns), * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Statistical analyses of the data were performed using the GraphPad Prism v.8.0 (La Jolla, CA). All the experiments were performed using at least three biological replicates.

LIMITATIONS

The dynamics of the observed phenotypic changes such as the number of YFP+ cells detected and MHC-I or Ki-67 levels through the infection can be slightly different depending on the precision of plasmid delivery, handling of the mice and processing the skin samples as described here. Other factors, such as varying the genetic background of mice, changing the dermabrasion method and/or timing may introduce further variation. Nevertheless, one should be able to observe the “infected” cells via their YFP expression and assess the dynamics of the effects of MmuPV1 on these cells by flow cytometry. Validation of some of the results obtained by FACS, as well as information regarding tissue topology of YFP+ cells can be obtained by microscopy approaches (not described here), although these approaches lack significantly in terms of quantification.

Another limitation of the model is the observation of these YFP expressing cells for a longer time points than the ones mentioned here. 30 days post-delivery, we see that the population of YFP+ cells are mostly gone and still in decline albeit still detectable. This limits the use of this protocol to

Table 2. Raw data of percentages measured by FACS

Sample	YFP+ cells	YFP+ Ki67+ cells	YFP+ MHC-I+ cells
Cre 1 d0	11	12.5	50
Cre 2 d0	9.09	6.45	87.1
Cre 3 d0	15.5	4.35	30.8
MmuPV1 1 d0	11.2	0	90
MmuPV1 2 d0	2.53	0	75
MmuPV1 3 d0	8.33	14.3	85.7
Cre 1 d5	14.8	2.7	46.8
Cre 2 d5	14.3	7.69	87
Cre 3 d5	8.82	5.97	47
MmuPV1 1 d5	23.4	16.6	13.6
MmuPV1 2 d5	26.5	21.9	21
MmuPV1 3 d5	14.1	25.9	16.9
Cre 1 d10	14.8	3.32	41.7
Cre 2 d10	12.3	2.03	19.6
Cre 3 d10	8.82	1.53	18.9
MmuPV1 1 d10	11.9	2.08	25.4
MmuPV1 2 d10	10.7	5.77	41.3
MmuPV1 3 d10	6.3	5.42	23.6
Cre 1 d30	3.09	6.4	35.6
Cre 2 d30	4.23	8.33	21.6
Cre 3 d30	5.1	9.6	28.3
MmuPV 1 1 d30	6.45	4.66	46.8
MmuPV 1 2 d30	10.2	9.52	40.1
MmuPV 1 3 d30	6.45	8.18	32.3

investigate longer infection time points (hard to detect the cells of interest in high enough numbers to perform reliable analysis).

This protocol describes the generation of a unique plasmid that has the genome of MmuPV1 with a lox-cre-lox reporter function. We previously validated that the constructed MmuPV1-lox-Cre-lox plasmid can indeed lead to reporter activation, Cre-excision, and MmuPV1 genome recircularization both *in vivo* and *in vitro*.¹ However, we previously were not able to observe any wart development at the later time points of the infection with the plasmid and we were also not able to re-infect a second set of mice from the swab samples of the tail skins of the first set of mice (not shown here). These observations indicate to a possibility that the resulting virions *in vivo* from the generated plasmid might not be generating enough infectious viral particles. In line with this observation, it is known that infection with MmuPV1 is mainly cleared by the functions of the adaptive immune system in immunocompetent mice such as C57BL/6 (which is the genetic background for the R26R-lox-STOP-lox-eYFP mice used here) in which, the pathologies due to the MmuPV1 infection, such as warts, are either absent or regressive in nature and mice are refractory to persistent infection.⁵ These observations render this model more suitable to investigate the early effects of papillomavirus on the target cells, transcriptional and phenotypic changes of the papillomavirus-harboring cells during the acute phase of the infection, and investigate the initial viral-host immune interactions. Further studies are required to determine the potential utility of this model to study long-term effects of the virus.

TROUBLESHOOTING

Problem 1

Low YFP+ cell numbers.

If the number of the YFP expressing cells is lower than expected during the flow cytometric analysis at steps 11f and 11g, there can be several different reasons and solutions.

Table 3. Example of calculation for absolute number

Sample	Recorded counting beads	Recorded YFP+ cells	Recorded YFP+ Ki67+ cells	Recorded YFP+ MHC-I+ cells
MmuPV1 1 d5	4511	969	1618	126
–	$5000/4511 = 1.108401685$	$1.108401685 \times 969 =$ 1074.041233	$1.108401685 \times 1618 =$ 1793.393926	$1.108401685 \times 126 =$ 139.6586123
Absolute Numbers:	–	1074.041233	1793.393926	139.6586123

Potential solution

- This may be due to an inefficient superficial dermabrasion of the tail skin and delivery of the plasmid at the step 5. Make sure that the solution containing the plasmid has not been subjected to repeated freeze-thaw cycles. Make smaller aliquots to use for plasmid deliveries. Keep the plasmid on ice until the delivery.
- Also, the dermabrasion should be done only superficially. Make sure to not use force and not cause big wounds on the skin which might trigger the effects of the local inflammation to the dynamics of the plasmid delivery.
- If the reason for low YFP+ cell numbers is the number of dead cells and generally low quality of cells at the FACS, then it might point to a problem in the preparation of the samples prior to acquisition by flow cytometer. At step 8, make sure that the separation of epidermis from dermis is performed adequately and the enzymatic digestion is done well.
- A different reporter mouse strain (using a different genetic background more susceptible to infection and/or a different fluorescent reporter) may also result in increased ability to detect the cells which have received this plasmid.

Problem 2

Assessing the success of plasmid delivery.

Related to the above problem with low number of YFP+ cells, the success of plasmid delivery and the consequent expression can be assessed before continuing to the Part 3 of tissue procurement to circumvent this problem.

Potential solution

- From each mouse, take swab samples from the surface of the tail skin and wash the swabs to remove cells prior to collecting the skin tissue for the above-mentioned assays. Isolate DNA from the swab samples and assessed the presence of the L1 gene of MmuPV1 via PCR as a way of confirming the presence of the viral genome on the skin surface. This technique can be used to make sure that the mice are indeed infected.

Problem 3

Increased numbers of dead cells.

Step 8e can be improved to decrease the number of dead cells during the preparation of single cell suspension.

Potential solution

- One can alternatively use the piston/plunger from a sterile 3 cc syringe to push the pieces through a cell strainer by using the flat end of the plunger to mince the sample.
- Keep the cells on ice throughout the procedures.
- Optimize numbers of samples processed simultaneously to minimize time between skin harvesting and flow cytometry.

Problem 4

Low number of cells to analyze.

If the number of cells is generally insufficient to perform the flow cytometric analysis at steps 11f and 11g, there can be different solutions.

Potential solution

- The area of the tail skin that is used to deliver the plasmids can be increased or the whole tail can be used for plasmid delivery which would increase the number of the cells to be analyzed overall. In this case the amount of plasmid should also be proportionally increased.
- More mice can also be used for the plasmid delivery to increase the overall numbers of cells to be analyzed.

Problem 5

Clogging during FACS.

As is common with freshly isolated cells, the epidermal cells can easily clump together and block the pores of the flow cytometer during acquisition at the steps 11f and 11g.

Potential solution

- Make sure to gently vortex the samples very well, pass the samples through a cell strainer just before the acquisition and also a second time during mid-acquisition if the sample is run for more than 10 min. This will avoid the clogging of the machine and losing precious samples. Also adjust the sample volume depending on the flow rate and number of events acquired per second. Try to optimize a well-diluted sample without causing a very long run time.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Katerina Strati, strati@ucy.ac.cy.

Materials availability

R26R-lox-STOP-lox-eYFP mice are commercially available at Jackson Laboratory <http://jaxmice.jax.org/strain/006148.html>, under the name B6.129X1-Gt (ROSA)26Sortm1(EYFP)Cos/J.

There is a restriction to the availability of lox-Cre-lox plasmid which is a unique reagent generated for this study and is not available commercially. This plasmid can either be generated by following the instructions provided previously elsewhere¹ and briefly mentioned here or can be accessed via contacting to the [lead contact](#) listed here.

Data and code availability

The published article includes all data analyzed during this study.

All data reported in this paper will be shared by the [lead contact](#) upon request.

This paper does not report original code.

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

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AUTHOR CONTRIBUTIONS

Formal analysis, V.Y., K.S.; investigation, V.Y., K.S.; methodology, V.Y., K.S.; validation, V.Y.; writing – original draft, V.Y., K.S.; writing – review and editing; V.Y., K.S.; data curation, K.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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