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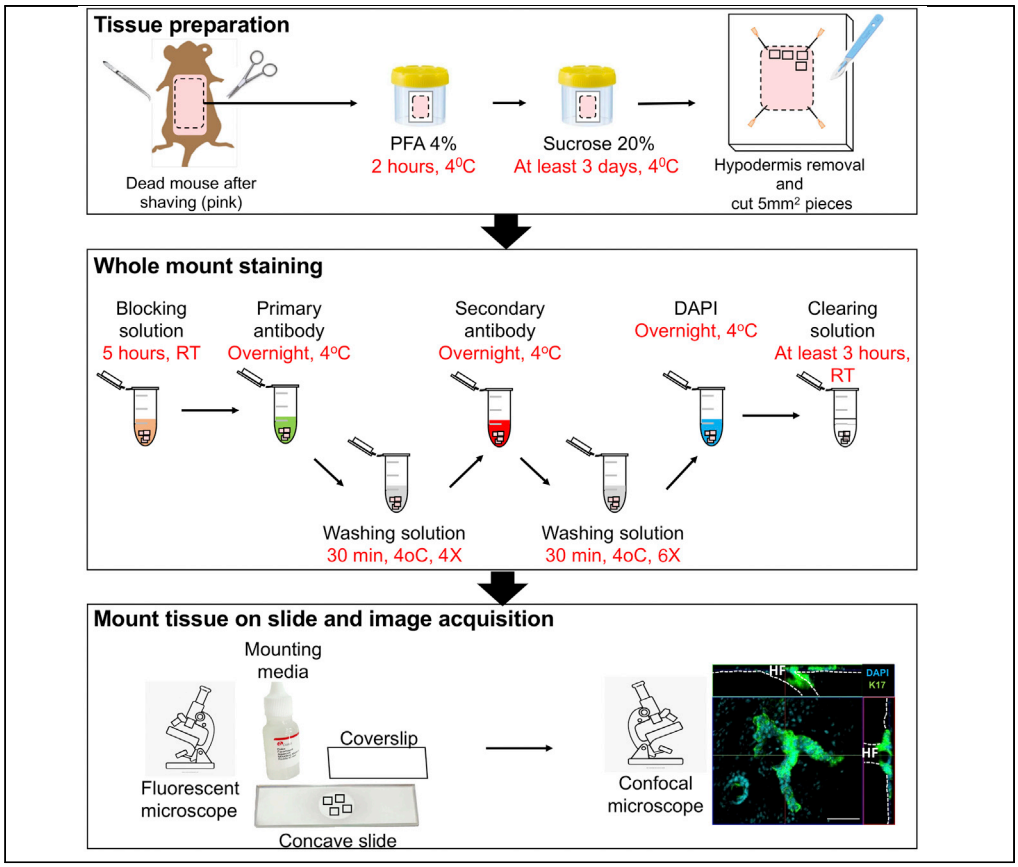
Protocol

Whole-mount staining coupled to a UV-inducible basal cell carcinoma murine model



The origin of basal cell carcinomas (BCC) remains elusive. This protocol combines the use of an ultraviolet (UV)-inducible BCC murine model (K14CrexPtch$^{wt/lox}$) and an optimized protocol for florescent whole-mount cytokeratin 17 immunostaining to address the spatiotemporal dynamics of BCC formation. The high-resolution three-dimensional confocal images are an excellent tool to visualize and quantify the distribution of skin cancers at a given time point.

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HIGHLIGHTS

Spatiotemporal dynamics of BCC formation to understand its origin

Combination of a UV-inducible BCC murine model and a whole-mount staining protocol

Visualize and quantify the distribution of skin cancers at given time points

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Whole-mount staining coupled to a UV-inducible basal cell carcinoma murine model

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SUMMARY

The origin of basal cell carcinomas (BCC) remains elusive. This protocol combines the use of an ultraviolet (UV)-inducible BCC murine model (K14CrexPtch$^{wt/lox}$) and an optimized protocol for florescent whole-mount cytokeratin 17 immunostaining to address the spatiotemporal dynamics of BCC formation. The high-resolution three-dimensional confocal images are an excellent tool to visualize and quantify the distribution of skin cancers at a given time point.
For complete details on the use and execution of this protocol, please refer to Roy et al. (2020).

BEFORE YOU BEGIN

Our previous protocols published in 2019 (Roy and Khosrotehrani, 2019) have focused on analyzing the spatiotemporal organization of epidermal stem cells, the current protocol is optimized and adapted for use with BCC murine samples.

KRT14Cre/ER x Ptch1wt/lox mice: tamoxifen induction and UVR treatments

Note: Treatments were adapted from a method previously described (Cozzi et al., 2012).

1. Prepare tamoxifen for intraperitoneal (IP) injection at 10 mg/mL in 90% corn oil and 10% absolute ethanol.
2. At 8 weeks of age (mix gender animals), inject 100 µL (1 mg) of tamoxifen intraperitoneally for 5 consecutive days using a 25-gauge needle. This allows the inducible Cre/lox system to invalidate one allele of the *Ptch1* gene.

Note: No BCC lesions were observed after the invalidation of one *Ptch1* allele.

3. Three days after the last injection of tamoxifen, mice are shaved using an electric shaver to remove all hairs from the back (Figure 2, step 1). Shave as close to the skin as possible while being careful to avoid nicks.
4. Start UV treatment by placing each mouse in an individual compartment (L × H=11.5 × 20 cm) of the box (Figure 1). The mice are 20 cm below the UV lamps receiving therefore a dose of 0.8 kJ/m^2 per minute. The treatment starts with 1.5 min of exposure and the time is increased 0.5 min every 2 weeks (Table 1).

Note: Animals are exposed to chronic UVB irradiation at a suberythemal dose three times per week to allow the accumulation of random mutations and consequently the remaining *Ptch1* allele will be invalidated.

Figure 1. UVB irradiation apparatus

The mice are placed in the individual compartments, the lamps are turned on, and the mice are irradiated as per Table 1.

(A) Six Philips TL100W 12RS UVB lamps.

(B) Wooden box with individual compartments utilized for UVB irradiation.

Note: Skin will become resistant to UVB after chronic exposure therefore exposure time has to be increased gradually during the experiment (Roy et al., 2020).

Animals are exposed or not (control mice) to chronic UVB irradiation at a suberythemal dose three times per week for 3, 5, 8, or 10 weeks (Table 1).

5. One day after the last UVB exposure, sacrifice animals and collect the dorsal skin as outlined in the following section, Collection and fixation of dorsal skin.

⚠ CRITICAL: If the skin become red or burned, do not treat mice with UVB further. However, the mice can be exposed to UVB again after the redness subsided. If this persists for more than 2 weeks, sacrifice the mice.

Collection and fixation of dorsal skin

Timing: total: 3 days and 2 h 30 min

Note: Steps 1–4, 20–25 min/mouse; steps 5 and 6, 2 h; steps 7 and 8, 3 days.

Table 1. Schedule of UVB exposure

	Exposure time of UVB		
	Day 1 (min)	Day 2 (min)	Day 3 (min)
Week 1	1.5	1.5	1.5
Week 2	1.5	1.5	1.5
Week 3	2	2	2
Week 4	2	2	2
Week 5	2.5	2.5	2.5
Week 6	2.5	2.5	2.5
Week 7	3	3	3
Week 8	3	3	3
Week 9	3.5	3.5	3.5
Week 10	3.5	3.5	3.5

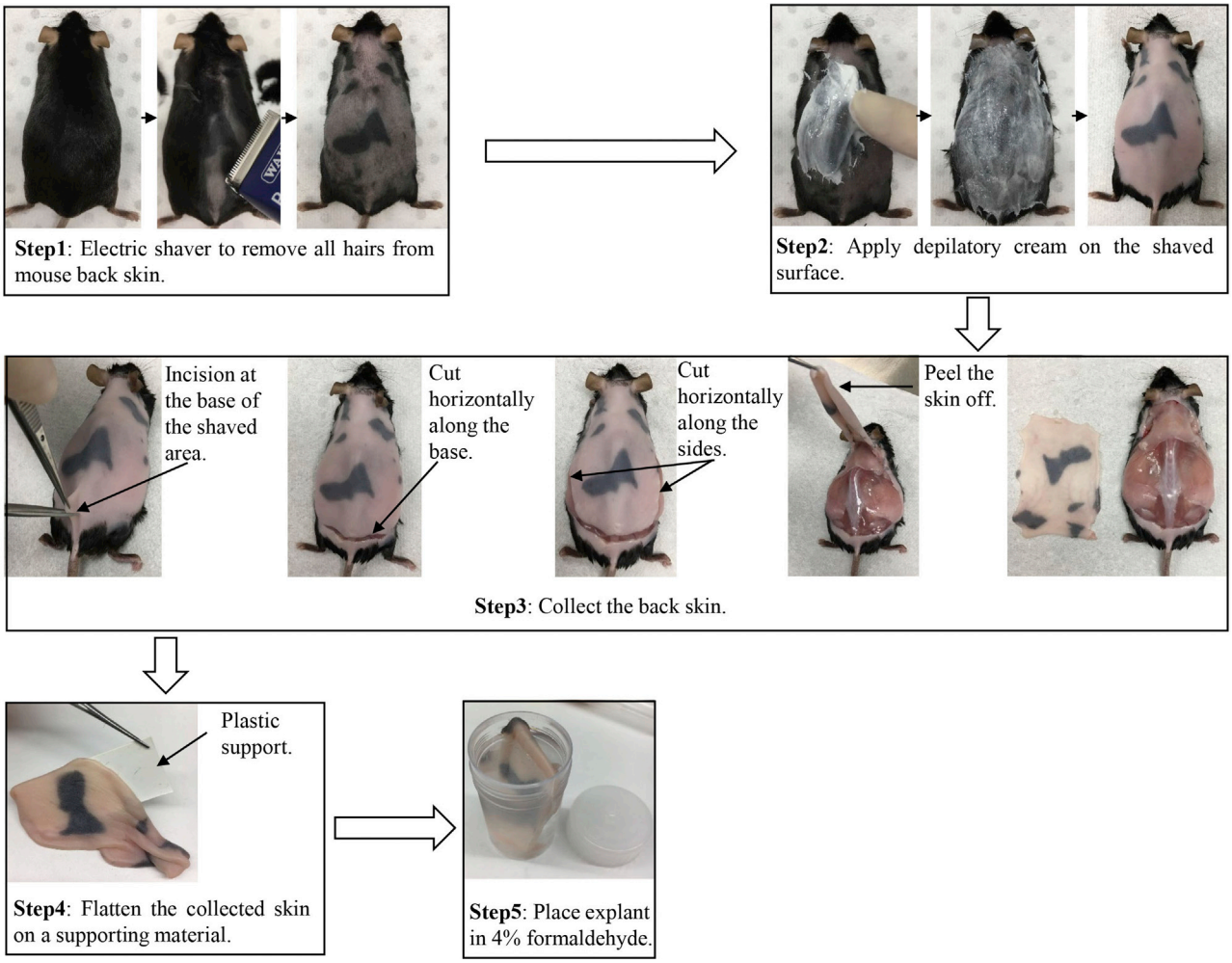

Figure 2. Collection and fixation of dorsal skin

Note: Figure 2 gives a visual description for the next 8 steps.

6. Sacrifice mice following standard laboratory protocol and use the electric shaver to remove all hair from the back of an adult mouse if necessary. Shave as close to the skin as possible while being careful to avoid nicks.
7. Apply depilatory cream on the shaved surface for 2 min. Rinse with water. Repeat if necessary to remove all the hair follicle. Pat dry gently the skin with a soft tissue.

⚠ CRITICAL: Do not leave the depilatory cream for more than 2 min to avoid damaging the skin. Reapply if necessary. A sensitive depilatory cream may be used to reduce the risk of damage.

8. With scissors, make incision at the base of the shaved area. Cut horizontally along the base and along the two sides. Use forceps to carefully peel the skin off in one piece.
9. Flatten the collected skin on a supporting material such as a piece of plastic.

⚠ CRITICAL: Fixation will cause tissue to harden. Unflattened skin will cause difficulty in mounting and imaging in later steps.

10. Place explants in at least 5 mL of 4% formaldehyde in 1× PBS buffer for a 9-cm^2 piece of back skin or until the entire tissue is covered.
11. Incubate at 4°C for 2 h

Note: Longer fixation time is not recommended. In our experience, tissue failed a routine staining after prolonged fixation (e.g., between 14 h and 16 h). Furthermore, longer fixation led to tissue hardening and difficulty in mounting as well as imaging.

12. Remove the skin from the fixation solution and wash with 1× PBS for 5 min to get rid of formaldehyde. With a tissue, pat dry the excess of PBS and place the skin in at least 5 mL of sucrose 20%.
13. Incubate at 4°C for at least 3 days.

Note: After at least 3 days in sucrose, the sample can be stored at −80°C after removing the excess of sucrose. The sucrose is not change over the 3 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-cytokeratin17 (1/100)	Abcam	cat# ab109725, RRID:AB_10889888
Rabbit monoclonal anti-cytokeratin14 (1/100)	Abcam	cat# ab181595, RRID:AB_2811031
Goat Anti-rabbit Alexa Fluor 647 (1/500)	Invitrogen	cat# A32733, RRID:AB_2633282)
Experimental models: organisms/strains		
K14–CreER^{tam}	Jackson Laboratories	JAX005107 RRID:IMSR_JAX:005107
C57BL/6	Australian Animal Resource Centre (ARC)	n/a
Ptch1 lox/lox	Prof. Brandon Wainwright's lab, IMB, Brisbane, Australia	n/a
Software and algorithms		
Olympus FV31S-SW	Olympus	n/a
Other		
Tamoxifen	Sigma	cat# T5648
Corn oil	Sigma	cat# C8267
Absolute ethanol	Sigma	cat# E7023
Veet cream	Supermarket/Pharmacy	n/a
RapiClear 1.52	SunJin Lab Co	cat# RC152
Normal goat serum	Vectors Lab	cat# S1000 RRID:AB_2336615
Tween 20	Sigma	cat# P2287
PBS tablet	Thermo Fisher	cat# 18912014
Paraformaldehyde power	Sigma	cat# P6148
Bovine serum albumin	Sigma	cat# A2153
Sucrose	Sigma	cat# S9378
Triton X	Chemsupply	cat# TL125
DMSO	Sigma	cat# D8418
4,6-Diamidino-2-phenylindole (DAPI)	Thermo Fisher	cat# D3571 RRID:AB_2307445
Maleic acid	Sigma	cat# M0375
Sodium hydroxide (NaOH)	Ajax Finechem	cat# 482-500

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fluorescence mounting medium	Dako	cat# S302380
Equipment		
Forceps	n/a	n/a
Scissors	n/a	n/a
Plastic supports	n/a	n/a
Dissection board	n/a	n/a
Electric shaver	n/a	n/a
Plastic jars	n/a	n/a
Scalpel	n/a	n/a
Single cavity concave slides 76 × 25 mm	Sail Brand	cat# 7103
Coverslips 2 mm × 32 mm	Menzel-Glaser	cat# 631-0852

MATERIALS AND EQUIPMENT

Fixation solution

Storage: −20°C for 6 months

	Final concentration	Amount
4% Paraformaldehyde powder	n/a	40 g
NaOH (1 N)	n/a	Until clear solution
HCl (1 N)	n/a	To adjust pH at 7
1× PBS	1×	Up to 1 L

Reagent sucrose 20%

Storage: 4°C for 1 month

	Final concentration	Amount
Sucrose powder	n/a	40 g
1× PBS	1×	Up to 200 mL

Reagent blocking solution

Storage: extemporaneous

	Final concentration	Amount
Triton X (10% in PBS)	0.05%	50 µL
Bovine serum albumin	n/a	0.2 g
Normal goat serum	n/a	2 mL
DMSO	n/a	100 µL
Maleic acid (pH 7.5) (1 M)	0.1 M	1 mL
1× PBS	1×	Up to 10 mL

Reagent diluent solution

Storage: extemporaneous

	Final concentration	Amount
Triton X (10% in PBS)	0.05%	50 µL
Bovine serum albumin	n/a	0.2 g
DMSO	n/a	100 µL
Maleic acid (pH 7.5) (1 M)	0.1 M	1 mL
1× PBS	1×	Up to 10 mL

Reagent washing solution

Storage: extemporaneous

	Final concentration	Amount
Triton X (100%)	0.5%	5 mL
Tween 20 (100%)	0.5%	5 mL
1× PBS	1×	Up to 1 L

Reagent counterstaining solution

Storage: extemporaneous		
	Final concentration	Amount
4,6-Diamidino-2-phenylindole (DAPI) (14.3 M)	0.0143 M	1 µL
1× PBS	1×	Up to 1 mL

⚠ CRITICAL: Paraformaldehyde is a flammable solid. It is also a carcinogen, sensitizer, irritant, a reproductive toxin. Eye contact can result in corneal damage or blindness. Follow all safety guidelines for this chemical.

Note: Microscope setup: The Olympus FV3000 confocal laser scanning microscope was used for imaging. The 20× objective was used to image. The FV3000 microscope was set to "line" sequential scan with an average of 2 scans per "line," and confocal aperture was set to 150 µm. The lasers used are listed in Table 2. Negative immunostaining controls were used to normalize the high voltage (HV), gain, and offset settings. For cytokeratin 17 (K17) staining, scans were carried out as a Z stack with boundaries set at the lower dermis and upper epidermis. Each Z stack image was taken at a 1.5 µm interval, with approximately 60 images per Z stack. Images were taken at all areas where K17 and DAPI co-localized across the samples.

Table 2. Lasers used with FV3000 confocal laser scanning microscope

Fluorophore	Stain	Laser excitation (nm)	Emission spectrum range (nm)	Detector
Blue	DAPI	405	430–480	GaAsp
Far red	Alexa Fluor 647	640	645–745	GaAsp

STEP-BY-STEP METHOD DETAILS

Buffer preparation

Preparation of solutions to perform whole-mount immunostaining.

1. Fixation solution: 4% formaldehyde. For 1 L of 4% formaldehyde, heat 800 mL of PBS to 60°C in a beaker and keep it on heat plate at 60°C in a ventilated hood. Add 40 g of paraformaldehyde powder to the heated PBS solution. Slowly raise the pH to 7 by adding 1 N NaOH dropwise from a pipette until the solution clears. Once the paraformaldehyde is dissolved, adjust the volume of the solution to 1 L with PBS. The solution can be aliquoted and frozen or stored at 2°C–8°C for up to 1 month.
2. 20% sucrose solution: For 200 mL of 20% sucrose, add 200 mL of 1× PBS to a glass beaker on a stir plate. Add 40 g of sucrose powder to the PBS solution. Stir until dissolved. Stored at 2°C–8°C for up to 1 month.

3. Blocking solution: 1× PBS, 0.5% Triton X, 2% bovine serum albumin (BSA), 20% normal goat serum (NGS), 1% dimethyl sulfoxide (DMSO), 100 mM maleic acid (pH 7.5). For 10 mL of blocking solution, dissolve 0.2 g of BSA in 6.85 mL of PBS, then add 50 μL of Triton X, 2 mL of NGS, 100 μL of DMSO, and 1 mL of 1 M maleic acid (pH 7.5).
4. Diluent solution: 1× PBS, 0.5% Triton X, 2% bovine serum albumin (BSA), 1% dimethyl sulfoxide (DMSO), 100 mM maleic acid (pH 7.5). For 10 mL of diluent solution, dissolve 0.2 g of BSA in 8.85 mL of PBS, then add 50 μL of Triton X, 100 μL of DMSO, and 1 mL of 1 M maleic acid (pH 7.5).
5. Washing solution: 1× PBS, 0.5% Triton X, 0.5% Tween 20. For 1 L of washing solution, add 5 mL of Triton X and 5 mL Tween 20 in 990 mL of PBS.
6. 4,6-Diamidino-2-phenylindole (DAPI) solution: For 1 mL DAPI solution, add 1 μL of DAPI in 999 μL of 1× PBS (1/1000 from a 14.3 M DAPI stock solution).

Whole-mount immunofluorescent staining

Timing: 4 days

Protocol for florescent whole-mount cytokeratin 17 immunostaining to address the spatiotemporal dynamics of BCC formation.

Note: we typically use the following timeline: day 1, steps 1–6; day 2, steps 7–11; day 3, steps 12–16; day 4, steps 17 and 18.

7. Collect skin from the storage place:
   a .After the excess of sucrose is removed, place the skin in a Petri dish
   b .If the skin was stored at −80°C, put the tissue in a Petri dish. Leave it between 20°C and 25°C for at 20 min or until the tissue is thawed.
8. Place the skin in a Petri dish. Use the scalpel to gently scrape away the fat and blood vessels covering the dermis until the dermis is clearly and uniformly exposed. Note that the dermis has a dull appearance whereas fat is shiny and reflective (Figure 3).
9. Using the scalpel, prepare small pieces of back skin (5 mm^2 maximum) for staining of specific antigens.
10. Place the samples in a 1.5 mL Eppendorf tube with 500 μL of blocking solution.
11. Gently mix the samples and solution by rocking the tube on a tube roller mixer between 20°C and 25°C for 5 h. Make sure that the samples are covered in solution before leaving to incubate. A minimal speed of the roller mixer is not required but the speed as to be adjusted to avoid the tubes failing from the roller mixer.
12. Transfer the samples to a new Eppendorf tube with 200 μL primary antibody diluted in diluent solution. Gently mix the samples and solution by rocking the tube on a tube roller mixer at 4°C between 14 h and 16 h.
13. Place the samples in a new Eppendorf tube with 300 μL of washing buffer.
14. Place tubes on a tube roller mixer between 20°C and 25°C for 30 min.
15. Repeat steps 13 and 14 three times.
16. Place the skin pieces in a new Eppendorf tube containing 300 μL secondary antibody diluted in diluent solution.

⚠ CRITICAL: From here forward, make sure tubes are placed in a dark chamber or covered with aluminum foil as the light can affect the fluorescence of the fluorochrome. Moreover, to avoid any damages of the tissue, collect the tissues with the minimum of pressure when using the forceps. Plastic forceps may also help. It is also recommended to collect the tissue by one of the corners to minimize the damaged surface of the tissue if you have damage.

17. Gently mix the samples and solution by rocking the tube on a tube roller mixer at 4°C between 14 h and 16 h.

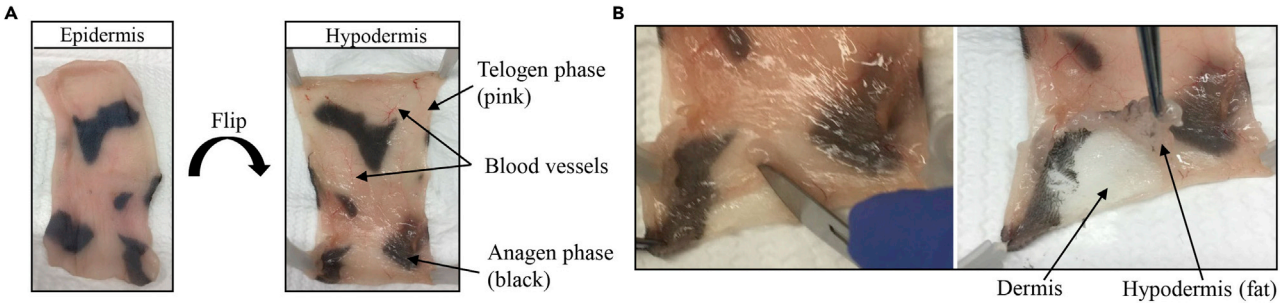

Figure 3. Preparing skin for whole-mount fluorescent staining step 2

(A) Epidermis has a dull appearance. Hypodermis (fat) is shinny and reflective.

(B) Gently scrape away the hypodermis (fat) and blood vessels using a scalpel. Dermis has a dull appearance.

18. Place the samples in new Eppendorf tube with 300 μL of washing buffer.
19. Place tubes on a tube roller mixer between 20°C and 25°C for 30 min.
20. Repeat steps 18 and 19 five times.
21. Place samples in a new Eppendorf tube with 300 μL of DAPI solution.
22. Gently mix the samples and solution by rocking the tube on a tube roller mixer at 4°C between 14 h and 16 h.
23. Place samples in a new Eppendorf tube with 100 μL of clearing buffer.
24. Leave the samples incubating for at least 3 h between 20°C and 25°C

⚠ CRITICAL: Prolonged incubation in clearing buffer will harden the tissue and lead to difficulty in mounting.

Mount tissue on concave slide

Timing: 5 min/slide

25. Remove the excess of clearing buffer with a tissue and put the skin pieces in the middle of a concave slide. Up to four pieces of 5 mm^2 can be mounted on a single slide (Figure 4).
26. Using a fluorescent microscope, make sure the epidermis is facing up (Figure 5, left image). Flip pieces that are not properly orientated.

Note: The orientation of the tissue can be chosen depending on the targeted area. Dermis can face up if you want to analyze dermis area, deep part of hair follicles, blood vessels (Figure 5).

27. Cover the samples with 2–3 drops of mounting media.

Note: Hardset mounting media is not recommended as hard tissue is difficult to mount and always results in air bubbles. It also prevents the reorientation of the tissue if acquisition of images from both epidermis and dermis is required.

28. Gently apply the coverslip at an angle to avoid bubbles. Add a light pressure on the coverslip to evenly spread the mounting media and remove bubbles from the tissue. It is fine if some bubbles persist on the side of the tissue.
29. Use nail polish to seal the coverslip on the slide.

EXPECTED OUTCOMES

A successful experiment should show a clear image of your staining from the top to the bottom of your tissue. A Z stack of 150 μm can be acquired without any problems. Most of the time a Z stack

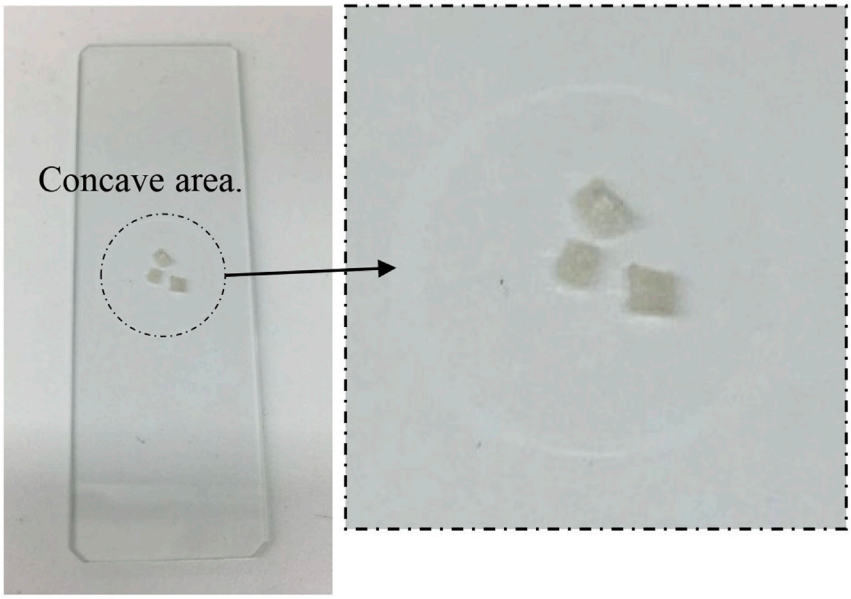

Figure 4. Up to four pieces of 5 mm^2 can be mounted on a single slide

of 200 μm should be easy to acquire. The background should be minimal. An example can be found in Figures 6 and 7.

Note that a very important step to reduce the background is the washing step. Also, the background is also minimized using the appropriate blocking solution. The origin (species in which it is produced) of the serum used in the blocking solution has to match with the animal in which the secondary antibody is produced. Here, the goat serum is used as the secondary antibody is produced in the goat.

If possible, it is also recommended to choose monoclonal antibodies to limit the background. The incubation of primary antibody plays also an important role in the background optimization. If background is observed, the incubation time of the primary antibody might be optimized. In this

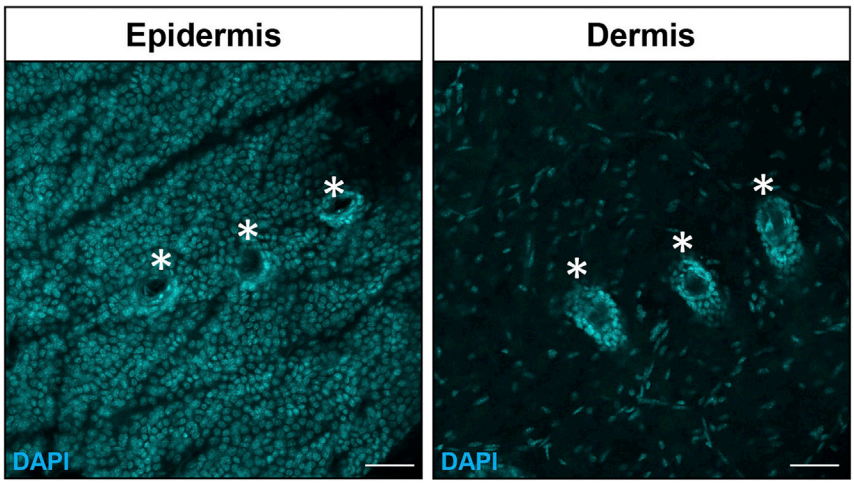

Figure 5. Representative images of skin counterstained with DAPI

Photomicrographs represents 2D optical section from a Z stack acquisition of whole-mounted skin displaying nucleus (DAPI). Left image: epidermis. Right image: dermis. Stars indicate the hair follicles (scale bar, 50 μm).

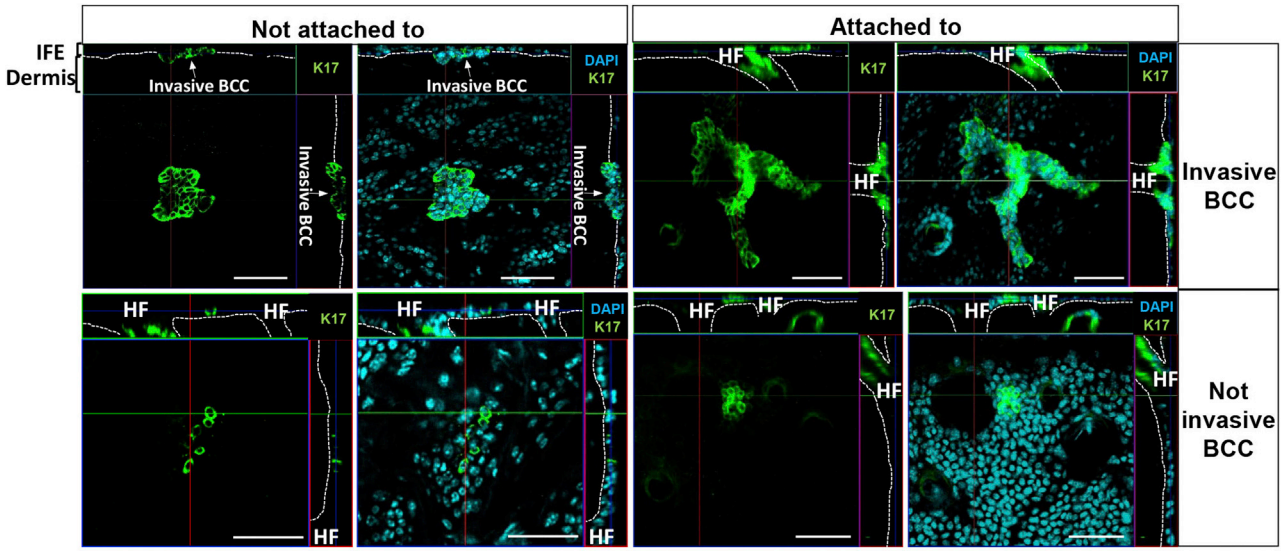

Figure 6. Two profile of K17 positive patches in epidermal skin upon UVB radiation

Photomicrographs represents 2D optical section from a Z stack acquisition of whole-mounted skin displaying representative K17+ patches (green) attached versus not attached to hair follicle (HF) and invasive versus not invasive. White dashed line represents the limit between the epidermis and the dermis (scale bar, 50 μm) (figure reprinted with permission from Roy et al., 2020).

protocol, a balance between a good staining and a minimum of background is obtained with an incubation between 14 h and 16 h of the primary antibody.

This protocol allows the visualization of BCC lesion size, their attachment to hair follicles, their characterization.

QUANTIFICATION AND STATISTICAL ANALYSIS

DAPI fluorescence stains for cell nuclei and anti-K17 antibody stains for BCCs (Anderson-Dockter et al., 2012). K17 is also a marker for hair follicles, however they are distinguishable from BCCs due to their morphology (Alessi et al., 2008). Co-localization of these markers (DAPI and K17) is indicative of BCC lesions.

NB: The investigators are blinded to allocation during experiments and outcome assessment.

1. Z stack images are taken, using FV3000 Olympus confocal microscope, across all skin samples of any area where DAPI and K17 appeared to co-localize, in order to determine total number of BCCs across the skin for each mouse.
2. Z stack images are combined using ImageJ, whereby individual image slices of skin are compiled in order to create a movie. This allowed for analysis across the whole depth of skin to identify BCCs.
3. The number of BCC lesions per Z stack movie are counted manually.
4. Data was pooled in order to determine the number of BCC lesions/mm^2.
5. To compare lesion size, the number of cells making up the BCCs identified within each sample of skin is counted manually using the Cell Counter plugin in Fiji software.
6. Data are analyzed through the GraphPad Prism v7.oa software. However, any statistic software may be used for this analysis. A p value of less than 0.05 is deemed significant.
   a. Lesion number, size, attachment to hair follicles and characteristics (ex: cycling status) are reported for each time point.

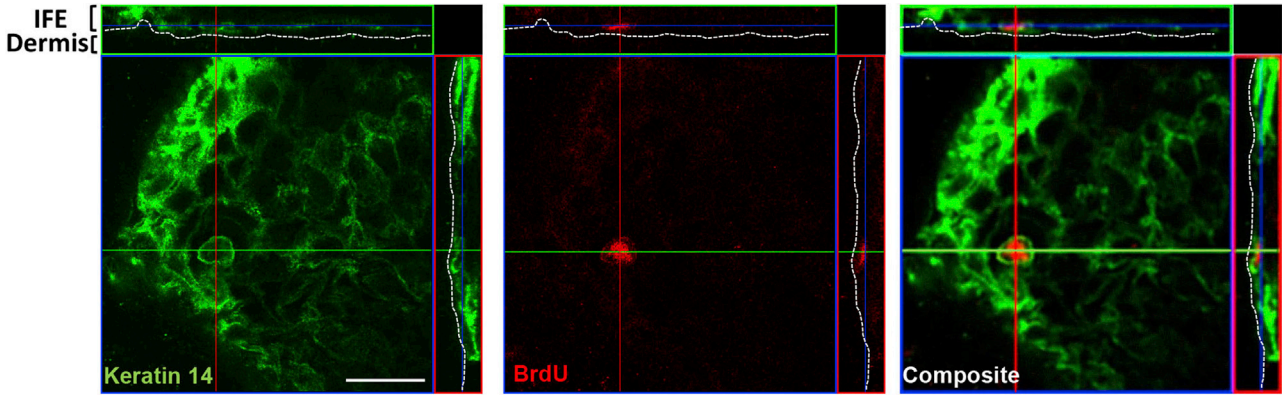

Figure 7. Epidermal label retaining cells are mostly distant from the HF

Photomicrographs represents 2D optical section from a Z stack acquisition of whole-mounted skin, irradiated for 2 weeks, displaying representative BrdU+ label retaining cell (red) co-stained with an anti-keratin 14 antibody (green) (scale bar, 50 μm) (figure reprinted with permission from Roy et al., 2020).

b. Lesion number/size are averaged and compared between groups using two-tailed t tests or Mann-Whitney test.
c. Lesion number/size distributions are compared between conditions using Chi square tests

Note: This staining protocol is not restricted to one primary antibody. Additional staining may be performed to further characterize the BCC lesions. [e.g., anti-cytokeratin 14 (basal keratinocyte marker), BrdU (proliferative or quiescent cell marker depending if proliferative or label retaining cell experiment is performed) Figure 7, Roy et al., 2020].

LIMITATIONS

Despite its benefits, clonal dynamics evaluation upon lineage tracing only offers a static view of the skin of different animals at different time points and models proposed so far, including ours, heavily rely on computer simulations and mathematical modeling that make unverifiable assumptions.

Lineage tracing model combined with the live imaging multiphoton microscopy to directly visualize dynamics of epidermal clones or tumors should strengthen our ability to better understand epidermal proliferation dynamics and tumor development without any assumption.

TROUBLESHOOTING

Problem 1

Exposure to depilatory cream can damage the surface of the skin and remove a part of the superficial epidermal cells. It will lead to the loss of information.

Potential solution

Use cream for sensitive skin and do not leave the depilatory cream for more than 2 min to avoid damaging the skin. Reapply if necessary. Rinse abundantly.

Problem 2

Fixation will cause tissue to harden. Wrinkled/unflatten skin will cause difficulty in mounting and imaging in later steps.

Potential solution

Flatten the collected skin on a supporting material such as a piece of plastic before putting the sample in the fixative solution. Avoid fixing the tissue more than 2 h will prevent this problem.

Problem 3

Prolonged incubation in clearing buffer will harden the tissue and lead to difficulty in mounting.

Potential solution

Regular checking (every half an hour) is important to prevent this problem. If the aspect of the tissue does not change between 2 observations, the tissue has to be removed from the clearing buffer and mounted on slide.

Problem 4

Skin tissue is difficult to clear when it is in anagen phase (Figure 3, the growing phase of the hair follicles) compared to the telogen phase (Figure 3, resting phase of the hair follicle).

Potential solution

The skin in anagen phase is thicker and require a longer incubation in the clearing solution. The use of multiphoton microscope can also help as the laser penetrate deeper in the tissue.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Edwige Roy (e.roy@uq.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

H.Y.W. and E.R. designed and performed the experiments. K.K. designed the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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