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Protocol

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Reproducible strategy for excisional skin-wound-healing studies in mice

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Supplementary Information – *Nature Protocols*

Supplementary protocol 1: Tail-base wound using biopsy puncher.

Timing: 30 minutes.

The following supplementary protocol aims to provide a brief description of performing tail-based wounds. For follow-up procedures, recovery, measurements, and additional steps, please refer to the main protocol.

Reagents:

- Buprenorphine (0.3 mg/ml, Richter Pharma).
- Isoflurane (Piramal).

Equipment:

- Alcohol prep pads (Covidien, cat. no. 6818-1).
- Spring surgical scissors: small, slightly curved, with a fine diameter tip (F.S.T, cat. no. 15023-10 or cat. no. 15011-12).
- Forceps: Curved (F.S.T, cat. no. 91197-00)
- 3-mm biopsy puncher with a plunger (Robbins Instruments, cat. no. RBP-30P)

Reagent setup:

- Please refer to the main manuscript.

1| Perform steps 11-16 as in the main protocol (setting up the recovery cages).

2| Perform steps 28-29 as in the main protocol (disinfecting the working area, preparation of tools, and analgesic treatment). Disinfect and have ready the 3-mm biopsy puncher.

3| Wipe the tail with an alcohol prep pad.

4| On the dorsal side of the tail, mark a dot at a distance of 1 cm from the tail base.

5| Center the 3-mm biopsy puncher with the mark and apply gentle pressure toward the skin while rotating the puncher clockwise and counterclockwise alternately.

6| Once you have penetrated the skin, remove the biopsy puncher with the excised piece of skin. If the skin to be excised is still loosely attached to the subcutaneous tissue, grasp it gently with forceps and use surgical scissors to remove the connective tissue remnants.

CRITICAL STEP: Bleeding of tail-base punching excision is more common than back-skin wounds. If needed, apply pressure with a sterile gauze to stop the bleeding.

7| Continue with day 0 wound size measurement, animals recovery, and follow-up as specified in the main protocol, step 36.

Supplementary protocol 2: Caudal-lateral wounds using a biopsy puncher.

Timing: 30 minutes.

The following supplementary protocol aims to provide a brief description of performing two caudal wounds at the lateral sides of the animal using a punch biopsy tool. For the follow-up procedure, recovery, measurements, and additional steps, please refer to the main protocol.

Reagents:

- Buprenorphine (0.3 mg/ml, Richter Pharma).
- Isoflurane (Piramal).

Equipment:

- Electric shaver: off-the-shelf, capable of trimming to less than 1 mm long.
- Alcohol prep pads (Covidien, cat. no. 6818-1).
- Spring surgical scissors: small, slightly curved, with a fine diameter tip (F.S.T, cat. no. 15023-10 or cat. no. 15011-12).
- Forceps: Curved (F.S.T, cat. no. 91197-00)
- 3-mm biopsy puncher with a plunger (Robbins Instruments, cat. no. RBP-30P)

Reagent setup:

- Please refer to the main manuscript.

1| Perform steps 1-16 as in the main protocol (hair removal and setting up the recovery cages). Remove the hair off the entire caudal portion of the dorsal skin.

2| Wipe the bare caudal skin gently with an alcohol pad.

3| While the mouse is lying with the dorsal side facing up, draw a fine line along the midline backbone. It is important to be precise when defining the midline, as this line will later be used to ascertain the location of the wounds.

4| Place the mouse on its side. Pull from the midline line to stretch the skin in a manner that results in symmetrical skin regions on both sides of the centered midline (one facing up and one down) (Supplementary Fig. 2A)

CRITICAL STEP: the stretched skin should be symmetrical to ensure the proper location of the wounds.

5| Apply pressure with the biopsy puncher on the stretched area. The punch biopsy tool should penetrate the skin from one side and continue to the lateral side. Rotate the punch biopsy clockwise and counterclockwise alternately while pressing (Supplementary Fig. 2B).

6| Once you have penetrated both sides of the skin (Supplementary Fig. 2C), remove the biopsy puncher with the excised skin pieces. The wounds should be symmetrically positioned at an equal distance from the midline (Main article, Fig. 1B middle panel)

7| Continue with day 0 wound size measurement, animal recovery, and follow-up as in the main protocol, step 36.

Supplementary protocol 3: Immunofluorescent staining of whole-mount epidermal tissue.

Timing: 5 hours.

Starting point: fixed samples, washed 3 times with PBS. **Step 74 in the main protocol**.

Reagent:

- Normal goat serum (NGS) (Abcam, cat. no. ab7481).
- Triton-X100 (Sigma, cat. no. T8787).
- Tween-20 (Fisher Scientific, cat. no. BP337).
- Primary antibody (1st Ab) of interest.
- Secondary antibody: anti the 1st Ab's host, conjugated to the desired fluorophore.
- PBS (See instructions on preparation in the main text).
- Bovine Serum Albumin Fraction V (BSA) (Merck, cat. no. 10735078001).

Equipment:

- Conical tube: 15 ml (Greiner bio-one, cat. no. 188261).
- Round bottom, 2 ml tube (Eppendorf, cat. no. 0030120094).
- Student Splinter Forceps (F.S.T, cat. no. 91156-11).
- Rotating mixer (ELMI, Intelli-Mixer™ RM-2L)
- Kimwipe (Kimberly-Clark, cat. no. 34120).
- Aluminum Foil (off-the-shelf product).

Reagent setup:

- **BSA, 10% (wt/vol).** In a 15 ml tube, add 0.5 gr of BSA to 5 ml of PBS. Allow particles to dissolve at 4 °C overnight or at very slow rotation for 30' at 4 °C. Avoid bubble formation in the solution. The 10% BSA stock can be stored at 4 °C for 3 days.
- **Triton-X100 stock solution, 10% (vol/vol).** In a 15 ml tube, add 1 ml of Triton-X100 to 9 ml of PBS. Rotate at RT overnight. Ttiton-X100 10% solution can be stored at 4 °C for 1 year.

  CAUTION: Tritox-X100 is a detergent. Wear protective equipment.

- **PBST 0.3% Triton-X100 (vol/vol).** In a 15 ml tube, add 0.3 ml of 10% Triton-X100 to 9.7 ml PBS. PBST (0.3% Ttiton-X100) solution can be stored at 4 °C for 1 year.

Blocking and Permeabilization:

1| A **blocking solution** is produced by combining the following reagents in a 15 ml tube. After the addition of all reagents, mix by inverting the tube several times:

Reagent	Stock percentage	Final percentage (vol/vol)	5 ml volume
PBS	1X	-	4.25 ml
NGS	100%	5%	0.25 ml
Triton-X100	10%	1%	0.5 ml

Calculate the required volume as 1 ml per sample (minimum 0.5 ml per sample). Scale up if necessary.

2| Fill 2 ml tubes (with round bottom) with 1 ml of **blocking solution** and gently place each tissue in a designated tube. Shake gently for 30' at RT (60°, 30 rpm if using a rotating device).

Primary Antibody Reaction:

3| **Antibody dilution buffer** solution is produced by combining the following reagents in a 15 ml tube. After the addition of all reagents, mix by inverting the tube several times:

Reagent	Stock percentage	Final percentage (vol/vol)	5 ml volume
PBS	1X	-	4.25 ml
BSA	10%	1%	0.5 ml
Triton-X100	10%	0.5%	0.25 ml

Calculate the required volume (such as 1 ml per sample). The exact volume should be defined based on the desired dilution of the antibodies (e.g., a 1:100 dilution indicates 10 μl of antibody per 990 μl of dilution solution). The volume per staining tube (sample) can be reduced to 200 μl per sample if the samples are completely covered by the liquid (see step 5).

4| In a new 15 ml tube (or 2 ml tube, if possible, for low volumes), prepare the primary antibody mix by diluting primary antibodies in the **antibody dilution buffer**. For antibodies used for the first time, a dilution of 1:100 to 1:500 is recommended.

CRITICAL STEP: When working with more than one antibody, it is important to use primary antibodies generated in different host species.

5| Split the mix into 2 ml tubes (with a round bottom); one tube per sample.

6| Gently lift the sample from the blocking solution, blot once on Kimwipe paper, and place a tube containing the desired antibody mix. Shake gently for 1 hour at RT (60°, 30 rpm if using a rotating mixer). Alternatively, the incubation can be performed overnight at 4° C.

7| Fill new 2 ml tubes with PBST (0.1% Tween-20), one per sample. Place the samples in the PBST for washing (30 rpm, 60°, 15' at RT if using a rotating mixer). Repeat twice.

CRITICAL STEP: PBST can be replaced between washes by pipetting. However, it is not recommended that inexperienced researchers perform this technique as the samples can accidentally adhere to the pipette and tear.

Secondary Antibody Reaction:

8| Prepare the **secondary antibody mix** solution by diluting fluorophore-conjugated antibodies in PBST (0.3% Triton-X100) according to the product datasheet (for untested antibodies, 1:250 dilution is recommended). Calculate the required volume as 1 ml per sample. Scale down to 200 µl per sample as necessary, as long as the sample is completely covered with liquid.

CRITICAL STEP: Each secondary antibody added to the mix should recognize the host species of a single primary antibody. Avoid spectral overlap of the fluorophores, utilizing a spectral viewer (for example, ThermoFisher online 'Fluorescence-SpectraViewer': https://www.thermofisher.com/order/fluorescence-spectraviewer#!/).

9| Split the secondary antibody mix into 2 ml tubes (with round bottom), one tube per sample.

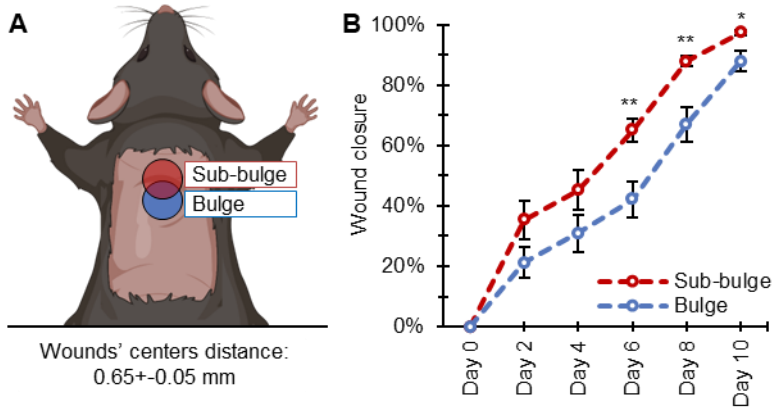
10| Gently lift the sample from the PBST (0.1% Tween-20) wash, blot once on a Kimwipe paper, and place the tissue in a tube containing the proper antibody mix. Shake for 1 hour at RT (rotating mixer, mode: 30 rpm, 60° if using a rotating mixer). Alternatively, incubation can be performed overnight at 4°C. Place an aluminum foil around the tubes in order to prevent photo-damage.

11| Perform DAPI staining, PBST (0.1% Tween-20) washes, and mounting at **step 75 of the main protocol** (Of note, keep working in 2 ml tubes instead of 35 mm dish as detailed in the main protocol).

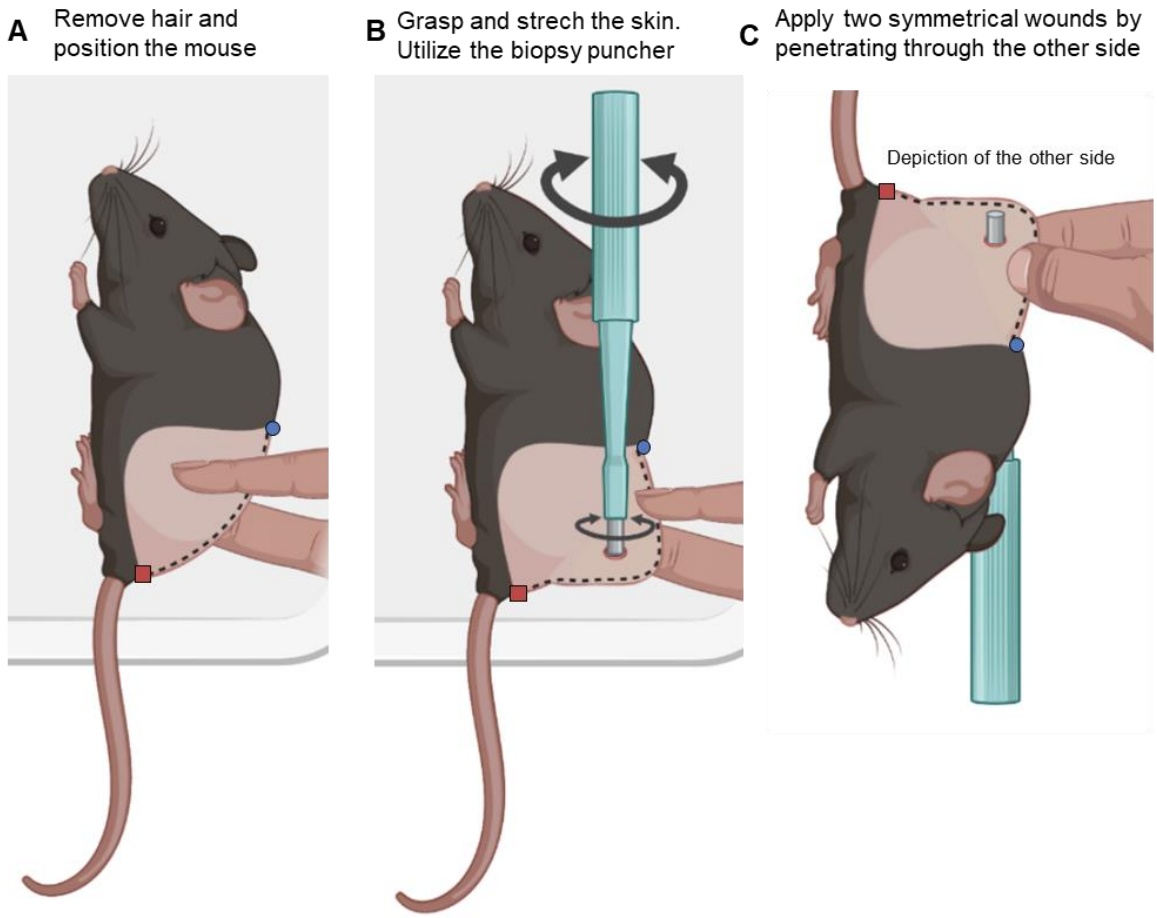
Supplementary Discussion: Considerations when performing fluorescence-based visualization of skin-whole mount using confocal microscopes.

In order to achieve high-resolution multi-color imaging of the entire scar area with magnification levels of 10x or greater, it is typically necessary to combine multiple "small" images stitched together (tiles-stitching). Additionally, to obtain a 3D z-stack image, the process can require a lengthy acquisition time. For instance, obtaining data from an entire scab region at 20x magnification may necessitate the collection of data from 5x5 to 9x9 tiles, with a 50 μm thickness and a z-stack interval of 5 μm (approximately the size of a single cell) requiring the acquisition of 891 images (9x9x11) for a single channel. As such, the duration of the imaging process can pose a limiting factor.

For this type of experiments, we recommend the use of a spinning disc confocal (SDC) system, which allows for faster acquisition times while reducing the potential for photo-damage to the tissue. Our experience with in-house microscopes has shown that the acquisition of large, stitched images with SDC (Eclipse Ti2, Nikon & CSU-W1, Yokogawa) is between 10 and 50 times faster than with laser-scanning confocal (LSM-880, Zeiss). However, for individual images, the optimal instrument will depend on the versatility of the optical properties required for the desired fluorophores.



Supplementary Figure 1: The precise location of the wound affects the wound healing process. A) The bulge area on the mouse's back skin can be determined and wounded. Misplacing the exact area may lead to a nearly indistinguishable wounding in the sub-bulge region. B) Wound closure analysis of mice wounded at the bulge (n=9) or sub-bulge region (n=7) reveals differences in wound closure kinetics.



Supplementary Figure 2: Utilizing caudal-lateral wounds using a biopsy puncher.

A) An anesthetized mouse is placed on its side. The midline (backbone line) is clearly defined and marked. **B)** Gently stretch the skin in a manner that the midline is aligned to the edge of the fold. Press a biopsy puncher tool through the stretched area while rotating the puncher clockwise and counterclockwise. **C)** An image depicting the penetration of the biopsy puncher tool through the two sides of the folded skin. Blue circles and red squares serve for lateral orientation between the panels.