bio-protocol

Published: Apr 20, 2022

Isolation of tdTomato Expressing Inter-follicular Epidermal Melanocytes or Keratinocytes from Mouse Tail Skin

Samuel Pop, Oscar Urtatiz and Catherine D. Van Raamsdonk*

Department of Medical Genetics, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver B.C., V6T 1Z3, Canada
*For correspondence: cvr@mail.ubc.ca

Abstract

The epidermis is the outermost layer of the skin. It is made up of mostly keratinocytes along with a small number of melanocytes and Langerhans cells. Melanocytes produce a pigment called melanin, which is transferred to the keratinocytes, and protects these cells from damage from UV radiation, as well as generating hair and skin colours. In this important relationship, keratinocytes exert control over melanocytes. Many questions regarding keratinocyte-melanocyte interactions have yet to be answered, and would benefit from study in model systems, to address diseases such as vitiligo and cutaneous melanoma. Most of the mouse is covered in fur and these areas lack the skin pigmenting inter-follicular epidermal (IFE) melanocytes. However, the mouse tail is pigmented analogously to human skin. Here, we present a method for isolating IFE melanocytes or keratinocytes expressing the tdTomato marker from the mouse tail, using fluorescence-activated cell sorting (FACS). The method involves firstly separating the tail skin epidermis from the dermis, and then digesting the epidermis to produce dissociated cells, which can then be sorted. These isolated cell populations can be studied using RNAseq or cultured *in vitro*. This protocol isolates IFE melanocytes or keratinocytes and immediately provides reasonable yields of cells, without the need to stain the cells for cell specific markers.

Keywords: Melanocytes, Keratinocytes, Mitf-cre, K14-cre, tdTomato, Skin, FACS, Dermis, Epidermis, Mouse tail, Inter-follicular epidermis

This protocol was validated in: eLife (2021), DOI: 10.7554/eLife.71825

Background

Melanocytes are the pigment producing cells of the skin. They are found in the basal layer of the epidermis in contact with the basement membrane, and in the hair follicles (Le *et al*., 2021). Melanin, the pigment produced by melanocytes, is transferred to keratinocytes in a specialized organelle called the melanosome (Natarajan *et al*., 2014). Melanin transfer in hair follicles pigments the hair, while melanin transfer in the inter-follicular epidermis (IFE) pigments the skin. The study of melanocyte and keratinocyte interactions in the inter-follicular epidermis is important for understanding normal skin pigmentation and diseases that affect melanocytes, such as vitiligo and cutaneous melanoma (Upadhyay *et al*., 2021). In mice, most of the skin is covered with dense fur and lacks skin pigmenting melanocytes (Fitch *et al*., 2003). However, the mouse tail is pigmented by IFE melanocytes, which are analogous to those in human skin (Van Raamsdonk *et al*., 2009; Deo *et al*., 2013; Tharmarajah *et al*., 2012, 2018 Urtatiz *et al*., 2018). Separately isolating IFE melanocytes and keratinocytes from mouse tail skin is useful for characterizing these cell types using molecular methods, and for studying them in primary cultures (Glover *et al*., 2015; Köhler *et al*., 2017; Urtatiz *et al*., 2021). A detailed protocol for the isolation of IFE melanocytes from the mouse tail skin epidermis has not been previously published.

In this paper, we describe our method for isolating IFE melanocytes or keratinocytes from mouse tail skin. There are several ways in which our protocol differs from others. For example, on the day before the procedure, the tail is waxed to remove the melanocytes in the hair follicle bulbs to focus on those that are in the IFE. In addition, a gentle scratching method is used to release cells specifically from the basal layer of the epidermis, where IFE melanocytes are located. Furthermore, tdTomato is used to label the cell population of interest via a conditional *Cre-loxP* system, so that the cells can be isolated by fluorescent activated cell sorting (FACS) (Nagy, 2000; Adan *et al*., 2016; McLellan *et al*., 2017). tdTomato is a red fluorescent protein with an emission wavelength of 581 nm (Shaner *et al*., 2004). It is a significantly brighter fluorescent molecule than some other markers, such as GFP, and provides increased sensitivity (Day and Schaufele, 2008). The use of intrinsic tdTomato expression makes it unnecessary to stain the cells for cell type specific markers prior to doing FACS, which is an advantage. This shortens the procedure, keeping cells healthier, and likely produces more consistent results and greater yields. However, incorporating two additional transgenes into the mouse breeding scheme will increase the amount of mouse breeding that must be done, and this could be impractical if a very large number of samples are required. This method permits downstream applications, such as RNA sequencing and cell culture, to be immediately conducted on isolated melanocytes or keratinocytes.

The *Cre-loxP* components that we used were *Rosa26-floxed stop-tdTomato*, with either *Mitf-cre* or *K14-cre* transgenes. In the *Rosa26-floxed stop-tdTomato* allele, the *tdTomato* cDNA is knocked into the ubiquitously expressed *Rosa26* locus (Madisen *et al*., 2009). Transcription of *tdTomato* is prevented by an upstream stop cassette, which is flanked by *loxP* sites (**Figure 1A**). When Cre recombinase is expressed in a cell from another transgene, the *loxP* sites are recombined by Cre, the stop cassette is excised, and *tdTomato* is permanently expressed (**Figure 1B**). By placing Cre expression under the control of a cell type-specific promoter, the excision can be limited to one cell type. For example, if Cre is expressed from the melanocyte specific *Microphthalmia transcription factor* promoter (*Mitf*), *loxP* excision will only occur in melanocytes (Alizadeh *et al*., 2008), while expressing Cre from the *Keratin 14* promoter (*K14*) will limit excision to keratinocytes (Hafner *et al*., 2004).

The process to obtain a single cell type from mouse tail skin consists of four main steps (**Figure 1C**), that are most similar to those used previously in Yang *et al*. (2017). First, the tail skin is removed from the tail bones. Then the skin is incubated in a dispase solution, to allow the separation of the epidermis from the dermis, the latter of which is discarded (Kormos *et al*., 2011). Next, the epidermis is incubated in a trypsin solution to obtain a single cell suspension (Tang *et al*., 2013). Finally, FACS is used to isolate the tdTomato expressing cells of interest from the mixed population (Adan *et al*., 2016). We recently used this technique to investigate how the $GNAQ^{Q209L}$ oncogene impairs the survival of IFE melanocytes (Urtatiz *et al*., 2021).

While this technique could likely be used at any age, here we describe the collection of cells from mice at 5 weeks old. The genetic background of the mice is C3HeB/FeJ. We prefer this strain because it has a functional copy of the *Agouti* gene, which plays an important role in melanocyte biology and pigmentation.

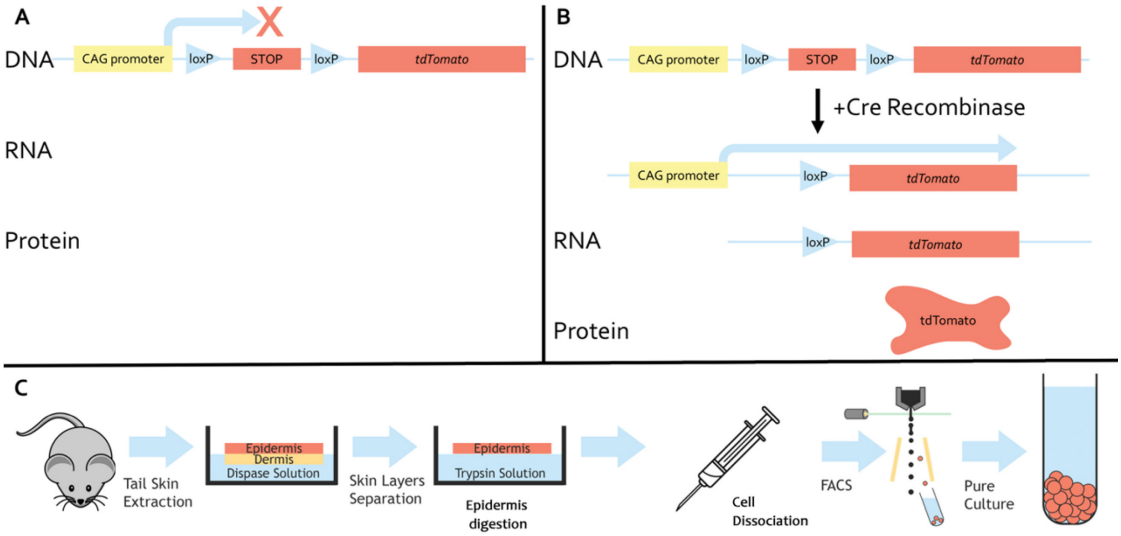


Figure 1. Overview of the method to isolate cells of the epidermis using fluorescent markers.
(A) When the *loxP* sites are in place, *tdTomato* is not expressed in *R26-floxed stop-tdTomato* mice. **(B)** When Cre recombinase is present, it recombines the *loxP* sites, which removes the stop cassette. This initiates the continuous expression of *tdTomato*, which is translated into a brightly fluorescent protein. **(C)** Experimental outline for isolating tdTomato-positive cells from tail skin epidermis.

Materials and Reagents

1. Disposable gloves
2. Nair® Wax Ready Strips for face (available at most drug stores or online)
3. 1 × Disposable scalpel (Fisher, catalog number: 02-688-79)
4. 1 × Ultra sharp general purpose razor blade (Fisher, catalog number: 13-812-236)
5. P20, P200, and P1000 Micropipette tips, sterile
6. 10 × 5 mL serological pipette (VWR, catalog number: 612-4945)
7. 4 × 10 mL serological pipette (VWR, catalog number: 612-4946)
8. 2 × 25 mL serological pipette (VWR, catalog number: 612-4947)
9. 4 × 15 mL conical tube (Corning, catalog number: 430790)
10. 3 × 50 mL Falcon® tube (Corning, catalog number: 352070)
11. 3 × 30 mL Petri dish (Sarstedt, catalog number: 82.1194.500)
12. 2 × 10 mL syringe (Fisher Scientific, catalog number: 14-817-54)
13. 1 × DMSO-Safe Acrodisc Syringe Filter, 0.2 µm pore size, sterile (Pall, catalog number: 4433)
14. 2 × 16 G needle (BD Precision glide Needle, catalog number: 305197)
15. 1 × Falcon® 40 µm Cell Strainer (Corning, catalog number: 352340)
16. 1 × 1.5 mL Eppendorf tube, sterile
17. 1 × Falcon® 5 mL Round Bottom Polystyrene Test Tube, without Cap, sterile (Corning, catalog number: 352052)
18. Dispase II protease (Sigma-Aldrich, catalog number: D4693-1G)
19. Trypsin (1:250), powder (Thermo Fisher, catalog number: 27250018)
20. EDTA (0.5 M), pH 8.0, RNase-free (Thermo Fisher, catalog number: AM9260G)
21. Minimum Essential Medium (MEM) Eagle with HBSS w/o L-Gln, ("MEM") (Lonza, catalog number: BE12-127F)
22. Fetal Bovine Serum ("FBS"), suitable for cell culture, sterile-filtered (Sigma-Aldrich, catalog number: F1051)
23. eBioscience® Fixable Viability Dye eFluor® 450 (Thermo Fisher, catalog number: 65-0863-14)

24. RiboLock RNAse Inhibitor (Thermo Fisher EO0381) (For downstream RNAseq only)
25. Dispase solution (see Recipes)
26. Trypsin solution (see Recipes)
27. MEM + EDTA + 10% FBS Solution (see Recipes)

Equipment

1. 1 × Heat pad (Sunbeam brand, standard style, 12 × 0.5 × 15 inches, 3 temperature settings)
2. 1 × 37°C (5% CO_2) tissue culture incubator (Nuaire, catalog number: NU-S810)
3. 1 × Ice bucket and ice (Fisher, catalog number: S66360)
4. 1 × Analytical balance (Mettler Toledo, catalog number: AL54)
5. 1 × 1,000 µL micropipette (Gilson, catalog number: F123602)
6. 1 × 200 µL micropipette (Gilson, catalog number: F123601)
7. 1 × 20 µL micropipette (Gilson, catalog number: F123600)
8. 2 × Dumont #55 fine straight forceps (Fine Science Tools, catalog number: 11255-20)
9. 1 × Stereomicroscope (Leica, catalog number: MS5)
10. 1 × Dumont #7 fine curved forceps (Fine Science Tools, catalog number: 11274-20)
11. 1 × Swing bucket centrifuge with temperature control from 4 to 18°C (Eppendorf, model: 5804R)
12. Mouse anesthesia and euthanasia equipment, as specified by the investigator's Institute's Animal Care Ethics board and with Institute approval
13. Biosafety cabinet, Class II Type B2, if cells are to be cultured following isolation (Nuaire)
14. A cell sorter with the required lasers and detectors: 460/50 (405) and 585/29 (561) (*e.g.*, Cytoflex LX Analyser, Beckman Coulter)

Procedure

Mouse Anesthesia Protocol

1. Obtain Institute approval before working with mice.
2. Use a reliable gas scavenging system to collect, remove, and dispose of waste anesthetic gases (fume hood, vacuum waste gas line, *etc.*) for the researcher's safety.
3. Place mouse into an inhalation chamber. Introduce 5% isoflurane in 1.0 L/min oxygen into the chamber, and observe the animal until it has lost its righting reflex.
4. Remove the mouse from the inhalation chamber, and attach a nose cone to continue to provide 5% isoflurane in 1.0 L/min oxygen. Place mouse on top of insulating material, such as a layer of paper towels, so that the mouse does not lose body heat as quickly, which happens faster if the work surface is made of metal.
5. Apply a corneal lubricant on a q-tip to the mouse's eyes, to protect them.
6. Once the breathing rate of the mouse is less than 60 breaths per minute, check for a toe pinch response. When there is no response, carry out the waxing procedure.
7. When finished waxing, remove the nose cone. Place the mouse into a prepared recovery cage. This is a clean cage lined with a clean absorbable substrate (*e.g.*, paper towel). The recovery cage should be placed on the heat pad set to low.

> *Note: Rodents who are recovering from anesthesia should not be placed directly onto normal cage bedding material. Placing unconscious mice onto bedding poses several risks, including asphyxiation due to inhalation of bedding pieces, and damage to the corneas until the return of the blink response.*

8. Monitor the mouse as it recovers from anesthesia, in a quiet area. Once it is fully recovered, the mouse can be returned to its home cage.

A. Waxing one day before cell collection

1. Turn the heatpad on at the lowest setting to warm up.
2. Cut four small rectangular pieces of Nair® wax strips (40 mm × 5 mm), leaving a wax-free border on one narrow end of each strip, in order to hold on to the strip without touching the wax.
3. Place mice under anesthesia for tail hair removal. We use a nose cone isoflurane inhalant system. For reference, our protocol for recovery anesthesia is included above. However, the exact method of anesthesia is not likely to affect the success of the experiment, and the procedure should be carried out according to the Animal Care Ethics board regulations that govern the institute where the research is being conducted.
4. Warm two wax strips slightly by placing the strips on the heat pad for 20 s. This should warm the strips to body temperature (37°C).
5. Gently remove clear plastic from the strips and place one strip on the top of the tail and one on the underside of the tail. The wax free area of the strip should be oriented so that it is placed towards the tip of the tail.
6. Smooth each strip gently downwards with your fingers to make sure that it is properly adhered.
7. Wait 20–25 s and then remove the wax strips with a quick gesture, in the direction that is towards the head of the mouse.
8. Repeat steps **A4–A7**, but this time place the remaining two wax strips on the lateral sides of the tail.

Note: The skin should be light pink, clean and without any injury. If some traces of wax remain on the skin, mouse self-grooming will remove it overnight.

B. Cell collection procedure

1. Sterilize forceps before use.
2. Make the dispase solution following the instructions located in the **Recipes** section.
3. Euthanize the mouse according to the Animal Care ethics regulations that govern the institute where the research is being conducted. At the University of BC, this is isoflurane anesthesia, followed by CO_2, then cervical dislocation. Using an alternative method of euthanasia is not likely to affect the success of the experiment. Process the skin as soon as possible after euthanasia.
4. Place the mouse on its back and, holding the tail base with forceps, use a scalpel to cut along the ventral midline from the tail base to the tail tip (**Figure 2A**) (**Video 1**).

Video 1. Tail skin incision technique.
This video was made at the University of British Columbia, according to guidelines from the University of British Columbia on Animal Care, and approved by the Animal Research Ethics Board of the University of British Columbia, under protocol #A19-0148.

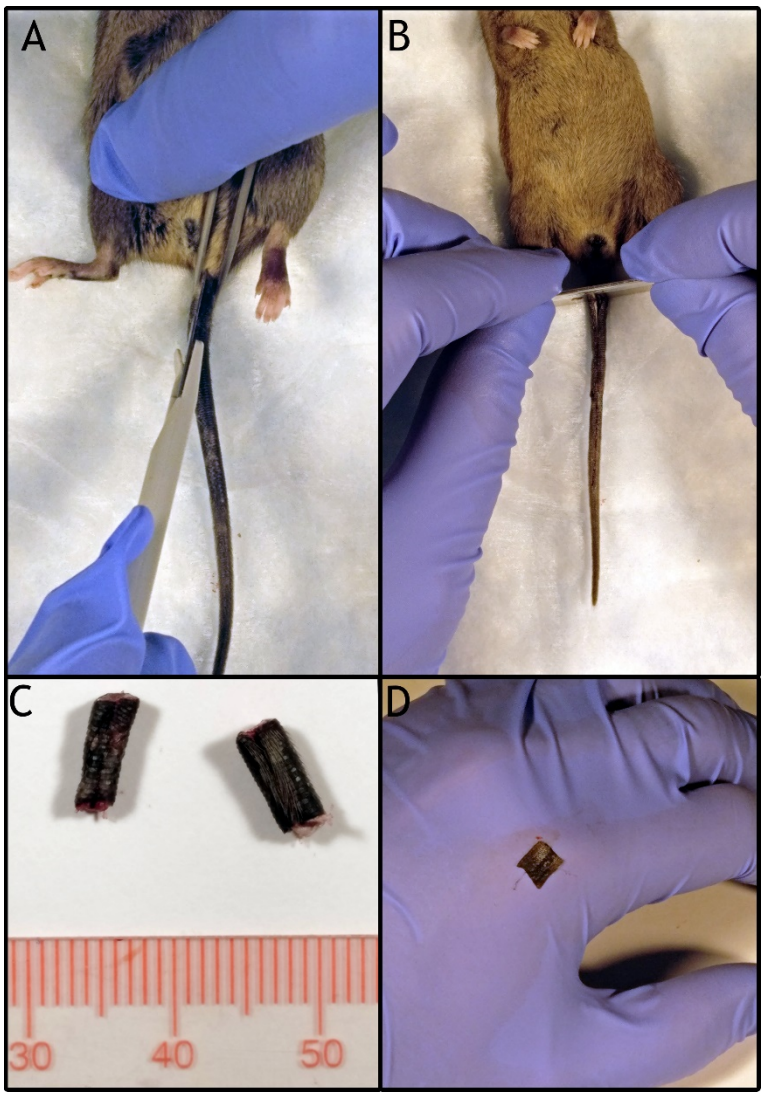


Figure 2. Isolating mouse tail skin from bone.
(A) Forceps and a scalpel are used to cut the mouse tail skin along the ventral midline. **(B–C)** A razor blade is used to remove the tail and cut it into smaller segments about 0.5 cm in length. Ruler shown in mm. **(D)** Tail skin is peeled off of the bone and briefly set on glove, to reduce curling.

5. Cut tail off and divide it into four segments (about 0.5 cm × 0.5 cm each) with a razor blade (**Figure 2B–C**). Discard the tip of the tail, where it becomes narrow.
6. Using the skin incision made in step **B3**, peel the skin from the tail bones.
   After being removed from the bone, tail skin tends to roll in on itself. In order to flatten it, it is recommended that the skin be placed flat for about 30 s (*e.g.*, on the back of a gloved hand) (**Figure 2D**).
7. Place flattened tail skins in the "dispase" Petri dish. Ensure the epidermis side of the skin is facing up, and the skin is floating and not submerged (**Figure 3**).

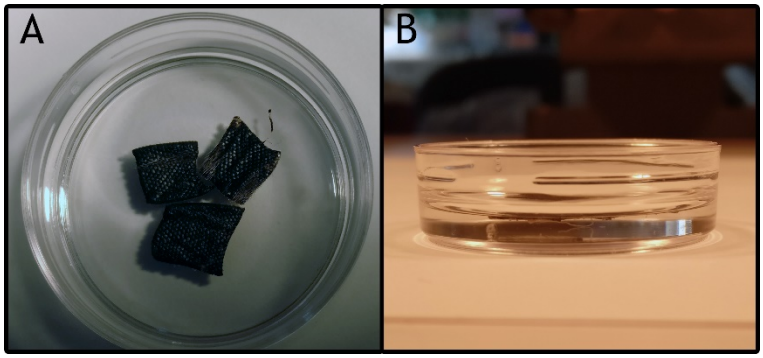


Figure 3. Tail skin floating in dispase solution.
(A) Birds eye and **(B)** horizontal view of tail skin floating in dispase solution.

8. Incubate the Petri dish with the tail skins in the dispase solution in a tissue culture incubator at 37°C (5% CO_2) for 35 min.
9. Meanwhile:
   a. Remove Fixable Viability Dye from -80°C and thaw it on ice.
   b. Add 2 mL of MEM to a Petri dish and label it “for trypsin”.
   c. Make the trypsin solution using the instructions located in the **Recipes** section.
   d. Make the MEM + EDTA + 10% FBS solution using the instructions located in the **Recipes** section, and keep on ice until use.
   e. Transfer 2 mL of the MEM + EDTA + 10% FBS solution to a 50-mL Falcon tube labeled “cells 1”, and place the tube on ice.
   f. Chill an empty 15-mL conical tube labeled "cells 3" on ice.
10. Remove the Petri dish with skin pieces in dispase solution from the incubator.
11. Using a 5-mL serological pipette, remove the dispase solution and discard.
12. Add, and then remove, 1 mL of MEM twice, to wash the skin pieces. They will float.
13. Add 1 mL of MEM to the dish.
14. Using a pair of fine forceps, split the epidermis from the dermis (**Figure 4**) as follows:
    a. Remove one piece of skin and place it in a new empty Petri dish. Use one pair of forceps to clamp onto the epidermis while the other forceps peels the white dermis layer away. Starting in a corner works best for peeling the entire segment in one motion.
    b. A stereomicroscope will aid in discerning epidermis from dermis.
15. Immediately after the dermis has been removed, transfer the epidermal layer to the Petri dish labelled “for trypsin,” currently holding 2 mL of MEM. The epidermis should be floating, with the underside of the epidermis in contact with the solution. Discard the dermis.
16. Repeat steps **B14** and **B15,** until the dermis from all skin pieces has been removed and the epidermis placed in the "for trypsin" dish.
17. Using two 5-mL serological pipettes, remove the MEM from the Petri dish holding the epidermis and replace it with 3 mL of the trypsin solution, making sure the pieces of epidermis are still floating.
18. Incubate the Petri dish with the pieces of epidermis in the trypsin solution in a tissue culture incubator at 37°C (5% CO_2) for 5 min.
19. Remove the Petri dish containing the epidermal pieces in trypsin solution from the incubator.
20. Inhibit trypsin activity by adding 3 mL of cold MEM + EDTA + 10% FBS solution to the dish, with a 5-mL serological pipette. Swirl to mix.
21. Then, use the same serological pipette to collect 4 mL of the solution from the dish and add it to the chilled conical tube labeled “cells 1” from step **B9**.
22. Use the following steps to release and collect cells from the epidermis:
    Pick up one piece of epidermis using a fine forceps #55 and scratch the underside of the scales once or twice using a pair of curved forceps #7. This will release some cells into the 2 mL of solution in the Petri dish. Epidermal cells at this stage appear white. Use a stereomicroscope to better visualize the process (**Video 2, before & after epidermal scratching**).

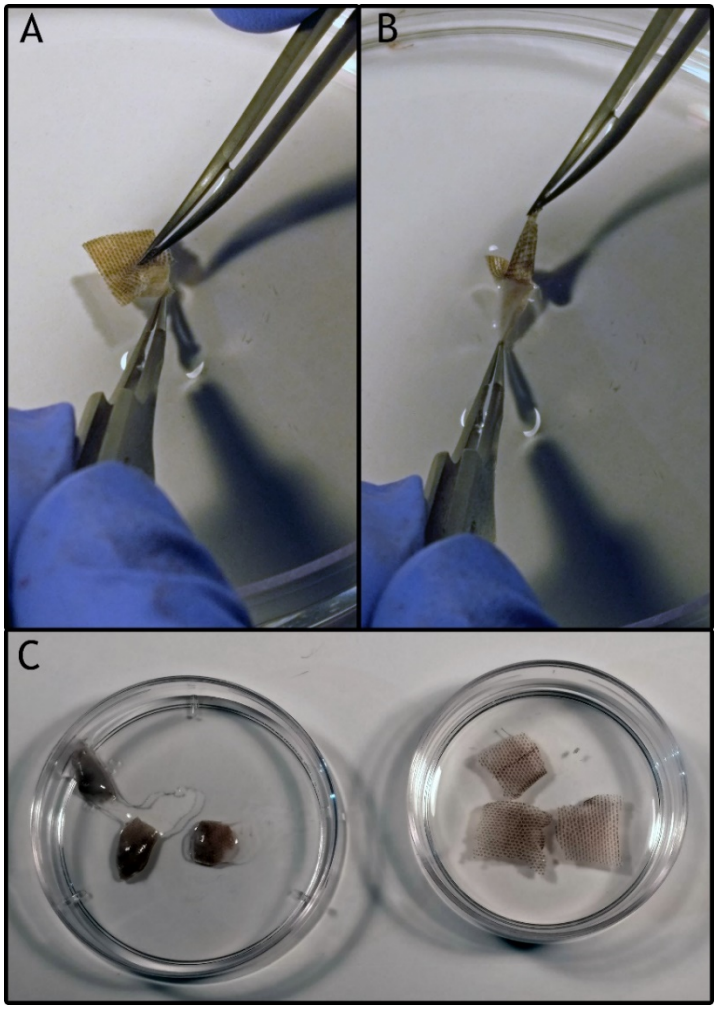


Figure 4. Splitting dermis from epidermis.
(A) A pair of fine forceps is used to split the dermis from epidermis starting in one corner and **(B)** following through in a single motion. **(C)** The end result, with dermis in the left Petri dish and epidermis on the right.

Video 2. Scale scratching technique

a. Use the 1,000 µL micropipette to transfer 1 mL of the solution in the Petri dish, including the released cells, to the Falcon tube labeled “cells 1”.
b. Immediately add 1 mL of fresh, cold MEM + EDTA + 10% FBS solution to the Petri dish.
c. Repeat this process: Scratch and transfer cells from each piece of epidermis three times. The final volume transferred into "cells 1" will be 14 mL (which will be added to the 4 mL already in the tube). If less volume is used, cell viability will suffer.

23. Using a 10-mL syringe and a 16 G needle, pipette the "cells 1" solution up and down 10 times, slowly, to limit the formation of air bubbles in the syringe. This process disrupts the cell clumps and separates the cells.
24. Pass the cell solution through a 40 µm cell strainer into a new 50-mL Falcon tube labeled "cells 2", to remove any clumps of cells that did not dissociate in step **B23**.
25. Using a 10-mL serological pipette, rinse the cell strainer mesh with 6 mL of cold MEM + EDTA + 10% FBS solution into the "cells 2" tube.
26. Centrifuge the "cells 2" tube at 300 rcf (× *g*) and 18°C for 3 min. A pellet should form.
27. Lower the temperature of the centrifuge to 4°C.
28. Gently remove as much supernatant as possible from "cells 2" without disturbing the pellet, using a 25-mL serological pipette. Discard the supernatant.
29. Add 1 mL of fresh cold MEM + EDTA + 10% FBS solution, and gently resuspend the cells.
30. Transfer cells to the pre-chilled 15-mL conical tube labeled "cells 3" from step **B9**.
31. Add 1 µL of Fixable Viability Dye to "cells 3" and gently mix.
32. Incubate "cells 3" on ice for 15 min.
33. Use a 10-mL serological pipette to add 7 mL of fresh, cold MEM + EDTA + 10% FBS to "cells 3".
34. Centrifuge "cells 3" at 300 rcf (× *g*) and 4°C for 3 min.
35. Using a 10-mL serological pipette, remove supernatant from "cells 3," leaving about 0.2 mL of liquid above the pellet.
36. Gently resuspend the pellet in the remaining 0.2 mL of liquid, and then transfer cells to an Eppendorf tube. Keep the Eppendorf tube with cells on ice until FACS sorting, which should be done as soon as possible.
37. Add 20 µL of fresh, cold MEM + EDTA + 10% FBS solution to a 15-mL round bottom polystyrene test tube, which will be used to collect the tdTomato-positive cells during FACS.
    a. If RNA sequencing is the final goal, also add 1 µL of RiboLock RNase Inhibitor.
38. FACS sort for tdTomato positive cells at 581 nm, while excluding Fixable Viability Dye (pacific blue) positive dead cells, which are fluorescent at 405 nm.

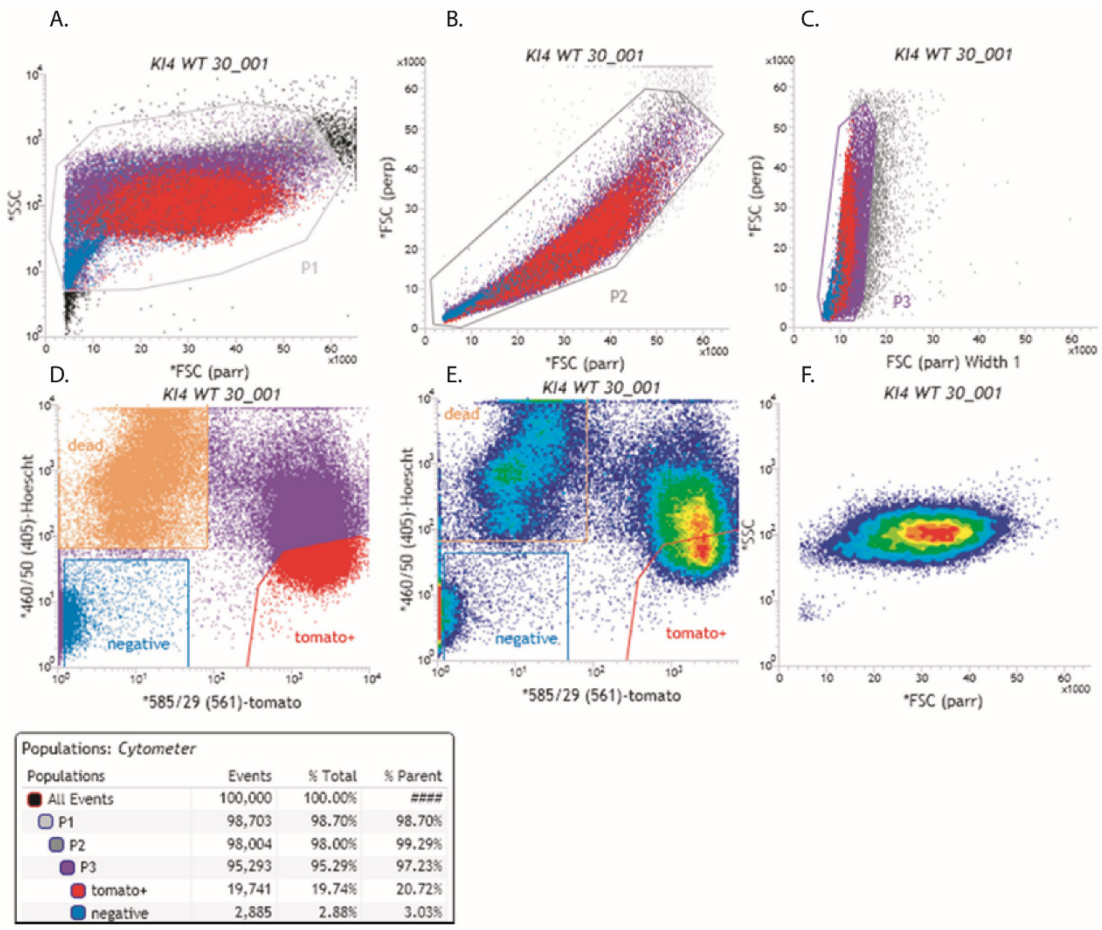
Notes

1. To succeed in separating the dermis from the epidermis with the dispase solution, tail pieces should be no larger than 0.5 × 0.5 cm.
2. If the cells are intended for cell culture, then whenever possible, the procedure should be conducted inside a biological safety cabinet using sterile technique. In our experiments, antibiotics were not added to the media until after cells were isolated by FACS and plated for cell culture. Others may wish to try adding antibiotics earlier, if there are problems with contamination in the culture.
3. In the first experiment, tdTomato-negative epidermis should be used as a control to set the gates for sorting tdTomato-positive cells.
4. Please see Urtatiz *et al.* (2021) for further details on our melanocyte primary culture conditions, if that is the desired application. Other published cell culture protocols will also be suitable.

Data analysis

Cell sorting from *K14-cre*; *tdTomato* mice yielded the results in **Figure 5**. The epidermis contains many dead keratinocytes, which are pushed upwards and shed as part of the normal process of skin renewal. Hence, there is a

large population of pacific blue-positive (dead) cells. The target population is shown in red. These are tdTomato-positive, pacific blue-negative cells (keratinocytes). The yield of these cells is about 400,000 per tail.



Populations: *Cytometer*

Populations	Events	% Total	% Parent
All Events	100,000	100.00%	####
P1	98,703	98.70%	98.70%
P2	98,004	98.00%	99.29%
P3	95,293	95.29%	97.23%
tomato+	19,741	19.74%	20.72%
negative	2,885	2.88%	3.03%

Figure 5. Example results of FACS performed on dissociated *K14-cre; tdTomato* mouse tail epidermis. This experiment shows the isolation of keratinocytes. **A–C)** Cells were first analyzed and gated using various forward scatter (FSC) and side scatter (SSC) properties, as is standard for FACS. The P1 and P2 gatings were used to remove debris. The P3 gating was used to remove cell doublets. **D)** The P3 population of cells (singlets) was then gated and analyzed for the pacific blue viability dye (y-axis) versus tdTomato (x-axis) fluorescence. The tdTomato-positive, pacific-blue negative cells (shown in red throughout Figure 5) are live keratinocytes, the target population. The pacific-blue positive cells (orange and purple) are dead cells, some of which are Tomato-positive (purple) and some of which are not (orange). Dead cells will eventually lose tdTomato fluorescence. The tomato-negative, pacific-blue negative cells (blue) are live cells that did not express tdTomato. These make up a small percentage. **E)** A heat map of the same cells shown in panel D. **F)** A FSC versus SSC heatmap of just the live, tdTomato-positive population, which was gated from the other cells in panels D/E.

Cell sorting from *Mitf-cre*; *tdTomato* mice yielded the results in **Figure 6.** The target population is shown gated as population P5 (green). These are tdTomato-positive, pacific blue-negative cells (melanocytes). There are far fewer melanocytes in the inter-follicular epidermis than there are keratinocytes. In this experiment, live tdTomato-positive melanocytes made up 0.64% of the cells. Previous studies have estimated that melanocytes account for ~1.5% of the total cells in the IFE (Glover *et al*., 2015). Typically, we process the epidermis from two mouse tails together to get a yield of ~25,000 melanocytes for downstream RNAseq or primary cell culture applications.

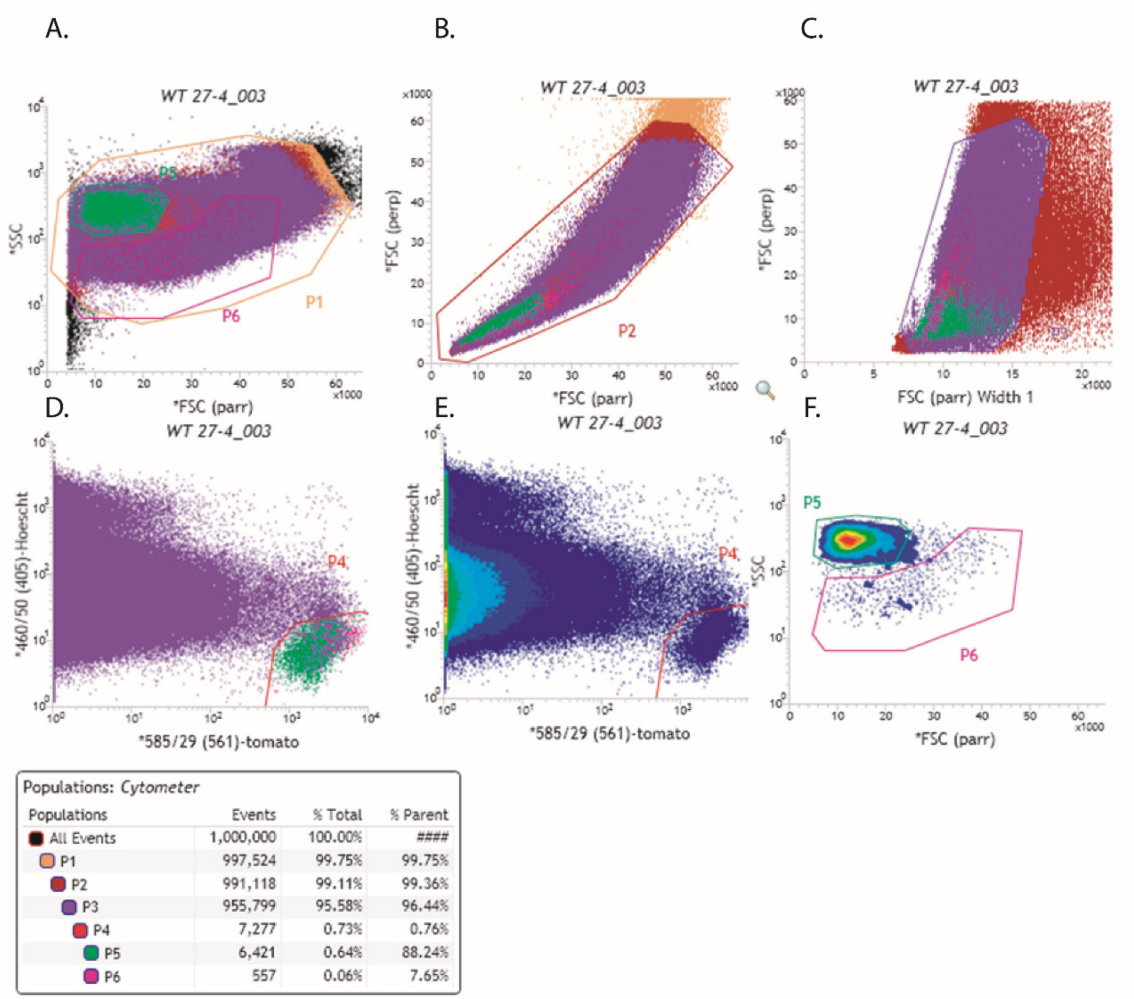


Figure 6. Example results of FACS performed on dissociated *Mitf-cre; tdTomato* mouse tail epidermis. This experiment shows the isolation of melanocytes. **A–C)** Cells were first analyzed and gated using various forward scatter (FSC) and side scatter (SSC) properties, as is standard for FACS. The P1 and P2 gatings were used to remove debris. The P3 gating was used to remove cell doublets. **D)** The P3 population of cells (singlets) was then gated and analyzed for the pacific blue viability dye (y-axis) versus tdTomato (x-axis) fluorescence. The tdTomato-positive pacific-blue negative population (P4) contains the target cells, but also some debris or doublets. The vast majority of cells are tdTomato-negative (purple). **E)** A heat map of the same cells shown in panel D. **F)** A FSC versus SSC heatmap of just the P4 population, which was gated in panels D/E. A tight grouping of cells forms the P5 population indicated in green throughout Figure 6. This is the target population of the experiment (live single melanocytes). The P6 population likely represents debris or doublets that were missed during the P1–P3 gating.

Recipes

1. Dispase solution

a. Add 5 mL of MEM to a 15-mL conical tube using a 5-mL serological pipette.
b. Weigh out 0.01 g of dispase and add it to the MEM (final concentration = 0.2%).
c. Mix by manually shaking the tube until the dispase is dissolved.
d. Transfer the dispase solution to a Petri dish.
e. Label the dish "dispase".

2. **Trypsin solution**
   a. Add 9.982 mL of MEM to a 15-mL conical tube.
      Use a 10-mL serological pipette to add 9 mL of MEM and a 1,000-µL micropipette to add the remaining 982 µL.
   b. Weigh out 0.01 g of trypsin and add it to the tube (final concentration = 0.1%).
   c. Mix by manually shaking.
   d. Add 18 µL of 0.5 M EDTA to the tube.
   e. Mix by manually shaking.
   f. Filter the trypsin solution into a new 15-mL conical tube using a 10-mL syringe with a syringe filter.
   g. Label the tube "trypsin solution".

3. **MEM + EDTA + 10% FBS Solution**
   a. Add 26.944 mL of MEM to a 50-mL Falcon tube.
      Use a 25-mL serological pipette to add 25 mL and a 1,000-µL micropipette to add the remaining 1,944 µL (1 × 1,000 µL and 1 × 944 µL).
   b. Add 54 µL of 0.5 M EDTA to the MEM using a 200-µL micropipette.
   c. Use a 5-mL serological pipette to add 3 mL of FBS to the MEM + EDTA.
   d. Mix by manually shaking.
   e. Keep on ice until use.

Acknowledgments

We thank Dr. Greg Barsh for the gift of the *Mitf-cre* mice. This work was supported by the Canadian Institutes of Health Research (MOP-79511 to C.D. Van Raamsdonk).
The original research paper for this protocol was published in *eLife*, DOI: 10.7554/eLife.71825 (Urtatiz *et al.*, 2021).

Competing interests

We have no competing interests to declare.

Ethics

This work was carried out under the approval of the UBC Animal Care Committee under A19-0148, approved on 7/30/2019, with annual renewals.

References

Adan, A., Alizada, G., Kiraz, Y., Baran, Y. and Nalbant, A. (2017). Flow cytometry: basic principles and applications. *Crit Rev Biotechnol* 37(2): 163-176.
Alizadeh, A., Fitch, K. R., Niswender, C. M., McKnight, G. S. and Barsh, G. S. (2008). Melanocyte-lineage expression of Cre recombinase using Mitf regulatory elements. *Pigment Cell Melanoma Res* 21(1): 63-69.
Day, R. N. and Schaufele, F. (2008). Fluorescent protein tools for studying protein dynamics in living cells: a review. *J Biomed Opt* 13(3): 031202.
Deo, M., Huang, J. L., Fuchs, H., de Angelis, M. H. and Van Raamsdonk, C. D. (2013). Differential effects of neurofibromin gene dosage on melanocyte development. *J Invest Dermatol* 133(1): 49-58.

Fitch, K. R., McGowan, K. A., van Raamsdonk, C. D., Fuchs, H., Lee, D., Puech, A., Herault, Y., Threadgill, D. W., Hrabe de Angelis, M. and Barsh, G. S. (2003). Genetics of dark skin in mice. *Genes Dev* 17(2): 214-228.

Glover, J. D., Knolle, S., Wells, K. L., Liu, D., Jackson, I. J., Mort, R. L. and Headon, D. J. (2015). Maintenance of distinct melanocyte populations in the interfollicular epidermis. *Pigment Cell Melanoma Res* 28(4): 476-480.

Hafner, M., Wenk, J., Nenci, A., Pasparakis, M., Scharffetter-Kochanek, K., Smyth, N., Peters, T., Kess, D., Holtkotter, O., Shephard, P., *et al*. (2004). Keratin 14 Cre transgenic mice authenticate keratin 14 as an oocyte-expressed protein. *Genesis* 38(4): 176-181.

Köhler, C., Nittner, D., Rambow, F., Radaelli, E., Stanchi, F., Vandamme, N., Baggiolini, A., Sommer, L., Berx, G., van den Oord, J. J., *et al*. (2017). Mouse Cutaneous Melanoma Induced by Mutant BRaf Arises from Expansion and Dedifferentiation of Mature Pigmented Melanocytes. *Cell Stem Cell* 21(5): 679-693 e676.

Kormos, B., Belso, N., Bebes, A., Szabad, G., Bacsa, S., Szell, M., Kemeny, L. and Bata-Csorgo, Z. (2011). *In vitro* dedifferentiation of melanocytes from adult epidermis. *PLoS One* 6(2): e17197.

Le, L., Sirés-Campos, J., Raposo, G., Delevoye, C. and Marks, M. S. (2021). Melanosome Biogenesis in the Pigmentation of Mammalian Skin. *Integr Comp Biol* 61(4): 1517-1545.

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., Lein, E. S. and Zeng, H. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* 13(1): 133-140.

McLellan, M. A., Rosenthal, N. A. and Pinto, A. R. (2017). Cre-loxP-Mediated Recombination: General Principles and Experimental Considerations. *Curr Protoc Mouse Biol* 7(1): 1-12.

Nagy, A. (2000). Cre recombinase: the universal reagent for genome tailoring. *Genesis* 26(2): 99-109.

Natarajan, V. T., Ganju, P., Ramkumar, A., Grover, R. and Gokhale, R. S. (2014). Multifaceted pathways protect human skin from UV radiation. *Nat Chem Biol* 10(7): 542-551.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E. and Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol* 22(12): 1567-1572.

Tang, J., Li, Q., Cheng, B. and Jing, L. (2014). Primary culture of human face skin melanocytes for the study of hyperpigmentation. *Cytotechnology* 66(6): 891-898.

Tharmarajah, G., Eckhard, U., Jain, F., Marino, G., Prudova, A., Urtatiz, O., Fuchs, H., de Angelis, M. H., Overall, C. M. and Van Raamsdonk, C. D. (2018). Melanocyte development in the mouse tail epidermis requires the Adamts9 metalloproteinase. *Pigment Cell Melanoma Res* 31(6): 693-707.

Tharmarajah, G., Faas, L., Reiss, K., Saftig, P., Young, A. and Van Raamsdonk, C. D. (2012). Adam10 haploinsufficiency causes freckle-like macules in Hairless mice. *Pigment Cell Melanoma Res* 25(5): 555-565.

Upadhyay, P. R., Ho, T. and Abdel-Malek, Z. A. (2021). Participation of keratinocyte- and fibroblast-derived factors in melanocyte homeostasis, the response to UV, and pigmentary disorders. *Pigment Cell Melanoma Res* 34(4): 762-776.

Urtatiz, O., Haage, A., Tanentzapf, G. and Van Raamsdonk, C. D. (2021). Crosstalk with keratinocytes causes GNAQ oncogene specificity in melanoma. *Elife* 10: e71825.

Urtatiz, O., Samani, A. M. V., Kopp, J. L. and Van Raamsdonk, C. D. (2018). Rapid melanoma induction in mice expressing oncogenic Braf(V600E) using Mitf-cre. *Pigment Cell Melanoma Res* 31(4): 541-544.

Van Raamsdonk, C. D., Barsh, G. S., Wakamatsu, K. and Ito, S. (2009). Independent regulation of hair and skin color by two G protein-coupled pathways. *Pigment Cell Melanoma Res* 22(6): 819-826.

Yang, F., Yang, L., Wataya-Kaneda, M., Yoshimura, T., Tanemura, A. and Katayama, I. (2018). Uncoupling of ER/Mitochondrial Oxidative Stress in mTORC1 Hyperactivation-Associated Skin Hypopigmentation. *J Invest Dermatol* 138(3): 669-678.