STAR Protocols

Protocol

Mouse tail skin wholemount staining and intravital calcium imaging

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Highlights

High resolution whole mount immunofluorescent analysis of mouse hair follicle

Intravital calcium imaging of hair follicle

Applicable to other cell types or featuress with different driver and reporter lines

Mouse tail skin offers multiple benefits for skin research. Here, we present a protocol detailing high resolution tail skin whole mount staining and *in vivo* calcium imaging of hair follicle stem cells (HFSCs) using *Sox9creERT2, GCaMP6s,* and *Ai14* mice. This approach enables the study of hair follicles and stem cells in different physiological and pathological conditions. The applications of the protocol include visualization of calcium signaling in other cell types with Cre/CreER lines or analyzing other cellular features with different reporter lines.

Protocol

Mouse tail skin wholemount staining and intravital calcium imaging

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SUMMARY

Mouse tail skin offers multiple benefits for skin research. Here, we present a protocol detailing high resolution tail skin whole mount staining and *in vivo* calcium imaging of hair follicle stem cells (HFSCs) using *Sox9creERT2, GCaMP6s,* and *Ai14* mice. This approach enables the study of hair follicles and stem cells in different physiological and pathological conditions. The applications of the protocol include visualization of calcium signaling in other cell types with Cre/CreER lines or analyzing other cellular features with different reporter lines.
For complete details on the use and execution of this protocol, please refer to Xie et al. (2022).

BEFORE YOU BEGIN

Unlike the mouse dorsal skin that has very thin epidermis, mouse tail skin epidermis thickness is similar to human skin epidermis. This unique feature enables us to easily separate the intact tail skin epidermis and attached hair follicles from the underlying dermis and makes high resolution immunofluorescent wholemount analysis feasible. Additionally, since mouse tail is physically distant from the torso region that contains the beating heart and breathing lung, tail skin is also ideal for stable intravital imaging without the need for special stabilization equipment.

The protocols below describe the specific steps for two different experiments: **Part A**. tail skin whole-mount staining of DAPI, Pcad, and cleaved-caspase-3; **Part B**. intravital calcium imaging of HFSCs using *Sox9^{creERT2}; GCaMP6s; Ai14* mice. GCaMP6s is a green fluorescent Ca^{2+} indicator that shows high sensitivity and slow decay kinetics (Chen et al., 2013). *GCaMP6s* is a reporter mouse line that expresses GCaMP6s from the *Rosa26* locus once activated by Cre (Madisen et al., 2015). *Ai14* is a reporter mouse line that expresses stable tdTomato from the *Rosa26* locus once activated by Cre (Madisen et al., 2010). *Sox9^{creERT2}* can specifically label HFSCs after tamoxifen injection at morphogenesis (Xu et al., 2015). Here, for our purpose of visualizing calcium signal in HFSCs, we used P11 female *Sox9^{creERT2}; GCaMP6s; Ai14* mice: after tamoxifen injection at morphogenesis, HFSCs express GCaMP6s and stable tdTomato. To visualize calcium signaling in other cell types, different cell type specific Cre/CreER mouse lines can be used. To visualize different cellular features other than calcium signaling, different reporter lines could also be used to follow our protocol.

Mouse experiments were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Biological Sciences, Beijing. All the animals were handled according to the guidelines of the Chinese law regulating the usage of experimental animals and the protocols (M0020) approved by the Committee on the Ethics of Animal Experiments of the National Institute of Biological Sciences, Beijing.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-cleaved-caspase-3 (1:1000)	Cell Signaling Technology	Cat#9661L; RRID: AB_2341188
Anti-P-cad (1:1000)	R&D	Cat#BAF761; RRID: AB_442232
Donkey anti Goat Cy3 (1:1000)	Jackson ImmunoResearch	Cat#705-165-003; RRID: AB_2340411
Donkey anti Rabbit Alexa Fluor 647 (1:1000)	Jackson ImmunoResearch	Cat#711-605-152 RRID: AB_2492288
Chemicals, peptides, and recombinant proteins		
Tamoxifen	Sigma-Aldrich	Cat#T5648
Sunflower Oil	Sigma-Aldrich	Cat#PHR2989
TritonX-100	Sigma-Aldrich	Cat# X100
DAPI	Sangon	Cat#D6584
Paraformaldehyde	Sigma-Aldrich	Cat#158127
Bovine serum albumin	Sigma-Aldrich	Cat#V900933
Normal Donkey Serum	Jackson ImmunoResearch	Cat#017-000-121; RRID: AB_2337258
EDTA	SINOPHARM	Cat#6381-92-6
Glycerol	Sigma-Aldrich	Cat#G5516
Isoflurane	RWD	Cat#R510-22-16
Experimental models: Organisms/strains		
Sox9^{creERT2}; GCaMP6s; Ai14(Cre+, fl/wt,fl/wt) P11 female	(Xie et al., 2022)	n/a
K14-H2BGFP P11 female	Dr. Elaine Fuchs	n/a
Software and algorithms		
GraphPad Prism	GraphPad Software	RRID:SCR_002798; https://www.graphpad.com/scientific-software/prism/
NIS-Elements AR	Nikon	5.11.01; https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research
Fiji	(Schindelin et al., 2012)	RRID:SCR_002285; https://imagej.net/software/fiji/downloads
Other		
Temperature Controller	LEIYEA	Cat#HB2
Animal Anesthesia System	VETEQIUP	Cat#V-1
Gas Filter Canister	RWD	Cat#R510-31
Stereo microscope	Leica Microsystems	Cat#M165
Nikon microscope	Nikon	Cat#A1R MP
Slide	CITOTEST	Cat#80302-2201
Coverslips	CITOTEST	Cat#80330-1630
24-well dish	Thermo Fisher Scientific	Cat#60444928
Orbital shaker	Kylin-Bell	Cat#TS-1

MATERIALS AND EQUIPMENT

Fixation solution		
Reagent	Final concentration	Amount
Paraformaldehyde	4%	40 g
1× PBS	n/a	Up to 1 L
Total	n/a	1 L

Heat to 60°C and adjust PH to 7.2–7.4 to dissolve.
Storage: −20°C for 6 months

Permeabilization solution

Reagent	Final concentration	Amount
TritonX-100	0.3%	3 mL
1× PBS	n/a	Up to 1 L
Total	n/a	1 L

Storage: room temperature (20°C–25°C) for 1 year

Blocking solution

Reagent	Final concentration	Amount
Bovine serum albumin	1%	0.25 g
Normal Donkey Serum	2%	0.5 mL
TritonX-100	0.3%	75 μL
1× PBS	n/a	Up to 25 mL
Total	n/a	25 mL

Storage: −20°C for 6 months

Tamoxifen solution

Reagent	Final concentration	Amount
Tamoxifen	10 mg/mL	10 mg
Sunflower oil	n/a	1 mL
Total	n/a	1 mL

Heat to 37°C, vortex at 200 rpm for 30 min to dissolve.
Storage: Use immediately after preparation.

Equipment

| Slide |
|---|
| Coverslips |
| 24-well dish |
| Orbital shaker |
| Temperature Controller |
| Animal Anesthesia System |
| Gas Filter Canister |

STEP-BY-STEP METHOD DETAILS

Part A. Tail skin wholemount staining

Collection and fixation of tail skin

⏱ Timing: 2.5 h

1. Use postnatal day 18 mice for the experiment demonstrated here. But mice of all ages could use this tail skin epidermis wholemount protocol. Sacrifice mice using CO_2.
2. Mark the middle one third of the dorsal tail along the anterior-posterior central axis (Figures 1A and 1B).
3. Mark the ventral midline of the mouse tail (Figure 1C).
4. Cut along the marked ventral midline with a pair of surgical scissors.
5. Remove the skin from the tail bone using surgical tweezers.
6. Use the middle one third of the dorsal side tail skin and cut it into smaller pieces with a scalpel (Figure 1D). The length of each piece is 0.5 cm, and width is 0.5 cm (Figure 1E).
7. Place the square skin pieces in at least 5 mL of 25 mM EDTA in 1× PBS buffer for 2 h at 37°C on an orbital shaker at 150 rpm (Figure 1F) (Xu et al., 2022).
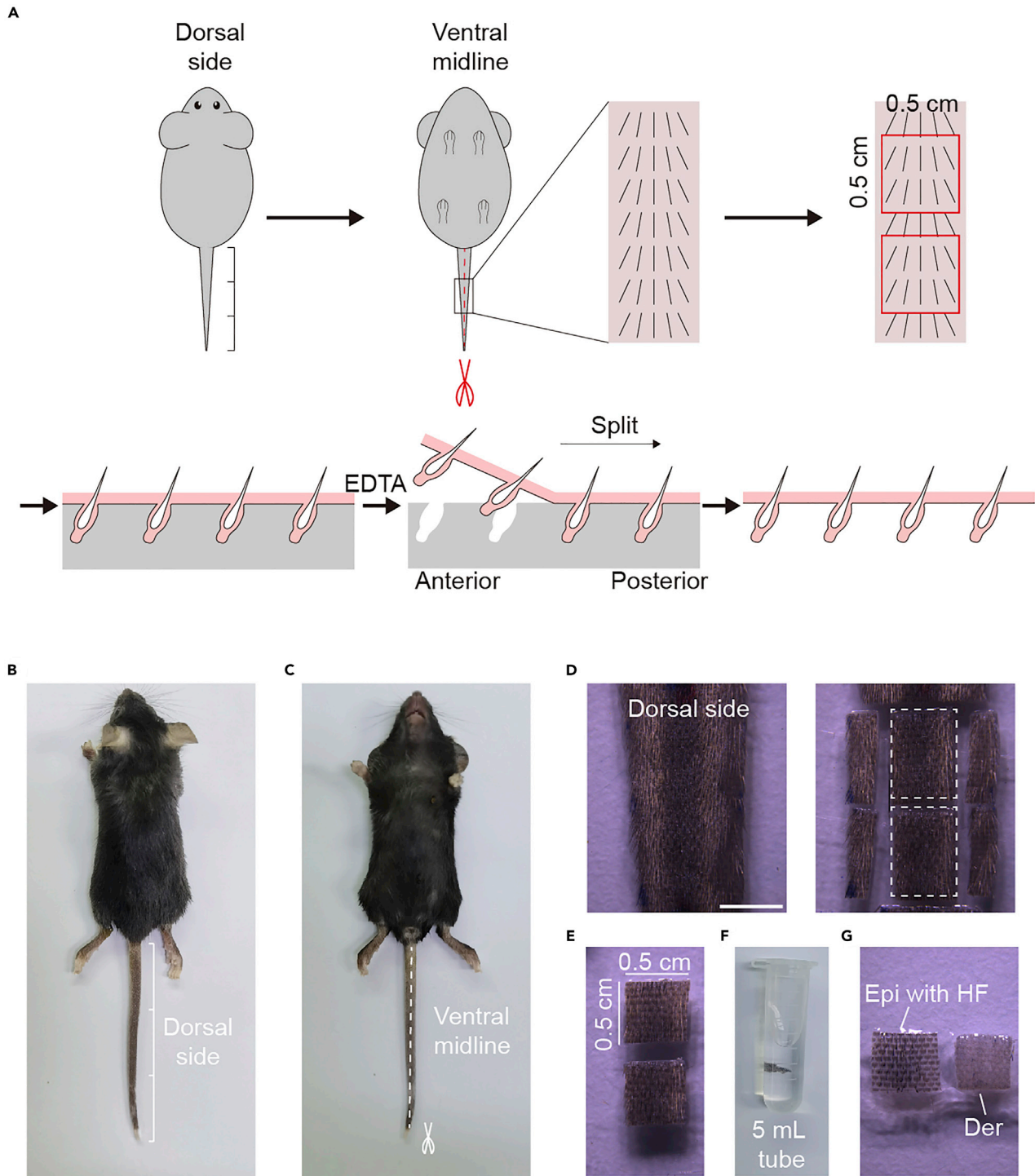
Figure 1. Collect epidermis with hair follicle of tail skin

(A) The workflow of collection of tail skin sample for wholemount staining.

(B) The middle-third dorsal side of tail skin is used for experimental consistency.

(C) Cut along the middle line of the ventral tail skin.

(D) Dorsal pieces of tail skin before EDTA treatment. Scale bar, 500 mm.

(E) Dorsal pieces of tail skin were clipping to 0.5 cm × 0.5 cm for EDTA treatment.

(F) EDTA treatment in 5 mL tube.

(G) Epidermis with hair follicles is separated from the whole tail skin after EDTA treatment.

Note: In tail skin wholemount staining, if one intended to make sure anagen hair follicles are separated along with the epidermis from the dermis, 2 h EDTA treatment needs to be increased to 4 h EDTA treatment.

8. Separate the epidermis with attached hair follicles from the dermis using a pair of fine-tipped tweezers.

   Note: an anterior-to-posterior peeling direction should be used to keep hair follicles attached to the epidermis (Figure 1G).

9. Place removed epidermis with attached hair follicles in 1 mL 4% formaldehyde in 1× PBS buffer for 7 min in 24-well dish at room temperature.

   Note: the hair follicle side should face down in the fixation solution.

10. Remove the epidermis with attached hair follicles from the fixation solution and wash with 1 mL of 1× PBS for 3 × 30 min in 24-well dish on an orbital shaker at 80 rpm at room temperature.

    Note: Because tail skin hair shaft is shorter than dorsal skin hair shaft, no need to use shaver or depilatory cream to remove tail skin hair shaft on the skin surface.

    ⚠ CRITICAL: The development of tail skin hair follicles varies slightly in both anterior-posterior axis and in dorsal-ventral axis. For the purpose of experimental consistency, only the middle one third of the tail from the center of the dorsal side was chosen for tail skin wholemount staining (Xu et al., 2015).

Immunofluorescent staining of tail skin

⏲ Timing: 2 days

Note: All subsequent staining procedures are carried out in 24-well plate, with the hair follicle side facing down in the well. For all incubation process, the plate is kept on an orbital shaker at 80 rpm. To change medium in the 24-well plate, use P1000 pipette to carefully remove and add solution, while avoid touching the tissue in the well.

11. To permeabilize skin sample, incubate tissues in 1 mL 0.3% TritonX-100 in 1× PBS for 10–20 min.
12. Incubate the epidermis with attached hair follicles in 1 mL blocking solution for 1 h at room temperature.
13. Incubate the epidermis with attached hair follicles with primary antibody (diluted in blocking solution) overnight (~16 h) at 4°C. Put one piece of tail skin in one well containing ~300 μL solution. Goat anti-Pcad 1:1000. Rabbit anti-cleaved-caspase3 1:1000.
14. Wash samples with 1 mL 1× PBS for 3 × 20 min at room temperature.
15. Incubate samples with secondary antibody and 1 μg/mL DAPI (diluted in ~300 μL blocking solution) for 1 h at room temperature. Donkey anti Goat Cy3 1: 1000. Donkey anti Rabbit Alexa Fluor 647 1:1000. Troubleshooting 1.
16. Wash samples with 1× PBS for 3 × 20 min at room temperature.
17. Next depending on the purpose of the experiment, removing unwanted hair follicles might be needed. For our study (Xie et al., 2022), we intended to study catagen-telogen hair follicles. In this case, the long anagen hair follicles will obscure the view of neighboring catagen-telogen hair follicles that are much shorter. So before mounting and imaging, use fine-tipped tweezers to remove anagen hair follicles one by one. Under stereo microscope, different stage hair follicles can be clearly distinguished by their length and morphological differences. Detailed representative images are provided in the expected outcomes below.

18. Place the tissue on the slide with the side of hair follicle facing the coverslip. Mount the tissue with 50% glycerol (diluted in PBS) with 1 μg/mL DAPI.
19. Apply coverslip and seal the edges with copious amount of clear nail polish.

    Note: In our experience no spacer is needed to preserve the 3-D structure of the hair follicle because the epidermis with attached hair follicles is not of significant thickness. We routinely add 50 μl mounting buffer for each 0.5 cm × 0.5 cm epidermis wholemount tissue, this amount of mounting buffer is sufficient to support the coverslip without it crushing the tissue. But for full thickness skin wholemount staining, which contains both epidermis and dermis, a spacer is needed to support the coverslip. In that case, we use Dow Corning High Vacuum Lubricant to circle the tissue and provide support for the cover slip.

    Note: Only use clear colorless nail polish to seal the edge of coverslip and avoid applying the nail polish on top of the skin samples.

Part B. Intravital calcium imaging
Tamoxifen induction for live imaging in vivo

◯ Timing: 7 days

20. Prepare tamoxifen for intraperitoneal (i.p.) injection at 10 mg/mL in 100% sunflower oil. Heat the solution to 37°C, vortex at 200 for 30 min to dissolve tamoxifen in solution. Use immediately after preparation.
21. At postnatal day 11, inject *Sox9^creERT2^; GCaMP6s; Ai14* mice intraperitoneally with tamoxifen at dosage of 40 μg/g body weight for 3 consecutive days. This allows the inducible GCaMP6s and tdTomato expression in HFSCs.
22. At postnatal day 18, the mice are ready for live imaging *in vivo.*

    Note: We chose this time point because we were analyzing catagen-telogen hair follicles in our study. Since using this genetic labeling strategy, HFSCs and their progenies will continue to express GCaMP6s and tdTomato for the lifetime of the animal, so long term intravital imaging can be carried out at any time points after tamoxifen induction. Specific observation time points should be determined for specific scientific questions.

Intravital imaging of calcium signal in tail hair follicle

◯ Timing: 2 h

23. Anesthetize mouse using isoflurane (Figure 2A). Troubleshooting 2.
24. Prepare the imaging station by immobilizing a 15 × 25 cm heating plate of the Temperature Controller on the microscopic table. For the duration of the imaging process, temperature of the heating plate is set at 37°C.
25. Immobilize the breathing apparatus of an Anesthesia System by tapes on one side of the heating plate.
26. After putting the anesthetized mouse nostril inside the breathing apparatus of the Anesthesia System, immobilize the mouse tail by tape on the heating plate, ready for imaging (Figure 2B). Around the imaging area of tail skin, use extra tapes to stabilize the tail skin and minimize movement during the imaging process. Troubleshooting 3.
27. Adjust microscopic lens to be positioned just above the intended imaging skin area.
28. Perform Ca²⁺ imaging using a Nikon 2-photon microscope with a 25× water-immersion objective lens with a numerical aperture of 1.02 (Olympus, UIS2) (Figure 2C) (Pineda et al., 2015; Xu et al., 2015).
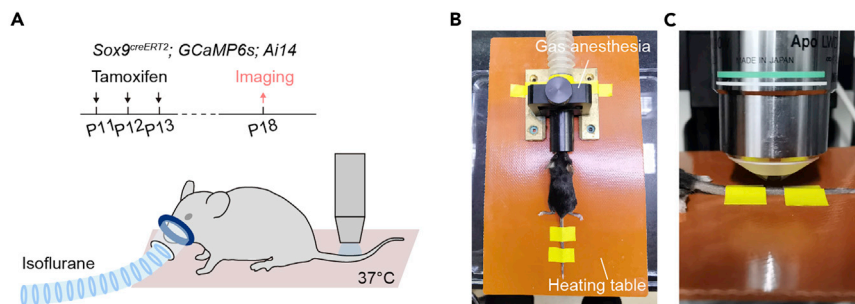
Figure 2. Mouse immobilization for live imaging *in vivo*
(A) *Sox9creERT2; GCaMP6s; Ai14* mice used for calcium imaging and tamoxifen injection timeframe.
(B) The custom table consisting of Anesthesia System and Temperature Controller.
(C) A zoom-in picture of tail and microscopic lens. Around the imaging area of tail skin, use tapes to immobilize the tail skin to avoid the movement caused by mouse breath.

29. To image GCaMP6s signals, tune the laser to the 910 nm wavelength with 40% intensity (Dana et al., 2019). To image tdTomato signals, tune the laser to the 1,040 nm wavelength with 10% intensity. GCaMP6s intensity represents calcium signal, and tdTomato provides stable internal control used to calculate calcium signal.

   Note: The appropriate laser intensity depends on the specific experiment and microscope, so it should be adjusted accordingly.

30. Acquire images at 1 frame per 10 s for 10 min using Nikon software.
31. For calcium flash quantification and analysis, refer to Quantification and statistical analysis part.

EXPECTED OUTCOMES

Wholemount staining of tail skin: A successful experiment of tail skin wholemount staining is shown in Figure 3. Before imaging, we used stereo microscope to distinguish the different hair cycle stages. Anagen hair follicles are much longer than catagen-telogen hair follicles; and anagen hair follicles contain enlarged matrix structure at the bottom, which is absent in catagen-telogen hair follicles (Müller-Röver et al., 2001). For the purpose of our study (Xie et al., 2022), we intended to study catagen-telogen hair follicles (outlined in white dotted line). In this case, the long anagen hair follicles (outlined in yellow dotted line) will obscure the view of neighboring catagen-telogen hair follicles that are much shorter. We used fine-tipped tweezers to remove anagen hair follicles one by one, so that they do not obscure the view of the intended catagen-telogen hair follicles. Here we provided representative images to show the difference of before and after removal of anagen hair follicles (Figure 3A). Depending on the purpose of specific experiment, this step may not be needed. Figure 3B showed after plucking of catagen hair shaft, HF at subsequent telogen has abundant cleaved caspase-3 signals in the bulge area, which was not observed in control telogen hair follicles. Troubleshooting 4.

Intravital imaging of calcium signaling in HFSCs using P11 *Sox9^{creERT2}; GCaMP6s; Ai14* mice: A successful experiment of live imaging of tail hair follicle is shown in Figure 4. The maximum imaging depth of a 2-photon microscope on intact tail skin limited our observation to the upper HF (Figures 4A and 4B). Here to clearly demonstrate the imaging depth of our method, we used *K14H2BGFP* mouse to show the outline of hair follicle under the lens of a 2-photon microscope, this reporter line was not used in the calcium imaging experiment. In Figures 4C and 4D, green fluorescent signal (expressed by *GCaMP6s* allele) is Ca^{2+} indicator, and red fluorescent signal (expressed by *Ai14* allele) serves as stable internal control for the imaging process. The representative images show after plucking of catagen hair shaft, HF at subsequent telogen has pulses of calcium flashes that are not observed in control HFs.
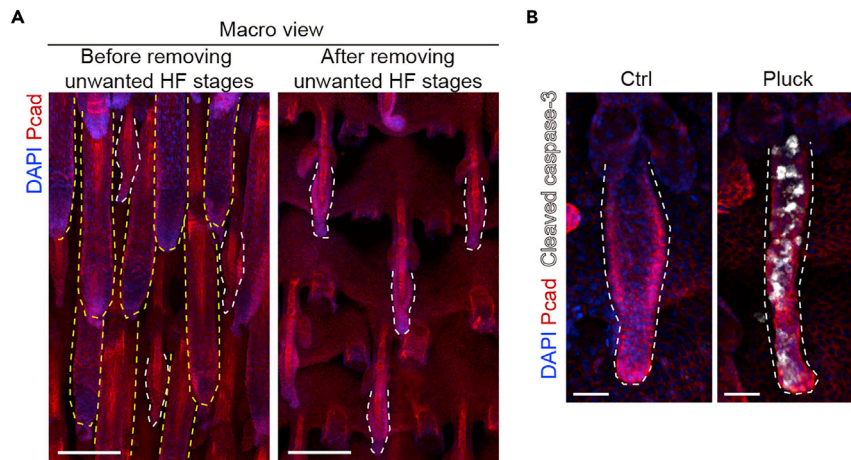
Figure 3. A successful wholemount staining of tail skin epidermis with hair follicle
(A) Macro view of wholemount staining before and after removing unwanted hair cycle stages. Before imaging, use tweezers to remove hair follicles in unwanted stages to avoid the influence. Yellow dash line, anagen hair follicles; white dash line, telogen hair follicles. Scale bar, 150 μm.
(B) A successful tail skin wholemount staining of DAPI, Pcad and cleaved-caspase3.Scale bar, 50 μm.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of calcium signaling

In ImageJ, outline individual cells in upper bulge and measure the GCaMP6s and tdTomato signal intensities of each cell in each frame (Figure 5A). Cell shape can be distinguished based on signals of GCaMP6s and tdTomato (Figure 5B). To calculate normalized GCaMP6s signal intensity, for each frame the background-subtracted GCaMP6s fluorescence value was divided by the background-subtracted tdTomato fluorescence value for the same region of interest using Excel (Figure 5C). The relative GCaMP6s signal change, $F/F_b(t)$, for each frame (t) was calculated (Rhoades et al., 2019; Scott et al., 2013). Baseline F_b was the mean value of the lowest 10th percentile of fluorescence intensities during the imaging period of the same cell (Figure 5D). A Ca^{2+} flash was defined based on an amplitude that was at least 2-fold above the baseline level. Ca^{2+} intensity track was analyzed using GraphPad Prism.

LIMITATIONS

The two-photon microscope we used limits the observation to within 200 μm depth, which reaches the upper hair follicle. If the cell type of interest is located in deeper skin, change to other skin region might be needed, or a different microscope might be needed.

TROUBLESHOOTING

Problem 1

The DAPI intensity of tail skin sample isn't uniform. The signal intensity is stronger at the sample's edge (step 15).

Potential solution

Stain tissues with DAPI together with secondary antibody for 1 h at room temperature will make sure all cells are stained with DAPI equally for the wholemount analysis.

Problem 2

The mouse died during imaging process (step 23).

Potential solution

Reduce and optimize the dosage of the isoflurane used in the Anesthesia System to reduce mortality in mice.
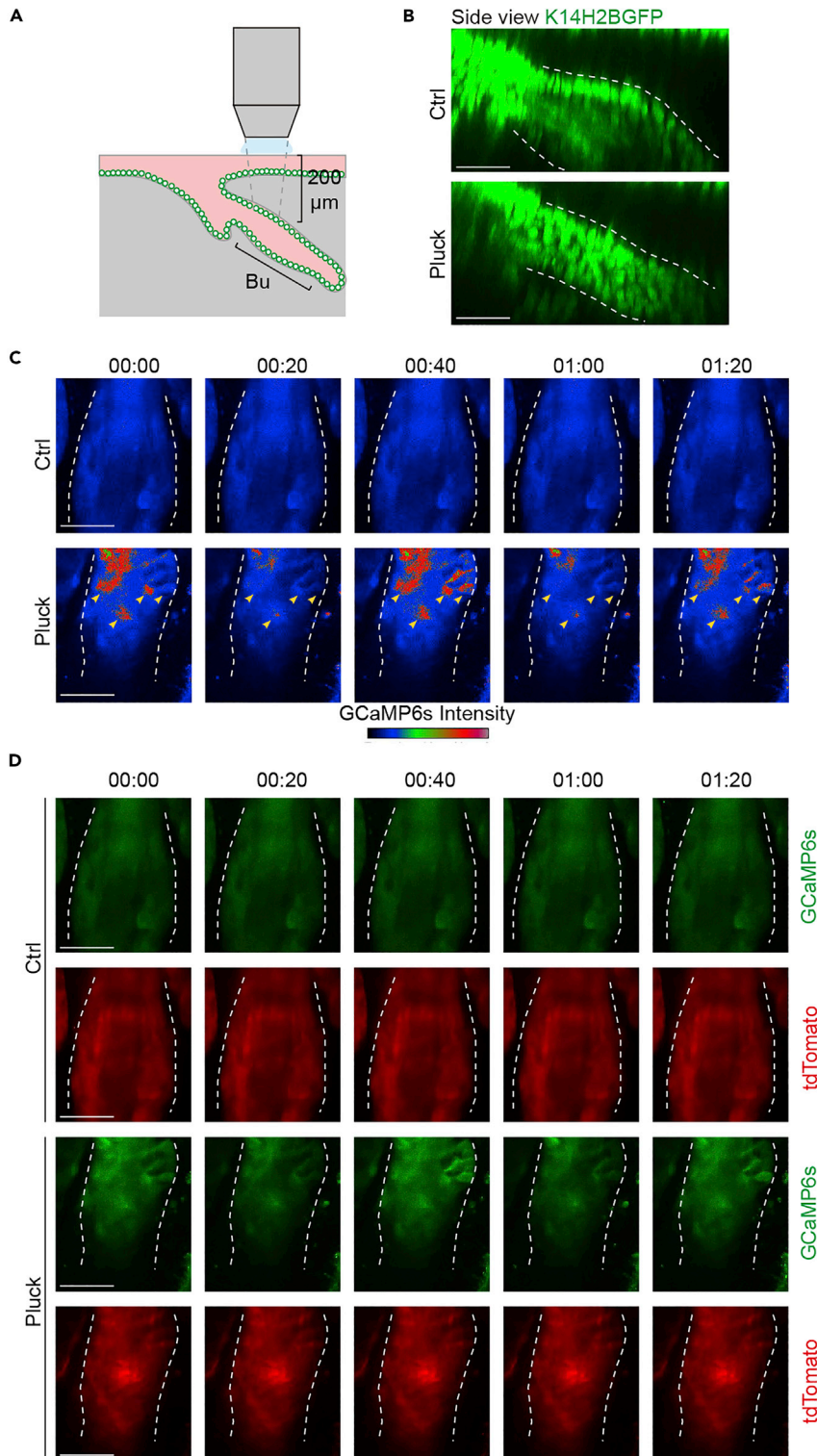
Figure 4. A successful live imaging of calcium of upper hair follicle *in vivo*

(A) Schematic diagram of two-photon calcium imaging experiment. Note the maximum imaging depth (200 μm) of 2-photon microscope on intact tail skin limits observation to the upper bulge.

(B) Side view of hair follicle in *K14H2BGFP* mouse. Note the lower part of hair follicle has weak signal. Scale bar, 30 μm.

Figure 4. Continued

(C) Representative images of *in vivo* time-lapse calcium imaging of control and plucked hair follicles. Pseudo color images represent normalized GCaMP6s intensity. Note plucked hair follicle showed calcium flash. Scale bar, 30 μm.

(D) Representative traces of calcium intensity of control and plucked hair follicles. Scale bar, 30 μm.

Problem 3

The imaging field keeps shaking (step 26).

Potential solution

Several factors could contribute to this problem: 1. The mouse's heartbeat and breathing caused body movement: apply extra tape to enhance immobilization of the torso and tail of the mouse to further minimize body movement. In addition, slightly increase isoflurane dosage of the Anesthesia System could helpful. 2. The imaging station itself is unstable: make sure the heating table of Temperature Controller on the microscope table is fixated securely.

Problem 4

No positive staining signal, or strong background signals are observed (expected outcomes).

Potential solution

First, the quality of primary antibody plays a dominant role in determining the outcome of the whole-mount staining experiment. So, make sure high-quality primary and secondary antibodies are used. However, if no ideal primary antibody is available, try decrease the dilution of primary antibody, and increase antibody incubation time to increase signal. Second, to increase the signal to noise ratio, increase dilution of antibodies and increase washing time to 3 × 1 h, to help reduce background signals. If using
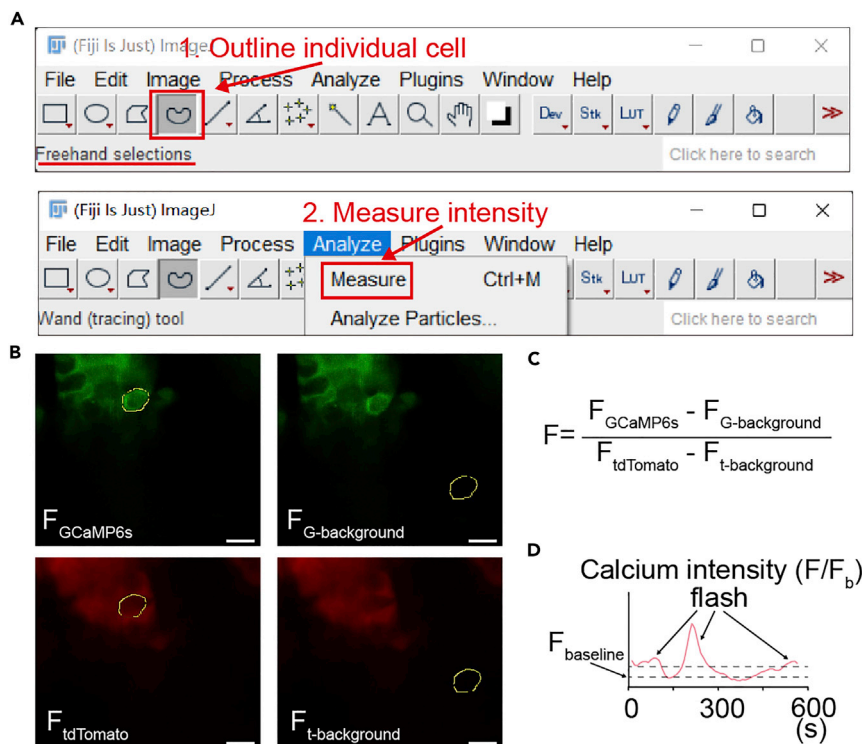
Figure 5. Quantification and analysis

(A) In ImageJ, outline individual cells in upper bulge and measure the GCaMP6s and tdTomato signal intensities.

(B) Example of quantification intensity of GCaMP6s and tdTomato of one cell in one frame. Scale bar, 10 μm.

(C) Calculation formula of relative intensity of calcium intensity of one cell in one frame.

$$F = \frac{F_{GCaMP6s} - F_{G\text{-background}}}{F_{tdTomato} - F_{t\text{-background}}}$$

(D) Ca^{2+} intensity track of one cell and calcium flash.

647 channel, strong background or signals could be observed sometimes. If possible, use another channel.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ting Chen (chenting@nibs.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

T.C. and Y.X. conceived the project, designed the experiments, and wrote the manuscript. Y.X. performed the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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