Video Article

CUBIC Protocol Visualizes Protein Expression at Single Cell Resolution in Whole Mount Skin Preparations

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Abstract

The skin is essential for our survival. The outer epidermal layer consists of the interfollicular epidermis, which is a stratified squamous epithelium covering most of our body, and epidermal appendages such as the hair follicles and sweat glands. The epidermis undergoes regeneration throughout life and in response to injury. This is enabled by K14-expressing basal epidermal stem/progenitor cell populations that are tightly regulated by multiple regulatory mechanisms active within the epidermis and between epidermis and dermis. This article describes a simple method to clarify full thickness mouse skin biopsies, and visualize K14 protein expression patterns, Ki67 labeled proliferating cells, Nile Red labeled sebocytes, and DAPI nuclear labeling at single cell resolution in 3D. This method enables accurate assessment and quantification of skin anatomy and pathology, and of abnormal epidermal phenotypes in genetically modified mouse lines. The CUBIC protocol is the best method available to date to investigate molecular and cellular interactions in full thickness skin biopsies at single cell resolution.

Video Link

The video component of this article can be found at https://www.jove.com/video/54401/

Introduction

The skin is essential for our survival. It consists of three main layers the outer epidermis, the dermis and the hypodermis. The epidermis is a highly regenerative tissue. It is a squamous stratified epithelium, consisting mostly of keratinocytes. Keratinocytes are born in the basal layer, and move upwards through the suprabasal layers while differentiating, and eventually they are shed in the outer cornified layer about a month after their birth. The epidermis develops a number of appendages including the hair follicles and sebaceous glands. The hair follicles also regenerate in a cyclical fashion throughout life[1]. The regenerative capacity of the epidermis is enabled by the presence of stem and progenitor cells that are located in the basal layer of the interfollicular epidermis and hair follicle[2].

Many signaling pathways have been implicated in epidermal development and regeneration. Some of these occur within the epidermis only, such as the Hedgehog pathway. Other signaling events take place between dermis and epidermis[3]. For instance, Wnt signals from the dermis are thought to be important for hair follicle development, and they are secreted by the dermal papilla at the onset of anagen to activate hair follicle bulge stem/progenitor cell proliferation and hair follicle growth[4]. It is important to understand the cellular and molecular mechanisms that control epidermal development and regeneration to better understand how they may be perturbed in regenerative skin disease such as skin cancer.

This article describes a Clear, Unobstructed Brain Imaging cocktails and Computational analysis (CUBIC) protocol[5-7] to clarify whole mount skin preparations, and visualize protein expression patterns in 3 dimensions at single cell resolution by confocal microscopy. The CUBIC method involves immersion of skin tissue in two aminoalcohol-based chemical cocktails. These solutions adjust the refractive indices in the skin sample, leaving the tissue transparent and the proteins intact, allowing immunodetection at single cell resolution.

Using this CUBIC protocol, the basal and proliferating keratinocyte populations in the interfollicular epidermis and in the hair follicle were imaged in full thickness skin biopsies of wildtype mice using anti-Keratin14 (K14) and anti-Ki67 antibodies. Sebaceous glands in wildtype skin biopsies were also visualized using Nile Red staining. Lastly, the basal keratinocyte populations in wildtype and hyperplastic YAP2-5SA-ΔC skin biopsies were compared[8].

This CUBIC protocol enables visual assessment of protein expression in full thickness skin biopsies at single cell resolution, and is an important tool to appreciate epidermal anatomy and morphological defects in the skin of genetically modified mice, and to investigate the cellular and molecular mechanisms underlying epidermal development and regeneration.

Protocol

Ethics Statement: All procedures involving animal subjects follow the guidelines of the Animal Care and Ethics Committee (ACEC) at UNSW Australia under approved ACEC protocol 13/64B.

1. Preparation of the Transparent Mouse Skin Tissue

Note: All mice used in this study were on a C57BL/6 genetic background

1. Collection of mouse skin tissue.
    1. Humanely euthanize the mice by cervical dislocation.
    2. Carefully remove hairs from the relevant skin area with a trimmer taking care not to wound the skin.
    3. Wash skin to decontaminate with 70% ethanol in Phosphate Buffered Saline (PBS).
    4. Lift dorsal neck skin with forceps and make incision with scissors.
    5. Dissect a large area of dorsal mouse skin (approximately 1.5 x 4 cm).
    6. Flatten skin dermis-side down on a filter paper, and make note of the anterior-posterior orientation of the sample.
    7. Trim filter paper around the dissected skin, and place in a 15 ml tube filled with freshly prepared 4% paraformaldehyde (PFA)solution in PBS.
    8. Fix for 1 hr at room temperature, or overnight in the refrigerator at 4 °C.
    9. Wash skin 2 x 5 min in PBS in a 15 ml tube.
    Note: The following (1.1.10 - 1.1.12) are optional steps for long term storage of tissue.
    10. Dehydrate dissected skin in PBS with increasing concentration of ethanol (25%, 50% 70%)in a 15 ml tube during 1-hr wash steps at room temperature.
    11. Store dehydrated skin in 70% ethanol in PBS in a 15 ml tube at 4 °C until further use.
    12. 4 hours before clearing, rehydrate skin tissue in PBS with decreasing concentration of ethanol (70%, 50%, 25%, 0%) in a 15 ml tube during 1-hr wash steps at room temperature.

2. Clearing the mouse skin biopsies
    1. Prepare the CUBIC1 clearing solution by dissolving 3.85 g urea and 3.85 g *N,N,N′,N′*-tetrakis (2-hydroxypropyl) ethylenediamine in 5.38 ml distilled water on a heater set to 60 - 70 °C. Use a hot stirrer.
    2. Add 2.31 g polyethylene glycol mono-p-isooctylphenyl ether/Triton X-100 to the solution once it is clear and has cooled down to room temperature.
    3. Cut the mouse skin with a sharp razor blade into biopsies of approximate dimensions 0.2 x 0.5 cm, and submerge in 5 ml of CUBIC1 clearing solution in a 15 ml tube. To optimize the visualization of hair follicles, ensure that the longer side of the biopsy is cut along the antero-posterior direction of the sample.
    4. Place on a rotating platform in a hybridization oven at 37 °C.
    5. Change the clearing solution after 7 days. Prepare fresh CUBIC1 solution prior to use.
    6. Check the transparency of the tissue after 7 days. If necessary, leave biopsies in CUBIC1 clearing solution until the tissue is completely transparent.
    7. Once the skin biopsy is transparent, remove the CUBIC1 solution, and add 4 ml of 1× PBS to wash the tissue 4 times for 6 hr at 37 °C.
    8. Wash the skin tissue in 20% w/v sucrose in PBS in a 15 ml tube for 4 hr at 37 °C.
    9. Freeze the tissue in mounting medium Optimal Cutting Temperature (OCT) Compound in a 15 ml tube overnight in a -80 °C freezer.
    Note: This step will increase the biopsy's permeability for antibody penetration in subsequent steps.

2. Immunofluorescence Staining

1. Thaw tissue from 1.2.9) to room temperature for 2 - 3 hr.
2. Wash tissue in the 15 ml tube with 5 ml of PBS for 8 hr at room temperature to remove OCT Compound.
3. Transfer biopsies to a 2 ml tube, and incubate tissue in 1 ml rabbit anti-Keratin14 or rabbit anti-Ki67 antibody, both diluted 1:100 in PBST (PBS + 0.1% Triton-X100) for 3 days on a shaker in a 37 °C oven.
4. Transfer biopsies to a 15 ml tube, and wash tissue 4 times for 6 hr in 5 ml of PBST on a shaker in a 37 °C oven.
5. Transfer biopsies to a 2 ml tube, add 1 ml anti-rabbit Alexa594 secondary antibody diluted 1:100 in PBST and incubate 3 days on a shaker in a 37 °C oven.
6. Transfer biopsies to a 15 ml tube, and wash tissue 4 times for 6 hr in 5ml of PBST on a shaker in a 37 °C oven.
7. Transfer biopsies to a 2 ml tube, add 1 ml 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain solution (1:1,000) in PBST and incubate overnight on a shaker in a 37 °C oven.
8. Remove DAPI counterstaining solution, and add 1 ml of PBST to the 2 ml tube to wash tissue 4 times for 6 hr on a shaker in a 37 °C oven.
Note: Optional step: Biopsies can be stored in the dark in 1x PBS with 0.02% sodium azide for at least 3 weeks.

3. Nile Red Staining

1. Dissolve Nile Red powder in dimethylsulfoxide (DMSO) to a final concentration of 1 mg/ml
2. Add 1 µl Nile Red solution to 1 ml PBS to a final concentration of 1 µg/ml.
3. Thaw tissue from 1.2.9) to room temperature for 2 - 3 hr, and wash with 5 ml PBS for 8 hr at room temperature.
4. Transfer biopsies to a 2 ml tube, and submerge tissue in 1 ml Nile Red staining solution for 2.5 hr at room temperature.
5. Wash the skin biopsies in the 2 ml tube with 1 ml PBST 4 times for 30 min at room temperature.

6. Remove PBST solution from the 2 ml tube, add 1 ml of DAPI nuclear counterstaining solution (1:1,000) in PBST and incubate overnight at room temperature.
7. Remove DAPI counterstaining solution, and add 1ml of PBST in the 2 ml tube to wash the skin tissue 4 times for 6 hr at room temperature.
Note: Optional step: Biopsies can be stored in the dark in 1x PBS with 0.02% sodium azide for at least 3 weeks.

4. Imaging

1. Prepare the CUBIC2 clearing solution, which contains 50% (w/v) sucrose, 25% (w/v) urea, 10% (w/v) 2,2′,2″-nitrilotriethanol, and 0.1% (v/v) Triton X-100.
2. Incubate the skin tissue in 1 ml CUBIC2 solution in a 2 ml tube on a shaker for 24 hr in a 37 °C oven. This step will even the refractive index of the tissue.
3. Check the clarity of the tissue. Once it is clear, position the entire skin biopsy (0.2 x 0.5 cm) on its longer side onto a glass coverslip, such that the direction of the length of the hair follicles is parallel to the coverslip surface (#1 24 x 60 mm)
Note: Optional step: Antibody-stained biopsies can be stored in CUBIC2 solution for about 7 days. Nile Red-stained biopsies can only be stored in CUBIC2 solution for up to 1 day, and should be imaged as soon as possible.
4. Prepare imaging chamber (**Figure 1**)
Note: Consumables required for the preparation of the imaging chamber are: blue tack, play dough or similar, and 2 coverslips (24 x 50 mm) (**Figure 1A**).
    1. Prepare two thin strips of blue tack (diameter approximately 1 mm x 2 cm), and two coverslips (**Figure 1B**).
    2. Place blue tack strips on one cover slip, allowing enough space for skin biopsy (**Figure 1C**). 4.4.3)
    3. Place skin biopsy in between strips on the coverslip in a drop of CUBIC2 solution (**Figure 1C**).
    4. Cover skin biopsy with the second coverslip (**Figure 1D**).
5. Place the imaging chamber with the mounted skin biopsy onto the stage of a confocal microscope and move the tissue into the light pathway.
6. Scan the sample using a mercury or halogen light source and standard epifluorescence filters (*e.g.,* DAPI/GFP/CY3/CY5) to identify fluorescently stained regions of interest.
7. Image regions of interest using a 10X and 20X objective (NA 0.75) and standard confocal fluorescence imaging techniques (*e.g.*, with DAPI stained samples illuminate with a 405 nm laser and collect fluorescence signal between 425 - 475 nm, and with Alexa Fluor 594 or Nile Red-stained samples illuminate with a 561 nm laser and collect fluorescence signal between 570 - 620 nm).
8. Generate image Z-stacks of each region of interest using microscope Z-stack image acquisition software (*e.g.*, using NIS elements imaging software 4.13 as described below).
    1. Ensure optimal laser power and PMT HV/Offset settings have been selected to collect sample fluorescence.
    2. Open the "ND Acquisition" toolbar from "Acquisition Controls".
    3. Select "Z series Setup" tab (ensure all other tabs are unselected).
    4. While live scanning, focus to the top of the sample and press the "Top" button.
    5. Focus to the bottom of the sample and press the "Bottom" button.
    6. Input required step size (or press optimized step size button).
    7. Press the "Run now" button.
    8. Save resultant Z-stacks as individual Tiff image stacks (1 fluorochrome per image Z-stack).
9. Use image Z-stacks to reconstruct 3D volume regions of interest using 3D analysis software (*e.g.*, using Imaris x64 7.2.3 as described below).
    1. Start analysis software.
    2. Choose "Surpass" button in toolbar for 3D volume generation.
    3. Open first color image Z-stack (*e.g.*, DAPI).
    4. Use the 'add channel' tool to add additional color channels, one channel per fluorochrome. Thus a sample containing both DAPI and Alexa Fluor 594 fluorochromes will require two channels.
    5. Use the "Image Properties" tool to set correct pixel (voxel) dimensions (XYZ), as determined in 4.7 above.
    6. Use "Display Adjustment" tool to change channel colors as needed (*e.g.,* set DAPI channel to blue, Alexa Fluor 594 channel to red).
    7. To position the 3D volume in the image window, use the computer mouse to click and drag volume as required.
    8. Use the "Snapshot" tool in the toolbar to generate screen shots of the 3D image.
    9. Use the "Animation" tool in the toolbar to generate movies of 3D sample rotation.

Representative Results

Full thickness dorsal skin biopsies of adult wildtype mice were clarified, stained with an antibody binding basal keratinocyte marker Keratin14 (K14), and nuclei were counterstained with DAPI staining solution (**Figure 2** and **Movie 1**).

DAPI-positive nuclei were visible throughout the sample (**Figure 2A, C**), and K14 staining was visible exclusively in the one-cell thick basal layer of the interfollicular epidermis, and outlining the sebaceous glands (black asterisks), the outer root sheaths of hair follicles, and in the secondary hair germs (**Figure 2B, C**), as previously published[9]. K14 staining intensity was high in the interfollicular epidermis, the distal hair follicle and the secondary hair germs (white asterisks), and it was relatively low in the bulge area of the hair follicle (Bu in **Figure 2C**). The dermal papillae were also clearly visible through DAPI labeling (arrow in **Figure 2C**).

To visualize proliferating cells, full thickness dorsal skin biopsies in telogen of adult wildtype mice were clarified and stained with an anti-Ki67 antibody, and nuclei were counterstained with DAPI solution (**Figure 3,** and **Movie 2**).

Proliferating keratinocytes were detected in the basal interfollicular epidermis (IFE in **Figure 3C**), and in the isthmus (Is in **Figure 3C**) of the hair follicles, but not in the bulge region (Bu in **Figure 3C**).

To visualize sebaceous glands, full thickness dorsal skin biopsies in anagen of adult wildtype mice were clarified and stained with Nile Red that labels the fat content of sebocytes, and nuclei were counterstained with DAPI staining solution (**Figure 4** and **Movie 3**). Nile Red-positive sebaceous glands were observed in the isthmus region of the hair follicles (SB in **Figure 4C**), showing that the CUBIC protocol does not significantly affect the morphology of these fat-containing structures. Unexpectedly, hair shafts were also labeled with Nile Red (HS in **Figure 4C**).

To visualize the morphological changes in the epidermis of YAP2-5SA-ΔC transgenic mice expressing a hyperactive YAP mutant protein in basal keratinocytes[8], full thickness dorsal skin biopsies of adult wildtype and YAP2-5SA-ΔC transgenic littermate mice were clarified and stained with the anti-K14 antibody, and nuclei were counterstained with DAPI solution (**Figure 5**, and **Movie 4** (wildtype) and **Movie 5** (YAP2-5SA-ΔC)).

In the YAP2-5SA-ΔC transgenic skin, the hyperplasia of the interfollicular epidermis (top double arrows in **Figure 4F**) was clearly visible, and the hair follicles were longer and displayed the K14-labeled cell masses proximally in the abnormal hair follicles (lower double arrows in **Figure 5F**), representing the enlarged stem cell populations, as previously described[8].

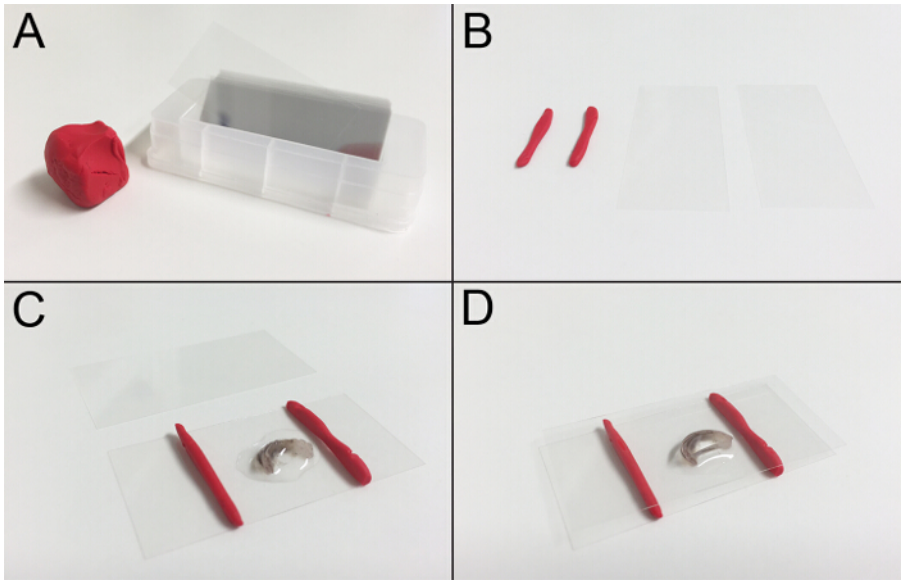


Figure 1: Preparation of the Imaging Chamber. A: Consumables required for the preparation of the imaging chamber are blue tack, play dough or similar, and 2 coverslips (24 x 50 mm). **B**: prepare two thin strips of blue tack (diameter approximately 2 mm x 2 cm), and two coverslips. **C**: Place blue tack strips on one cover slip, allowing enough space for the skin biopsy. Place the skin biopsy in a drop of CUBIC2 solution in between the two strips. **D**: Place second coverslip on blue tack strips to cover the skin biopsy. Please click here to view a larger version of this figure.

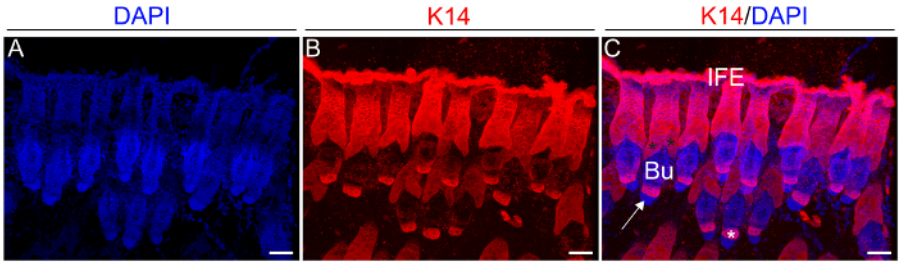


Figure 2: K14/DAPI Labeling of Dorsal Skin in Telogen of Adult Wildtype Mice. 3D volume reconstruction of a dorsal skin biopsy in telogen of an adult wildtype mouse labeled with a K14 antibody (Red in **B**, **C**), and DAPI nuclear counterstain (blue in **A**, **C**). K14 staining is strong in the interfollicular epidermis (IFE), the isthmus and the sebaceous glands (black asterisks), and the secondary hair germs (white asterisk), and relatively low in the bulge area (Bu). Scale bars 50 µm. Please click here to view a larger version of this figure.

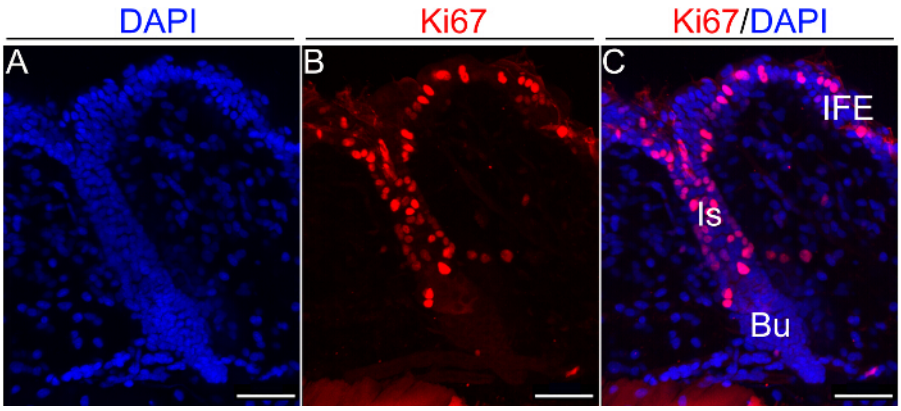


Figure 3: Ki67 Labeling of Dorsal Skin in Telogen of Adult Wildtype Mice. 3D volume reconstructions of a dorsal skin biopsy of an adult wildtype mouse labeled with a Ki67 antibody (Red in **B**, **C**), and DAPI nuclear counterstain (blue in **A**, **C**). Proliferating Ki67-positive keratinocytes are evident in the basal interfollicular epidermis (IFE), and in the isthmus region (Is), but not in the bulge (Bu) of the telogen hair follicle. Scale bars 50 µm. Please click here to view a larger version of this figure.

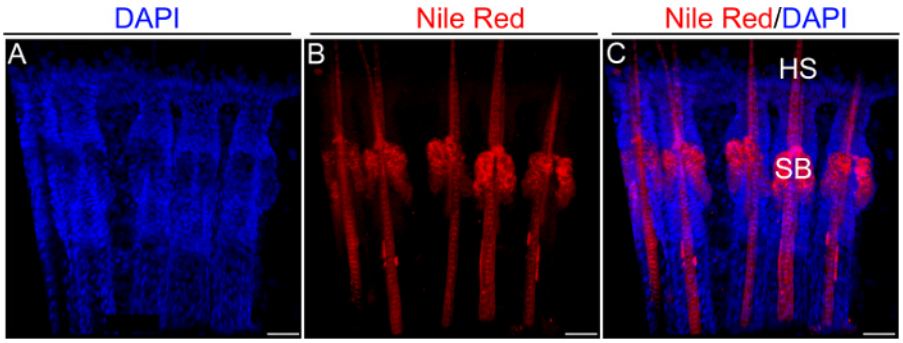


Figure 4: Nile Red Staining of Dorsal Skin in Anagen of Adult Wildtype Mice. 3D volume reconstruction of a dorsal skin biopsy in anagen of an adult wildtype mouse labeled with Nile Red (red in **B**, **C**), and DAPI nuclear counterstain (blue in **A**, **C**). Nile Red staining is visible in the sebocytes (SB) and in the hair shaft (HS). Scale bars 50 µm. Please click here to view a larger version of this figure.

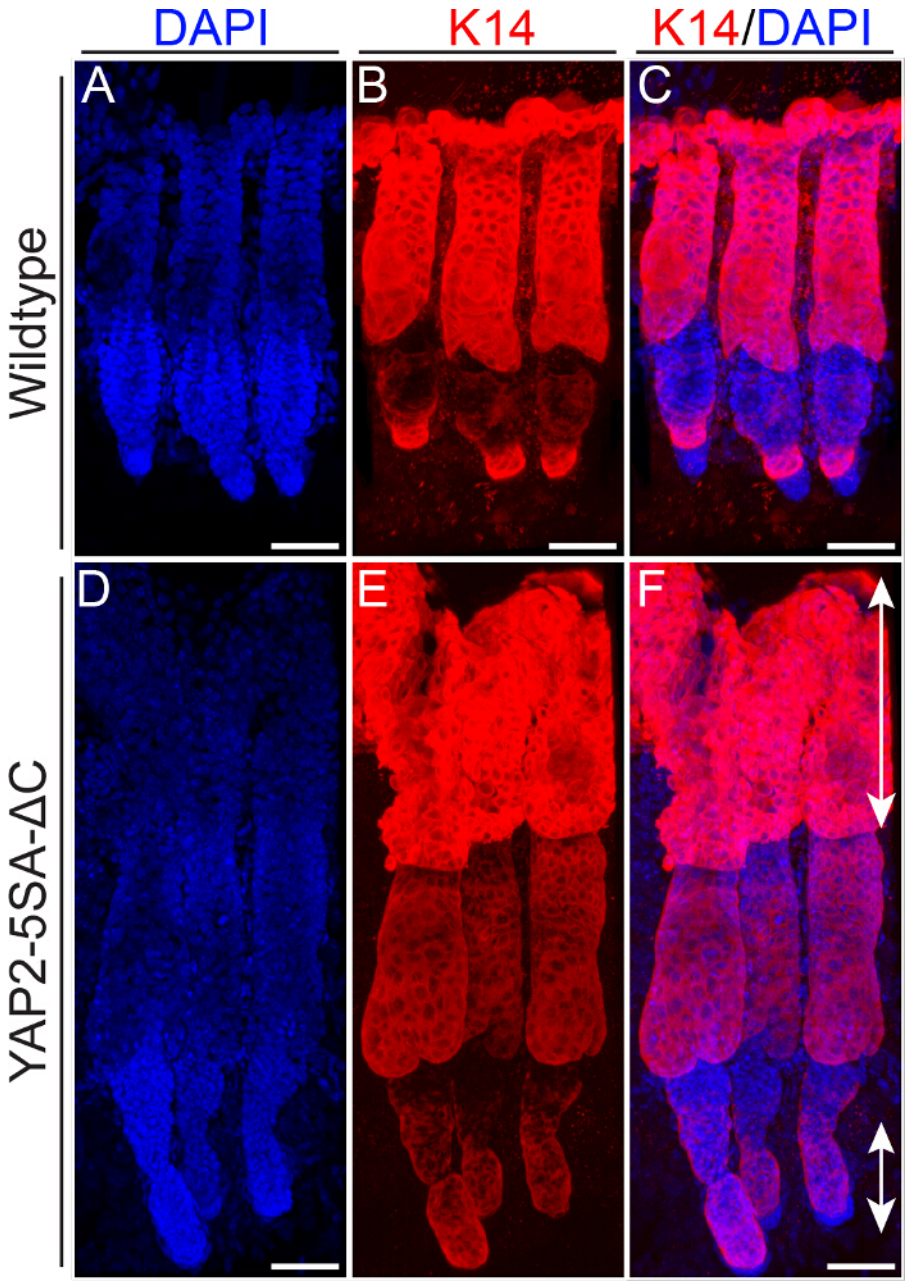
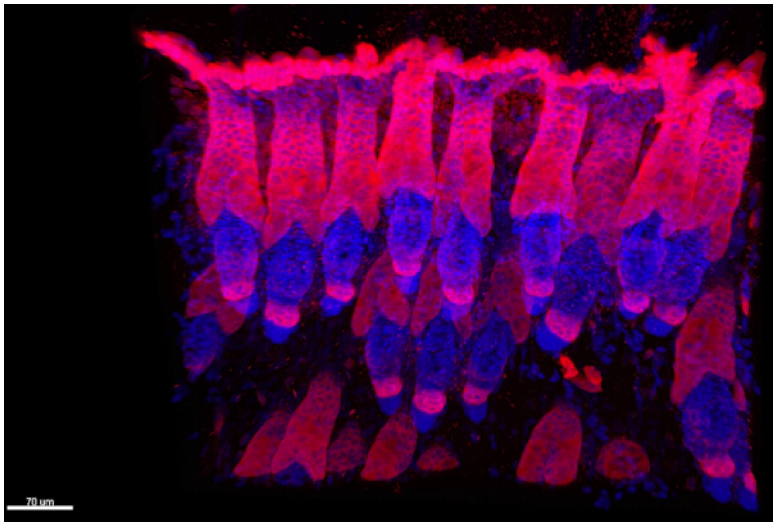
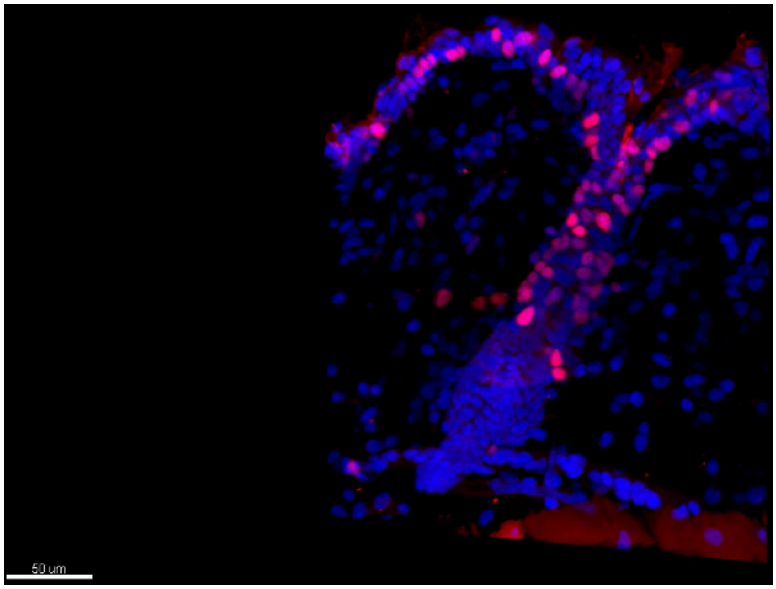


Figure 5: Morphological Abnormalities in the YAP2-5SA-ΔC Epidermis. 3D volume reconstructions of dorsal skin biopsies of adult wildtype (**A**-**C**) and YAP2-5SA-ΔC transgenic littermate mice (**D**-**F**) labeled with a K14 antibody (red in **B**, **C**, **E**, **F**), and DAPI nuclear counterstain (blue in **A**, **C**, **D**, **F**). The epidermal hyperplasia of the YAP2-5SA-ΔC epidermis is apparent in the interfollicular epidermis (top double arrows in **F**) and in the hair follicles, which display the characteristic stem/progenitor cell masses proximally (lower double arrows in **F**)[8]. Scale bars 50 µm. Please click here to view a larger version of this figure.



Movie 1. Please click here to download this movie.



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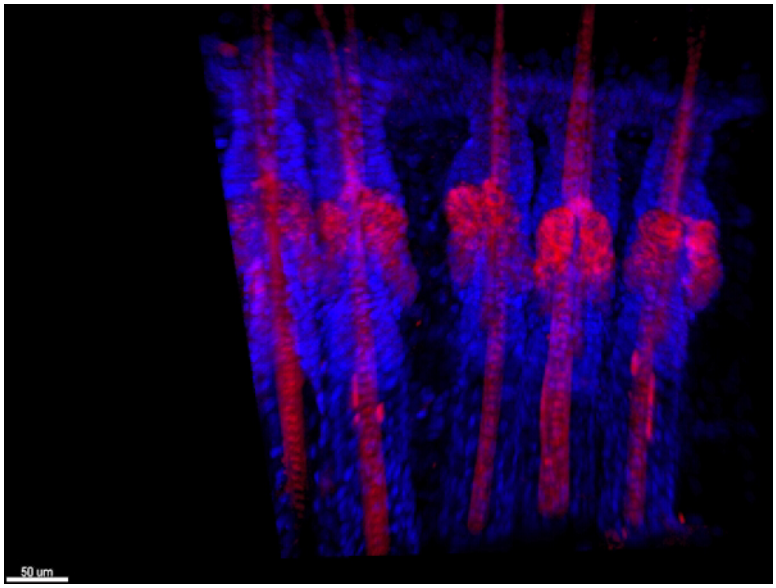

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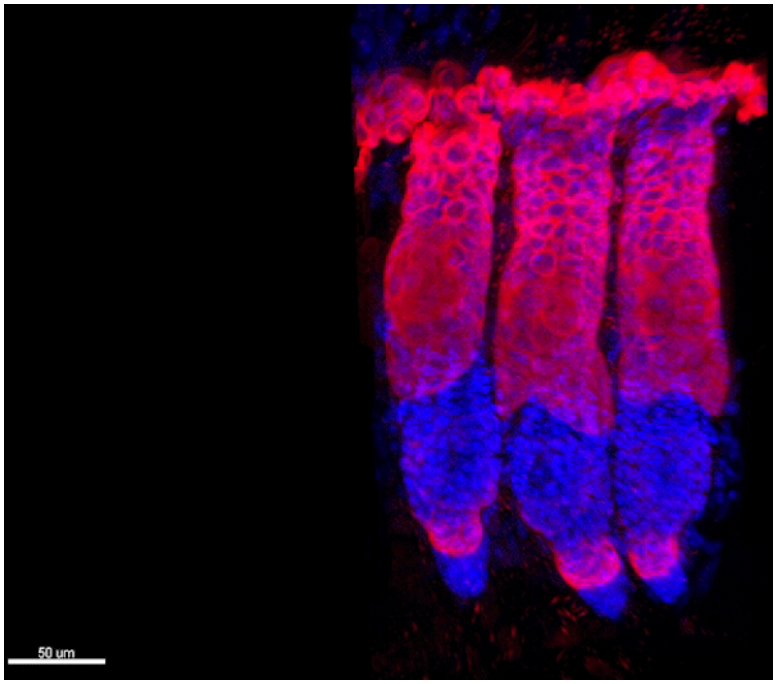
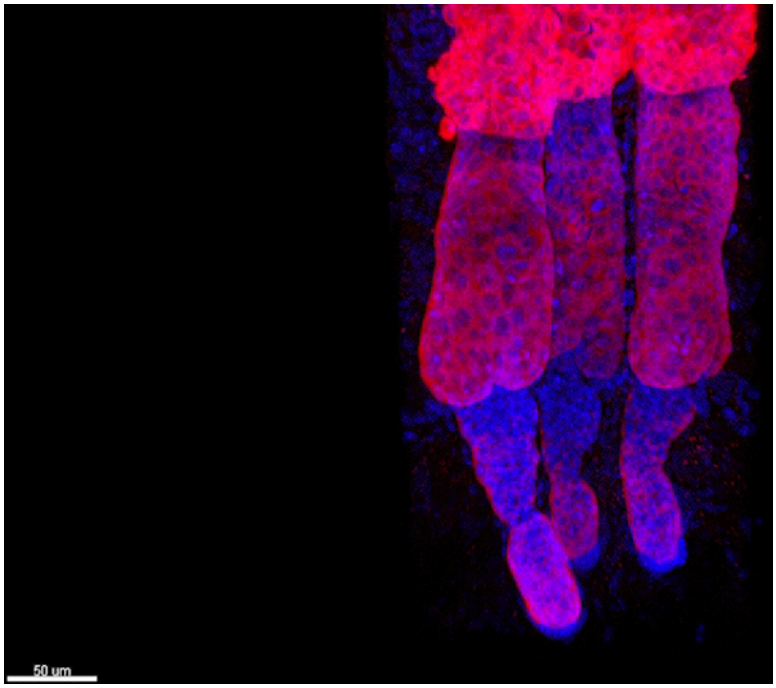


Movie 4. Please click here to download this movie.



Movie 5. Please click here to download this movie.

Discussion

The regulatory mechanisms controlling skin development and homeostasis are most commonly studied in 2D using tissue sectioning and histological staining or labeling with antibodies, which enables only a restricted appreciation of skin morphology, cell populations or protein expression. A number of methods have been developed to improve visualization of the spatial organization of cells and proteins at single cell resolution in 3 dimensions in epidermal whole mounts[10-13]. Some of these however involve separation of the epidermis from the dermis, which is technically challenging especially using hairy skin, and often results in tissue damage and rupture of the hair follicles. Also, separation of epidermis and dermis impairs studying the cellular and molecular interactions that occur between them during embryonic development and tissue homeostasis.

The 'flat mount approach' is another method where full thickness mouse skin is dissected and immunostained, and then clarified using benzyl benzoate and benzyl alcohol (BBBA) while preserving immunofluorescent signals[14]. This method does not require the technically challenging separation of the epidermis from the dermis. However, BBBA is highly toxic, it denatures some fluorescent proteins, and it contaminates microscope objectives, not allowing for the high-resolution imaging of the samples required to investigate cellular and molecular interactions in the tissue.

This report describes a CUBIC protocol[5-7] to clarify full thickness mouse skin biopsies from any desired anatomical region and age using relatively safe and cheap reagents. This technically simple protocol allows visualization of protein expression and proliferation patterns by immunofluorescence in full thickness skin biopsies counterstained with DAPI in three dimensions at single cell resolution, enabling unprecedented and highly accurate qualitative assessment and quantification of protein expression, proliferation, tissue morphology and hair follicle dimensions. This method is also suitable for visualization of sebocytes using Nile Red staining despite the removal of tissue lipids during the clarification procedure. An important limitation of this method is the availability of antibodies efficiently binding and allowing visualization of their respective target proteins using this skin clarification process. In addition, the generated image and movies files are large, and good IT resources such as computers with powerful processors and ample data storage space are essential.

A critical step in the procedure is the preparation of the skin biopsies. To visualize intact hair follicle anatomy, the orientation of the skin biopsy should be taken into account, and the length of the biopsies should be cut parallel to the direction of the length of the hair follicles. In addition, small and very thin skin biopsies are difficult to mount correctly for microscopy. Larger biopsies will require more time to clear, and may incur antibody penetration problems resulting in antigen detection artefacts. It is therefore advisable to cut, clear and stain multiple biopsies per experiment.

It is also important to tightly seal the tubes while preparing the CUBIC solutions to prevent evaporation of the contents during the heating process, which results in changes in reagent concentrations and may cause impaired tissue clearing. The next important step is that the tissue will be clarified at 37 °C to accelerate the clearing process. After 7 days, the CUBIC1 solution will need to be prepared fresh to replace the old one.

In the future, more antibodies will be identified that are compatible with the CUBIC skin clarification procedure, and analyses may be extended to visualizing and quantification of epidermal and melanocyte (stem) cell markers and dermal proteins. This technique will also play a fundamental role in 3D studies of skin pathology and anatomy, and aid in elucidation of cellular and molecular mechanisms underlying abnormal and normal

epidermal biology in skin of mutant mice and transgenic reporter mouse models, including signaling interactions between the dermis and the epidermis.

The CUBIC protocol[5-7] is likely to be applicable to skin of other vertebrate animal models, and to human skin punch biopsies. Light sheet microscopy will enable visualization of epidermal anatomy, protein expression patterns, and progression of the hair follicle cycle stages in larger skin samples.

Disclosures

The authors have nothing to disclose.

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