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Alicia DSouza
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Iowa City, July 8th, 2020

Dear Dr. DSouza,

Please find enclosed our revised manuscript JoVE61616 by Rhea and Dunnwald entitled "*Protocol for a Murine Excisional Wound Healing Model and Histological Morphometric Wound Analysis*".

We thank the editor and the reviewers for their relevant comments. We have answered all of them and provide a detailed, point-by-point answer to each of the comments in the next pages. As instructed, we tracked our changes, which are indicated by the blue font in the revised document. For clarity, however, we did not indicate formatting changes in the protocol and minor wording alterations.

Thank you for your consideration and I look forward hearing from you about our manuscript soon.

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TITLE:

Murine Excisional Wound Healing Model and Histological Morphometric Wound Analysis

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KEYWORDS:

wound healing, excisional wound, histology, morphometry, skin, mouse, surgery, tissue repair

SUMMARY:

This protocol describes how to generate bilateral, full-thickness excisional wounds in mice and how to subsequently monitor, harvest, and prepare the wounds for morphometric analysis. Included is an in-depth description of how to use serial histological sections to define, precisely quantify and detect morphometric defects.

ABSTRACT:

The murine excisional wound model has been used extensively to study each of the sequentially overlapping phases of wound healing: inflammation, proliferation and remodeling. Murine wounds have a histologically well-defined and easily recognizable wound bed over which these different phases of the healing process are measurable. Within the field, it is common to use an arbitrarily defined “middle” of the wound for histological analyses. However, wounds are a three-dimensional entity and often not histologically symmetrical, supporting the need for a well-defined and robust method of quantification to detect morphometric defects with a small effect size. In this protocol, we describe the procedure for creating bilateral, full-thickness excisional wounds in mice as well as a detailed instruction on how to measure morphometric parameters using an image processing program on select serial sections. The two-dimension measurements of wound length, epidermal length, epidermal area, and wound area are used in combination with the known distance between sections to extrapolate the three-dimension epidermal area covering the wound, overall wound area, epidermal volume and wound volume. Although this detailed histological analysis is more time and resource consuming than conventional analyses, its rigor increases the likelihood of detecting novel phenotypes in an inherently complex wound healing process.

INTRODUCTION:

Cutaneous wound healing is a complex biological process with sequentially overlapping phases. It requires the coordination of cellular and molecular processes that are temporally and spatially

regulated in order to restore the barrier function of the damaged epithelium. In the first phase, inflammation, neutrophils and macrophages migrate into the wound, mobilizing local and systemic defenses[1]. Following and overlapping the inflammatory phase is the proliferation stage. Fibroblasts begin rapidly proliferating and migrating into the granulation tissue. Keratinocytes away from the leading edge directionally proliferate towards the wound as differentiated keratinocytes in the leading edge migrate to re-epithelialize the wound[2]. Finally, the remodeling and maturation phase begins, during which fibroblasts in the granulation tissue start to synthesize and deposit collagen. The remodeling and organization of the new matrix can last up to 1 year following injury[3]. Due to the complexity of overlapping events involving cross-talk between multiple cell types, and despite years of research, many of the cellular and molecular mechanisms underlying wound healing remain poorly understood.

The mouse model is the predominant mammalian model for investigating mechanisms of wound healing due to their ease of use, relatively low cost and genetic manipulability[1,4,5]. Although different types of wounds have been described in the murine model, the most common is an excisional wound (either bilateral punch or direct punch biopsy), followed by incisional wound models[4]. The excisional wound model has a distinct advantage over the incisional model as it inherently generates control tissue that has not undergone the healing process. The punch biopsy tissue that is excised as part of the surgical protocol can be processed in the same manner as the wounded tissue and used to establish the homeostatic conditions for a desired criterion. Excised control tissue may also be useful if assessing the effects of a skin pretreatment or confirming successful gene alteration at the time of injury[4].

Healing parameters can be assessed by many different techniques, including planimetry or histology. However, planimetry can only evaluate visible characteristics of the wound, and due to the presence of a scab, often does not correlate to measurements of healing that are visualized by histology, thereby making histology the "gold standard" of analysis[4]. Despite histological analysis being the gold standard, it is most often performed on an arbitrary subset of the wound[6,7]. For instance, cutting the wound in "half" prior to embedding and sectioning the wound is currently common practice to reduce the time and resources spent on sectioning materials and data analysis. The method of morphometric analysis described in this protocol was developed to encompass the entire wound tissue, to accurately reflect the morphological characteristics of the wound, and to increase the likelihood of detecting wound healing defects with a small effect size. In this protocol, we detail a surgical method for generating the most commonly studied murine wound, the bilateral full-thickness excisional wound, as well as a detailed and rigorous method for histological analysis such is rarely used in the field.

PROTOCOL:

All experiments were completed in accordance and compliance with federal regulations and University of Iowa policy and procedures have been approved by the University of Iowa IACUC.

1. Animals and husbandry

1.1. Use adult mice of the desired mouse line at 8-10 weeks of age when the hair follicle stage is

in telogen.

1.2. On the day of surgery, separate mice into clean cages and individually house to minimize wound disruption.

2. Surgery

NOTE: It is unnecessary to maintain sterile surgical conditions. While care should be taken to maintain sterility between animals, the punch biopsy itself is done on a clean, but nonsterile surface. The surgery duration per animal is between 10 and 15 min.

2.1. Anesthetization

2.1.1. Anesthetize the animal for 1-2 min in an induction chamber with the isoflurane vaporizer set to a 4-5% flow rate and the oxygen flow meter set at 1 liter per minute. See **Discussion** for alternative anesthesia options.

2.1.2. Confirm proper anesthetization before beginning the procedure. The depth of anesthesia can be confirmed by a firm toe pinch.

2.1.3. Transfer the mouse from the induction container to a nose cone and reduce the isoflurane flow rate to 1.5% and the oxygen flow meter to 0.5 L/min.

2.1.4. Apply ophthalmic ointment to both eyes as the procedure exceeds 5 min.

2.1.5. Maintain normal body temperature using a thermal pad.

2.2. Preparation of the wound site

2.2.1. Use an electric razor clipper in a rostral caudal motion to remove the fur on the back of the mouse at the shoulder level. Remove hair lower on the back as needed if performing more than two wounds.

2.2.2. Remove the remaining hair by using a razor blade in a caudal rostral motion held at 20˚ from the back of the mouse to closely shave the clipped area (**Figure 1A**).

2.2.3. Clean the shaved area with a povidone-iodine swab.

2.2.4. Wipe the skin with a sterile 70% isopropyl alcohol prep pad to reduce potential cutaneous irritation from the iodine swab.

2.3. Wounding

2.3.1. Pinch the skin between the shoulder blades along the dorsal midline and pull the

sandwiched skinfold away from the body (**Figure 1B**).

2.3.2. Position the mouse on its side with the skinfold on a flat surface draped with a clean paper-based towel or equivalent. Use a sheet of dental wax underneath the towel to protect the underlying surface from damage (**Figure 1C**).

2.3.3. Place the biopsy punch of desired size as close to the body as possible and allow the skin to relax. Do not stretch the skin, or the wound size will be larger than the designated punch size (**Figure 1D**).

2.3.4. Punch the skin by pressing down, a rocking motion may be used to ensure all layers of the skin on both sides have been penetrated (**Figure 1E**). Use a new biopsy punch for each animal.

2.3.5. Remove the punch biopsies from the wounds (**Figure 1F**). If there are still sites of attachment use sterile scissors and tweezers to free the punch from the surrounding skin. Process the punch biopsy control tissue as required based on downstream plans for wound healing analyses (see **Discussion** for suggestions).

2.3.6. Take macroscopic photographs from an equal distance to the wound sites or with a ruler in the frame in order to measure the initial wound area and eliminate outliers from analysis.

2.3.7. Administer analgesia for a minimum of 24 h in accordance with an approved animal protocol.

2.3.8. Monitor the mouse as it comes out of anesthesia until it maintains an upright posture and is walking normally around the cage.

3. Post wound monitoring

3.1. Monitor mice daily for experimental endpoints as determined by the investigator and in accordance and compliance with institutional protocols. Examples include: infection, visible weight loss, or a hunched posture.

3.2. Take daily macroscopic photographs in a controlled manner as was done after the initial surgery.

4. Harvesting wounds

4.1. Euthanize mice at the desired time point post-wounding in accordance with an approved animal protocol.

4.2. Take macroscopic photographs of the wound sites in a controlled manner consistent with previous photograph acquisition (**Figure 2A,C**).

4.3. Cut a wide rectangle around the wound sites using a scalpel (**Figure 2D,E**).

4.4. Free the rectangular piece of tissue using scissors and tweezers to peel back and cut the skin away from the underlying tissue and place in a Petri dish (**Figure 2F,G**).

4.5. Harvest the wounds, trim down to 2 mm of unwounded tissue surrounding all sides of the wound (**Figure 2H,I**). See **Discussion** for alternative options to harvest the wound.

4.6. Process the wounds as required for subsequent studies. Reserve at least one wound per mouse for paraffin embedding and histological analysis.

5. Wound fixation and embedding

5.1. Fix the wound

5.1.1. Fix the wound tissue in a freshly prepared 4% paraformaldehyde solution[8] for 3 h at room temperature then transfer to 4 ˚C overnight. Electron microscopy (EM) grade paraformaldehyde and solution filtration is not required.

5.1.2. Wash the wounds twice for 30 min in 1x PBS.

5.1.3. Replace PBS with 70% ETOH and store at 4 ˚C until embedding. Process tissues within 24-48 h to avoid antigen loss or within 1-2 weeks if only evaluating histological characteristics.

5.2. Process and embed the wound

5.2.1. Transfer each wound to an embedding cassette. Label embedding cassettes in pencil as the process will remove inks.

5.2.2. Process the tissue either manually or using an automated processor by dehydrating the tissue with increasing ethanol percentages, clearing with xylene, and then infiltrating the tissue with paraffin wax (**Table 1**).

5.2.3. Embed the wound 90˚ (“standing”) from the horizontal surface of the embedding mold (**Figure 3A,B**).

6. Day 0 wound area analysis

6.1. Download NIH-Image J or NIH-Fiji free software (https://imagej.net/Fiji/Downloads).

6.2. Open a file with a photograph of day 0 wounds.

6.3. Check the box for “**Area**” under **Analyze | Set Measurements**.

6.4. Select "**Set Scale**" under **Analyze**. Enter the distance in pixels, the known corresponding distance and the unit of the distance (= unit of length) if macroscopic measurements are part of the study or skip this step if only relative measurements are required.

6.5. Select "**Freehand selections**" on the Fiji toolbar.

6.6. Outline the perimeter of the wound.

6.7. Click **Measure** under **Analyze**.

6.8. Create a spreadsheet to keep track of the measurements per animal per wound.

6.9. Copy the measurement of the wound area in the spreadsheet.

6.10. Calculate the mean area and the standard deviation of all wounds for a given experiment.

6.11. Exclude any wounds outside two standard deviations of the mean from histological analysis.

7. Serial sectioning

7.1. Chill the paraffin-embedded wound blocks at 4 ˚C overnight.

7.2. Insert the paraffin block on the block holder of the microtome and orient so the blade will cut straight across the block. Orient the block such that the tissue "stands" at 90˚ allowing the simultaneous sectioning of the epidermis and dermis (**Figure 3C,D**).

7.3. Make 2-4 ribbons of 20-30 paraffin sections of 7 µm each.

7.4. Use a dry paint brush and a dissection teasing needle to transfer each ribbon to a firm yet manipulatable surface such as a firm black plastic sheet.

7.5. Detach the top section of each ribbon with a razor blade and place on a microscope slide.

7.6. Observe the unstained sections under a brightfield microscope to determine which ones contain wounded tissue, which can be identified by absence of hair follicle, changes in the appearance of the connective tissue or the epidermis, and/or the presence of a scab (**Figure 4A,B**).

7.7. Discard unwounded sections up to 20 sections before the beginning of the wound.

7.8. Section through the wound by repeating steps 7.3 and 7.4 until no wound is detected in unstained sections.

8. Mounting of paraffin sections

8.1. Separate paraffin sections every 5 sections with a razor blade, starting with the first ribbon (**Figure 3E**).

8.2. Label microscope slides with both the slide number and all the section numbers.

8.3. Grab the group of 5 sections with a wet paint brush and float them on the surface of the water of a warm water bath (40-45 ˚C) to flatten them out.

8.4. Pick the group of 5 sections out of the water bath using one of the labelled microscope slides (**Figure 3F**) and place on a slide warmer set at 37 ˚C for up to 24 h.

8.5. Store the slides upright in a slide box.

9. Histological staining

9.1. Transfer every 8th microscope slide (equivalent to every 40th paraffin section) to a staining rack and stain with hematoxylin and eosin.

10. Microscopic imaging

10.1. Acquire images using a bright field microscope equipped with a 4x objective and digital acquisition capabilities. Record the scale at which the image is taken.

10.2. Image the entire wound of the top section of each stained slide and make sure to include some unwounded tissue on either side. Take multiple overlapping pictures if the wound is larger than the frame of a single picture.

10.3. Save the file including the section number for morphometric analysis. Use the section number followed by a, b, c, etc. for overlapping pictures of the same wound.

11. Morphometric analysis

NOTE: When the wound spans multiple pictures, sum the measurements taken from the individual pictures to obtain **one value** per metric per wound section to record in the spreadsheet.

11.1. In Image J, open a digital file of a stained wound picture. Do not use stitched pictures for analysis. Perform measurements on zoomed-in overlapping pictures by finding landmarks to leave off and pick up measurements from picture to picture.

11.2. Set the scale and measurement preferences.

11.2.1. Select "**Set Scale**" under **Analyze**. Enter the distance in pixels, the known corresponding

distance and the unit of the distance (= unit of length). The scale should appear in the window and should correspond to the scale the image was acquired at.

11.2.2. Check the box "**Global**" to keep the scale the same for each open image.

11.2.3. Repeat steps 11.2.1 to 11.2.2 every time Image J is closed and reopened.

11.2.4. Check the box for "**Area**" under **Analyze | Set Measurements**.

11.3. Measure the wound length

11.3.1. Select "**Freehand selections**" on the Fiji toolbar.

11.3.2. Measure starting from the last hair follicle of the uninjured tissue on one side of the wound to the first hair follicle of the uninjured tissue on the other side of the wound (**Figure 4A,B**).

11.3.3. Trace along the dermo-epidermal junction to reach these two landmarks. If the epidermis does not cover the entire wound, follow the dermo-epidermal junction on one side of the wound and where the migrating tongue ends continue following the superior aspect of the granulation tissue or the junction between the granulation tissue and the scab until you reach the migrating tongue and then finally the first hair follicle of the uninjured tissue on the other side (**Figure 4E,F**).

11.3.4. Under **Analyze**, click **Measure**. The length of the measurement will appear in the same units as set in the scale.

11.3.5. Create a spreadsheet to keep track of the measurements (**Supplementary Table 1**).

11.3.6. Copy the wound length into the spreadsheet.

11.4. Measure the epidermal length

11.4.1. If the epidermis covers the entire wound, the epidermal length is the same as the wound length.

11.4.1.1. Copy the "wound length" measurement into the "epidermal length" column in the Excel spreadsheet and skip to step 11.5.

11.4.2. If the epidermis does not cover the entire wound, select the "**Freehand selections**" and measure the distance between each epidermal leading edge following the superior aspect of the granulation tissue or the junction between the granulation tissue and the scab to the first hair follicle (**Figure 4C,D**).

11.4.2.1. Under **Analyze**, click **Measure**.

11.4.2.2. Subtract this measurement from the wound length and record the number under "epidermal length" in the Excel spreadsheet.

11.5. Measure the wound area

11.5.1. Select "**Freehand selections**" on the Fiji toolbar.

11.5.2. Measure starting from the last hair follicle of the uninjured tissue on one side of the wound to the first hair follicle of the uninjured tissue on the other side of the wound (**Figure 4A,B, E,F**).

11.5.3. Trace along the superior aspect of the epidermis (do not include the scab) or the superior aspect of the granulation tissue if the wound is not fully covered by the epidermis.

11.5.4. Continue to trace vertically along the hair follicle into the granulation tissue once the opposite hair follicle is reached and until adipose tissue or muscle is reached. Follow the inferior border of the granulation tissue to the opposite side of the wound and join the starting point along the hair follicle to close the area (**Figure 4E,F**).

11.5.5. Under **Analyze**, click **Measure**.

11.5.6. Copy the wound area into the spreadsheet under "wound area measured."

11.6. Measure the epidermal area

11.6.1. Select "**Freehand selections**" on the Fiji toolbar.

11.6.2. If the wound is fully epithelialized:

11.6.2.1. Trace along the superior aspect of the epidermis until the opposite hair follicle is reached and complete the area by "returning" to the starting point following the dermo-epidermal junction between the epidermis and dermis (**Figure 4D**).

11.6.2.2. Under **Analyze**, click **Measure**.

11.6.2.3. Copy the epidermal area into the Excel spreadsheet under "epidermal area measured" and skip to step 11.7.

11.6.3. If the wound is not fully epithelialized:

11.6.3.1. Trace along the superior aspect of the epidermis until the leading edge and return to the starting point following the dermo-epidermal junction (**Figure 4C**).

11.6.3.2. Under **Analyze**, click **Measure**.

11.6.3.3. Repeat step 11.6.3.1 and 11.6.3.2 on the opposite side of the wound.

11.6.3.4. Under Analyze, click Measure.

11.6.3.5. Sum the two numbers obtained in steps 11.6.3.2 and 11.6.3.4 and enter the result under "epidermal area measured" in the spreadsheet.

11.7. Repeat steps 11.3 to 11.6 on every 40^{th} sections (every 8^{th} slide).

11.8. Calculate the epidermal area of the entire wound.

11.8.1. Create a new column in the spreadsheet "epidermal area calculated" next to "epidermal length."

11.8.2. Multiply the number for "epidermal length" by 280 for each section except the last one (7 µm thick section x 40 sections).

11.8.3. Multiply the number for "epidermal length" by 7 for the last section (thickness of the section).

11.8.4. Sum the value of the "epidermal area calculated" for each section to obtain the epidermal area of the entire wound.

11.9. Calculate the wound area of the entire wound.

11.9.1. Repeat steps 11.8.1 to 11.8.4 using the wound length measurements.

11.10. Calculate the epidermal volume of the entire wound.

11.10.1. Repeat steps 11.8.1 to 11.8.4 using the "epidermal area measured" measurements.

11.11. Calculate the wound volume of the entire wound.

11.11.1. Repeat steps 11.8.1 to 11.8.4 using the "wound area measured" measurements.

11.12. Calculate the percentage of epidermal volume in the wound.

11.12.1. Divide the total epidermal volume by the total wound volume and multiply by 100 to obtain the percentage (do not do this ratio for each section).

11.13. Calculate the percentage of epidermal area among the wound area.

11.13.1. Divide the total epidermal area by the total wound area and multiply by 100 to obtain the percentage. If a wound is fully epithelialized, this number should be 100.

REPRESENTATIVE RESULTS:

Figure 5 depicts the range in measured and calculated values obtained by performing morphometric analysis on wild-type wounds generated in different mouse strains by multiple surgeons and analyzed by different individuals. Wild-type mice from different strains can display statistical differences as described both in our studies and in the literature[9,10]. Based on these representative results, we recommend that, within one study, mice from only one strain be used. Although we recommend that the same individual perform all the wounds within a particular study, multiple individuals could act as surgeons as long as the area of the wounds at day 0 is not statistically different between individual's work. Finally, because the morphometric analysis described in this protocol can be extensive, multiple individuals could analyze parts of the same experiment, but only if the results of their analysis of two samples are within 5% of each other. However, it is preferable to have a single individual analyzing the wounds in a blinded manner to avoid bias.

Figure 6 displays a meta-analysis comparing wound measurements obtained by following the protocol described in this study[11] with measurements obtained from the "middle" of the wound and 40 surrounding sections. In **Figure 6A**, the epidermal area and wound area were calculated from the measurement of the epidermal and wound lengths on wound sections followed by the calculation of the percentage of the epidermal area among wound area (sometimes referred to as "percentage of closure" or "percentage of epithelialization"). Similarly, in **Figure 6B**, the percentage of the epidermis in the wound was obtained as the ratio of the epidermal volume (calculated from the measured epidermal area) over the wound volume (calculated from the measured wound area). For both parameters, the analysis of the whole wound showed strong statistical differences between groups (up to $P < 0.001$ following One-way Anova). However, the significance was decreased (up to $P < 0.01$ following One-way Anova) when only 40 sections in the middle of the would were analyzed. These results demonstrate a decrease in the level of significance when only a subset of the wound is analyzed. These data suggest that defects with a small effect size will likely only be detected when performing morphometric analysis on the entire wound, and that more mild wound healing phenotypes will be missed from a "middle of the wound" type of analysis.

Common practice for analyzing in vivo wound healing involves measuring the area of the wound on histological sections chosen somewhere in the wound[12]. With that in mind, the average of the measured wound area from the serial sections of entire wounds was compared with the one obtained from the middle subset sections. The results showed no significant difference between experimental groups and between method of analysis (**Figure 6C).** However, the current protocol uses the measured area (shown in **Figure 6C**) over the whole wound to calculate the wound volume. As shown in **Figure 6D**, the calculated wound volume (which can only be calculated using the analysis of the whole wound) is significantly different between the experimental groups. In sum, these representative results demonstrate the importance of in-depth histological analysis of wound healing parameters described in this protocol in order to detect phenotypes that would

otherwise have been missed using a more traditional wound healing analysis.

FIGURE AND TABLE LEGENDS:

Figure 1: Bilateral excisional wound procedure. (**A**) Representative photograph of a mouse after clipping and shaving hair from the surgical area. (**B**) The skin was pinched between the shoulder blades along the dorsal midline. (**C**) The mouse was positioned on its side with the skinfold laid flat. (**D**) Representation of the biopsy punch placement. (**E**) Punched skinfold. (**F**) The mouse with two bilateral wounds at day 0 and the excised punch biopsy control tissues as indicated by the white arrows.

Figure 2: Wound harvest procedure. (**A-C**) Macroscopic photographs of 6 mm excisional wounds after 4 days (**A**), 7 days (**B**) and 11 days (**C**). (**D-E**) With a scalpel blade, a cutaneous incision was made in the shape of a wide rectangle that includes the wounds and surrounding unwounded tissue. (**F**) The skin was released from underlying tissue with forceps and scissors. (**G**) Representation of harvested bilateral wounds. (**H**) The dotted white line represents the border of the tissue that would be harvested following the use of a punch biopsy (this method allows for standardized amount of tissue harvested). (**I**) The dotted white line represents the border of the tissue that would be trimmed for histology (rectangular shape allows for easier embedding).

Table 1: Sample processing procedure for paraffin embedding. RT = room temperature. N/A = not applicable.

Figure 3: Wound embedding and sectioning. (**A**) Paraffin-processed wound lying flat in an embedding mold. (**B**) The wound was held at 90˚ ("standing") from the horizontal surface of the mold for embedding. (**C**) Paraffin-embedded wound properly oriented for sectioning (**D**) Paraffin block mounted on the microtome was sectioned into ribbons of about 20 sections (**E**) Paraffin ribbons cut in 5-section increments as indicated by the white brackets. (**F**) Serial sections laid flat in a warm water bath (40-45 ˚C) were mounted on a microscope slide. The cartoon in (**A-C**) represents the wound (orange) in the skin (blue) with the proper orientation of the epidermis (e) and the dermis (d) for each step.

Figure 4: Histological characteristics of wounds and illustration of morphometric parameters. (**A, B**) Histological features of a day 4 (**A**) and a day 7 (**B**) 6mm wound. (**C-F**) Representation of the different measurements used to perform the quantitative morphometric analysis: length of the epidermis (**C**, **D**, dotted black line), measured area of the epidermis (**C**, **D**, yellow shaded area), length of the wound (**E**, **F**, dotted yellow line) and measured area of the entire wound (**E**, **F**, gray shaded area). HF = hair follicle; scale bar = 1 mm.

Figure 5: Morphometric characteristics of representative 6 mm wild-type wounds at day 4, day 7 and day 11 post-wounding. Scattered plots represent data obtained from morphometric analyses of 6 mm wild-type wounds generated in different mouse strains by multiple surgeons and analyzed by different individuals. (**A**) wound volume, (**B**) epidermal volume, (**C**) percentage of epidermis in the wound, (**D**) wound area (calculated), (**E**) epidermal area (calculated), and (**F**)

percentage of epidermal area among wound area demonstrate the range of variation in morphometric values.

Figure 6: Meta-analysis comparing parameters obtained from whole wound with middle of the wound identifies new defects with higher significance. Wound healing morphometry was performed on day 7 wounds injected with saline (control), transforming growth factor beta 3 (Tgfb3), Tgfb3 with neutralizing antibody (Tgfb3 + NAB) and neutralizing antibody alone (NAB). Measurements were performed on serial sectioned wounds ("whole") or on 40 sections from the middle of the wound ("middle") and used to calculate the percentage of epidermal area over wound area (**A**), the percentage of epidermis in the wound (**B**), and the wound volume (**D**). (**C**) represents the average of the wound area measured on the whole or middle of the wound. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant, One-way Anova). This figure has been modified using data from Le et al.[11].

Supplementary Table 1: An example of a spreadsheet for recording morphometric measurements.

DISCUSSION:

The bilateral excisional wound model is a highly customizable procedure which can be used to study many different aspects of wound healing. Before beginning a wound healing project, investigators should perform a power analysis to determine the number of wounds needed to detect a defect of a particular effect size. Inconsistencies exist within the literature on whether individual mice or wounds should be used as biological replicates, however, a recent study showed that there is no significant correlation between two wounds on a single animal[4]. This suggests that wounds from a single animal are independent from each other and can be considered as biological replicates, reducing the number of mice required to detect a defect. This is relevant when considering small effect sizes for which a high number of wounds is required to reach significance[4]. In addition, the results of the power calculation may influence how many wounds are performed on each animal, with 2 and 4 wounds per animal being most common.

The surgical protocol itself is also highly flexible. While we recommend anesthetization by isoflurane vaporizer due to the short procedure length and quick recovery (10-15 min per mouse), investigators without access to a vaporizer can use injectable anesthesia including ketamine/xylazine or pentobarbital. Selection of an appropriate analgesic is particularly critical, as it pertains to the inflammatory phases of wound healing. The use of nonsteroidal anti-inflammatory drugs (NSAIDS) such as Flunixin meglumine or Meloxicam should be used with caution as they can decrease inflammation. Opioids are, therefore, preferred in studies where inflammation is being investigated. We recommend analgesics (e.g., Buprenorphine Sustained-Release) which provides up to 48 h of analgesia and eliminates the need for repeated, additional doses. All surgical procedures should be executed in accordance with federal guidelines and supported by an approved animal protocol.

Wound healing is a process that encompasses several phases, each of which being characterized by different biological processes involving distinct cell types[3]. The inflammatory phase occurs

between days 0 and 5, with the early migration of polymorphonuclear neutrophils (PMN) and macrophages to the site of injury[13]. The proliferative phase occurs between 3 and 14 days with re-epithelialization taking a varying amount of time based on the size of the wound[14]. In this protocol, we used a 6 mm biopsy punch and most wounds were re-epithelialized by 7 days. However, this time frame would need to be shortened if smaller punches were used (they are available as small as 1 mm). In combination with earlier time points, these smaller wounds may be preferable to reduce the amount of histological analyses[15]. Finally, the remodeling and maturation phases occur after 7 days and up to a year following the injury[16]. These later time points may be required to investigate the maturation of the wound or to investigate wound healing delays in experimental animals. Therefore, the investigator will need to determine the critical time points required to investigate particular phases of wound healing based on their particular hypothesis[5].

The analysis of wound healing is often not restricted to histological analyses. Unstained paraffin sections can be used for additional analyses such as immunofluorescence or Masson's trichrome for collagen deposition. The processing of control tissue (punch biopsies) and the harvesting steps of the tissue will all depend on how, in addition to histological analyses, wound healing is assessed. As part of the surgical protocol, punch biopsies are removed in order to generate the excisional wound. These punches can serve as unwounded control tissue for downstream applications such as protein (for western or cytokine profiling), DNA or RNA extraction and should be processed accordingly. It is recommended that at least one punch be saved for histological analysis, especially in cases where skin is treated prior to wounding (for example, application of tamoxifen for induction of Cre-Lox recombination[17]). Examination of the punch can determine the effect of the treatment on cutaneous morphology or simply allow the assessment of baseline cutaneous morphology of the particular mouse model being used. Wounds not used for histology can be harvested with a punch biopsy that encompasses the size of the wound. This procedure has a few advantages, including harvesting the same amount of tissue regardless of the wound size (larger wounds have less surrounding healthy tissue). Finally, we describe use of 4% paraformaldehyde as the fixative and paraffin as the material for preserving and embedding tissues, respectively. Other fixatives may be required for certain applications and can be substituted (for example, Carnoy's or Bouin's fixatives). For best immunofluorescent staining freezing and embedding of the wound in Optimal Cutting Temperature compound followed by frozen sectioning remains the method of choice.

Sectioning of wounded tissue can present many challenges, particularly day 4 wounds because of the presence of the scab. To generate high-quality sections, it is recommended to verify that all parts of the microtome are tightly fastened, the block holder is retracted to its initial position, the blade holder is set between 0 and 10˚ and the blade is tightly fastened but not overtightened. Although the paraffin block is chilled, the tissue may still shred. If shredding continues, a piece of ice can be placed on the block for 5 minutes while still mounted. Once sectioning has begun, it is highly recommended to avoid removing the block from the microtome to prevent the loss of paraffin sections. It is imperative to record any lost paraffin sections, both their numbers and their ranking in the serial sectioning. This will affect the morphometric analysis and needs to be considered. After initiating sectioning, the identification of wounded tissue on unstained sections

may be difficult, especially if not familiar with histology. When in doubt, it is recommended to be conservative and keep all the sections that contain tissue. Once the histological stain is performed, the tissue organization will be more evident and the wound more distinct. Occasionally, hair follicles may not be clearly visible or may be far from the wound edge. If this is the case, other key characteristics of the wounded tissue can be used to establish the boundaries of the wound including an abrupt increase followed by immediate decrease in epidermal thickness and sharp changes in the organization of the connective tissue (**Figure 4A-B**).

Digital imaging is a critical step in the morphometric analysis. The morphometric analysis should be performed on individual images using a landmark on each image to avoid repeated measurements. However, it is possible to stitch all the frames together using digital software and perform the analysis on a single image. Although this seems easier at first, manipulation of a large image may slow the analysis process down. The choice of the section is also critical for analysis. Although we recommend to digitally acquire the top section of every 8th slide, the quality of the section should be prioritized and the best of the 5 sections on that slide should be imaged. Sections with small folds could still be analyzed by estimating the area/length of the fold. The number of that particular section should be recorded both in the digital file and in the Excel spreadsheet, such that the distance between the previous and the next analyzed section can be adjusted accordingly.

Cutaneous wounds affect an estimated 6.5 million people with treatment costing over $25 billion annually[18] and are an inherent component of surgical procedures in addition to being secondary to many other health concerns, including diabetes and obesity. The mouse has been used as a convenient model to study human diseases because of the ease in manipulating its genome, despite often exhibiting a subtle phenotype compared to the human disorder. The bilateral excisional wound model and subsequent morphometric analysis described in this protocol is significant because of its ability to address the difficulty in detecting minor defects in an inherently complex wound healing process[19]. Because of its greater sensitivity and reduced variability, this protocol provides opportunities to use fewer animals to obtain the same experimental sensitivity. The protocol is highly customizable and can be used to study all stages of tissue repair. Detailed histological morphometric analysis in combination with phenotypic characterization of the tissue have great potential to increase the knowledge needed to further the understanding of critical factors modulating tissue repair.

ACKNOWLEDGMENTS:

We are grateful to all the members of the Dunnwald Lab who have contributed to the optimization of this protocol over the years, and to Gina Schatteman whose persistence in promoting the use of serial sectioning for wound analysis made its creation possible. This work was supported by funding from NIH/NIAMS to Martine Dunnwald (AR067739).

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Developmental Dynamics. **249** (4), 509-522 (2020).

Figure 1

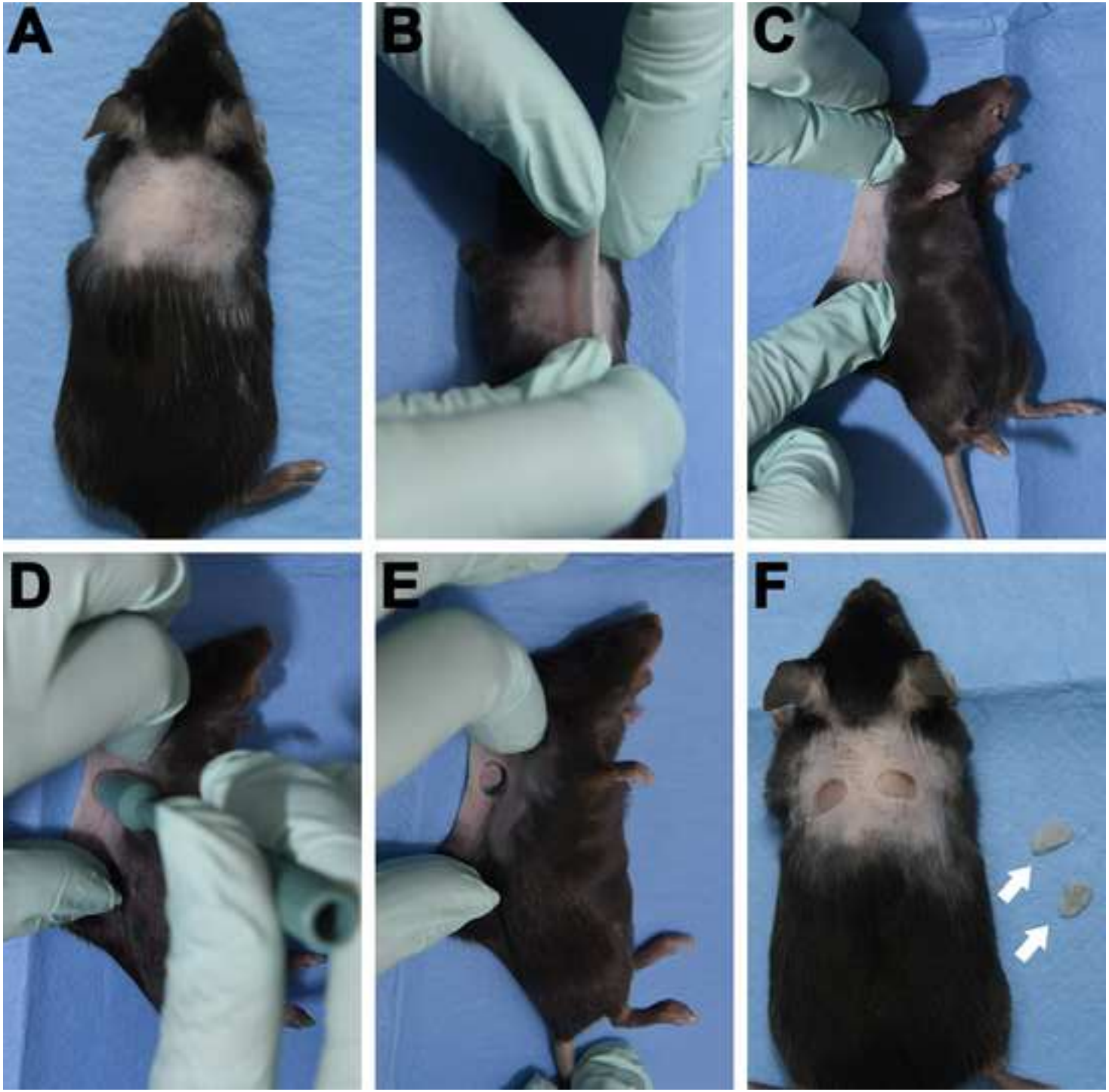

Figure 2

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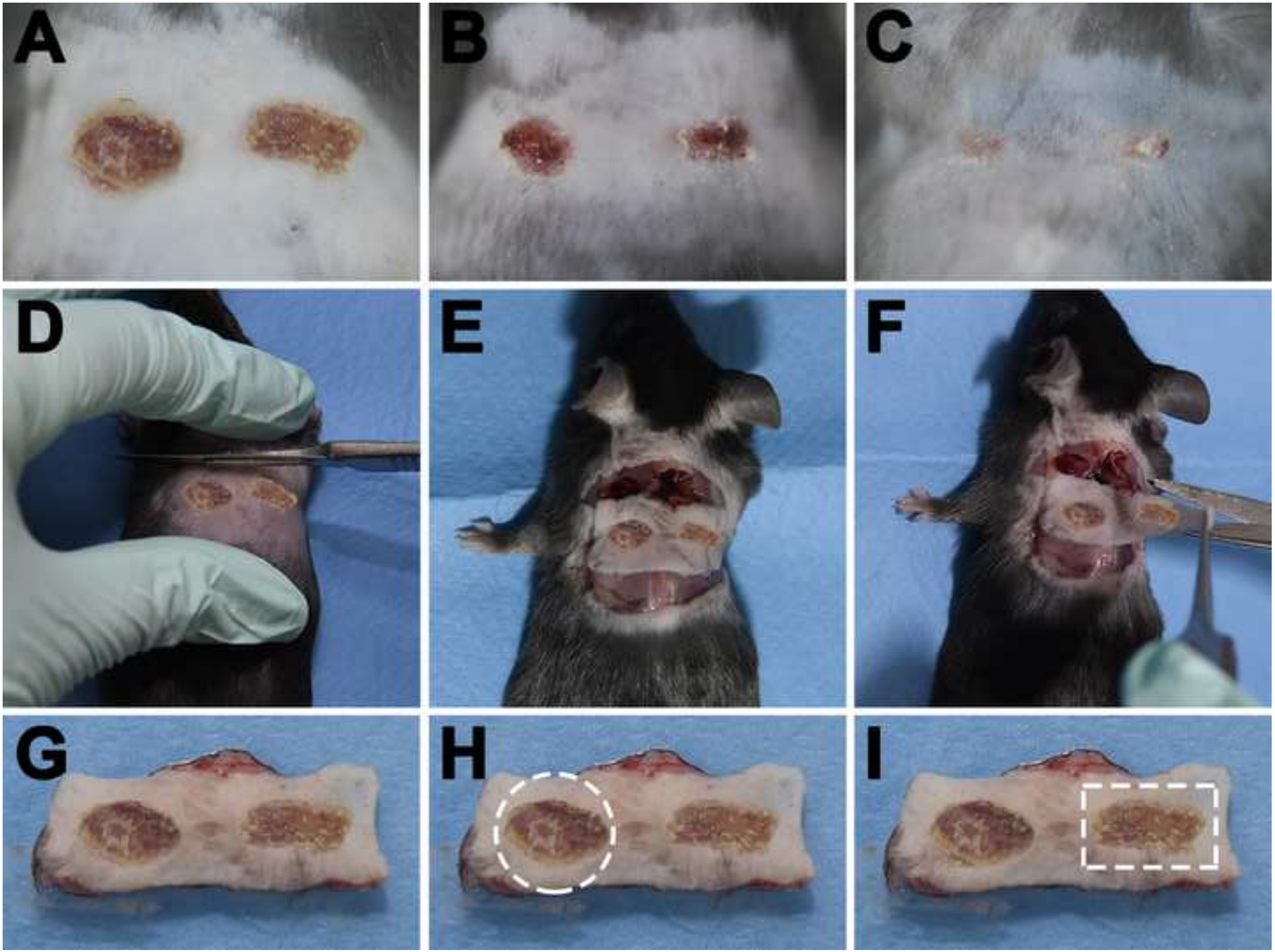

Figure 3

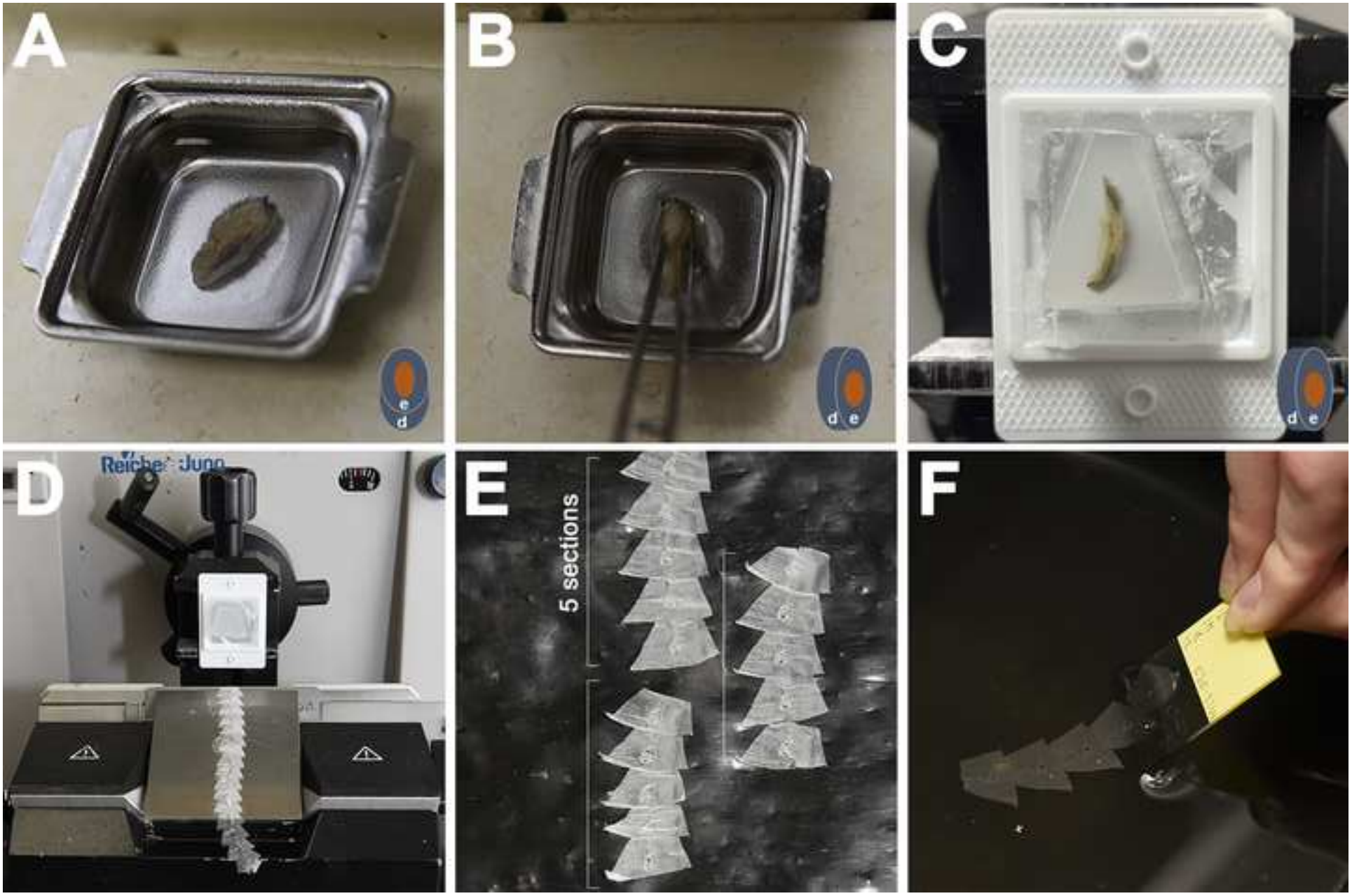

Figure 4

Click here to access/download;Figure;Figure 4 v2R1.psd

Figure 5

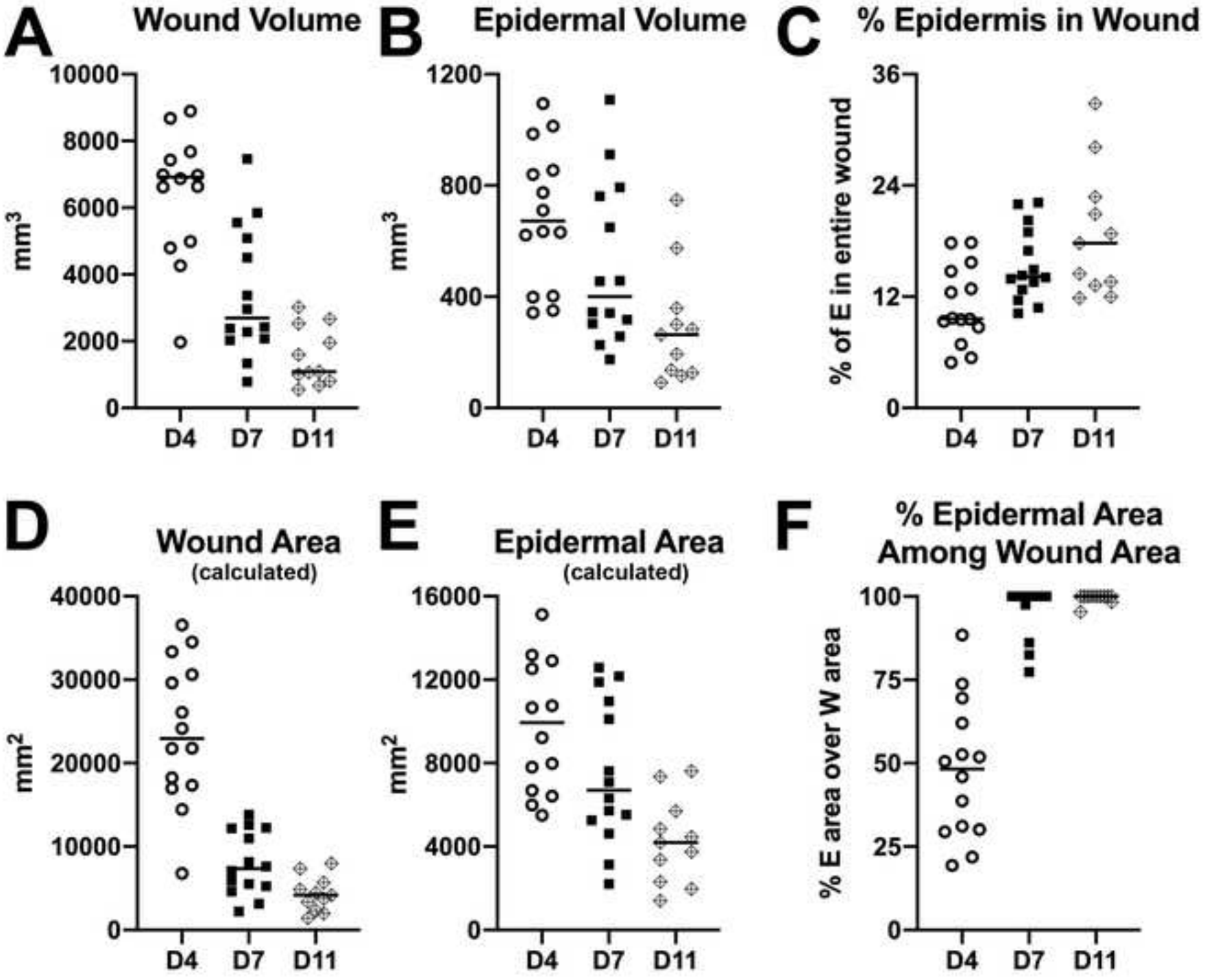

Figure 6

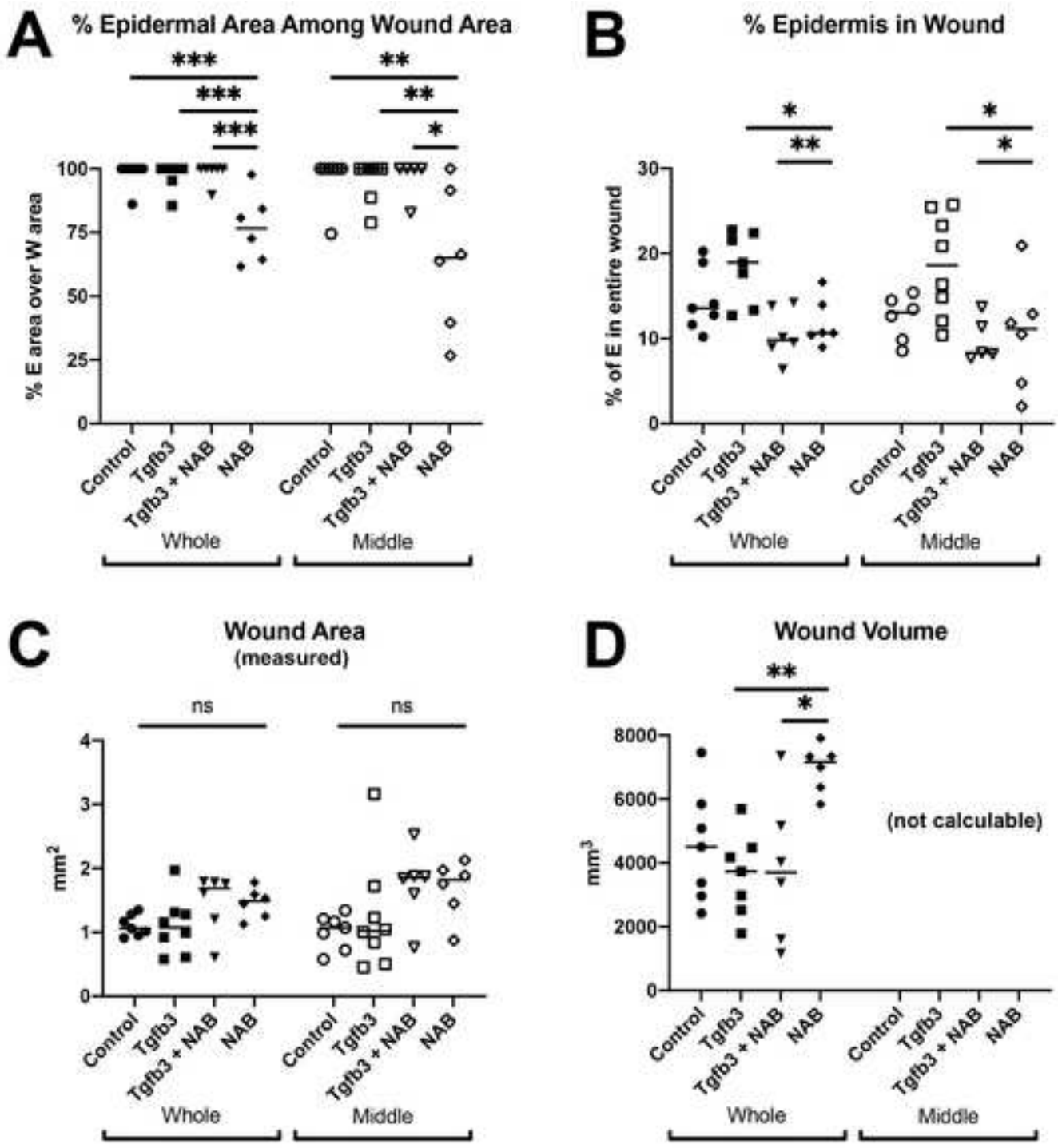

Table 1

Solution	Time (minutes)	Temperature (Celsius)	Pressure (kPa)
80% ETOH	30	RT	N/A
95% ETOH	30	RT	N/A
100% ETOH	30	RT	N/A
Xylene	30	RT	N/A
Xylene	30	RT	N/A
Paraffin	30	60	20
Paraffin	30	60	20

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials rev.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
100% ethanol			
70% ethanol			
80% ethanol			
95% ethanol			
Alcohol Prep	NOVAPLUS	V9100	70% Isopropyl alcohol, sterile
Ammonium hydroxide			
Biopsy pads	Cellpath	22-222-012	
Black plastic sheet			Something firm yet manipulatable about the size of a sheet of paper
Brightfield microscope			With digital acquisition capabilities and a 4X objective
Cotton tipped applicators			
Coverslips			22 x 60 #1
Dental wax sheets			
Digital camera			Include a ruler for scale, if applicable
Dissection teasing needle (straight)			
Embedding molds			22 x 22 x 12
Embedding rings	Simport Scientific Inc.	M460	
Eosin Y			
Glacial acetic acid			
Hair clipper			
Heating pad	Conair		Moist dry Heating Pad
Hematoxylin			
Microtome			
Microtome blades			
Paint brushes			
Paraffin Type 6			
Paraformaldehyde			
Permount			
Phosphate buffer solution (PBS)			
Povidone-iodine	Aplicare	82-255	
Processing cassette	Simport Scientific Inc.	M490-2	
Razor blades	ASR		.009 Regular Duty
Scalpel blades #10			

Scalpel handle			
Sharp surgical scissors			sterile for surgery
Skin biopsy punches			Size as determined by researcher
Slide boxes			
Slide warmers			
Superfrosted microscope slides	Fisher Scientific	22 037 246	
Temperature control water bath			
Tissue embedding station			Minimum of a paraffin dispenser and a cold plate
Tissue processor			Minimum of a oven with a vacuum pump
Triple antibiotic opthalmic ointment			
tweezers, curved tip			sterile for surgery
tweezers, tapered tip			sterile for surgery
WypAll X60	Kimberly-Clark	34865	
Xylene			

Point by point response to the Editor and Reviewers.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
The authors have proofread the manuscript and found no errors at this time.

•**Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
The authors have revised the protocol language where applicable to be in the imperative voice/tense.

•**Protocol Detail:**
1) 2.1.1. mention anesthesia method.
The authors have specified the preferred method of anesthesia in the protocol (isoflurane), however, this method requires special equipment not accessible to all investigator teams. Therefore, we have directed readers to the discussion section of the manuscript for alternative options.

•**Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
The authors have adjusted the protocol numbering and formatting accordingly.

•**Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

The authors have modified the highlighted text to meet these requirements.

•**Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The authors have reorganized the discussion to better follow the suggested order of topics. Unnecessary details have been removed and condensed to a 6-paragraph section.

• **References:** Please spell out journal names.

The authors have spelled out all journal names.

•**Table of Materials:** Sort alphabetically.

The authors have sorted the Table of Materials alphabetically.

•If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures are original, however, Figure 6 is an original figure generated by re-analyzing previously published data, hence the note “This figure has been modified using data from Le et al.[11].”

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

The protocol described were concise and can be easily reproduced/replicated.

The methodology is adequate and analytical process is comprehensive.

Major Concerns:

The parameters that were recorded and measured indicates that the researchers laid more emphasis on wound closure and re-epithelialization rather than wound strength and collagen fiber deposition on the dermis/wound bed.

The authors agree with the reviewer. The current protocol only describes morphometric analysis on sections stained with Hematoxylin and Eosin, which is not conductive to the evaluation of the collagen fiber deposition. However, the current protocol will generate many unstained paraffin sections which can be used for additional analyses of the healing process, including immunofluorescence and Masson’s trichrome staining. The authors have added

examples in paragraph 4 of the discussion to help emphasize the overall utility of the protocol and offer suggestions for supplementary analyses.

Minor Concerns:
The duration of the experimental study was not indicated.
The authors have added the duration of the surgery per animal in section 2 of the revised protocol. However, the actual duration of the experimental study depends on the time post-wounding that is considered for the study. These times can range from 1 to 11 days post-wounding, or longer. The rationale for the different time points are provided in the second paragraph of the discussion.

Certain parameters on the micrograph which were mentioned in the text were not labelled in the micrograph e.g. migration tongue
Figure 4 has been revised to include the mention: migrating tongue.

The Researchers should explain further why the 8th serial section in particular was chosen for analysis.
When the current protocol was designed, the initial analysis of the wound was performed on every 10^{th} stained paraffin section. The labor-intensive analysis led the investigators to compare morphometric analyses of every 10^{th}, 20^{th}, and 40^{th} stained paraffin sections. The authors found that the same data were obtained within 5% margin error when analyzing every 40^{th} stained paraffin section (or every 8^{th} microscope slides when 5 sections are placed on one microscope slide). Hence the current protocol described in this manuscript.

A depilatory cream could equally be used for minute hair removal
Although depilatory creams have been used to remove hair in the mouse, they do create a local micro inflammation that may adversely affect the wound healing outcome https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4578994/. For this reason, the authors do not recommend such product for a wound healing study (or any cutaneous studies), but favor the use of mechanical shaving.

Reviewer #2:
Manuscript Summary:
Adequate and informative

Major Concerns:
None

Minor Concerns:
None

Reviewer #3:
Manuscript Summary:

This is an outstanding and urgently need methods manuscript in the field. The authors should be commended on their attention to detail and willingness to share their considerable experience. The manuscript is well written and the figures are excellent.

Major Concerns:
None

Minor Concerns:
I have just a few suggestions for small changes/considerations:

1) The authors describe the punch-through-punch methods to generate 2 excisional wounds simultaneously on a mouse. It should be mentioned that direct biopsying (i.e. punch biopsy of each wound separately on a prone mouse) is also a popular method.
The authors have clarified different methods of excisional wound generation in the introduction.

2) Biopsy punches quickly become blunted. I would suggest including direction to change punch between animals (or regularly).
The authors agree with the reviewer and have added clarifying language to the protocol in section 2.3.4.

3) Figures 5 & 6 are excellent. For figure 5 is it possible to color code the data points that are related (e.g. same strain / surgeon?). This would give an idea of inter- and intra- variability.
While the authors like this idea, it would make for a very complicated graph/figure without additional panels evaluating these variables separately. We clarified the figure legend to include that these morphometric values were from 6 mm wounds.

4) The authors suggest (and provide data to show) that using their whole wound volumetric histology delivers greater statistical significance (sensitivity) in an experimental situation. This is VERY important and will, as the authors suggest, permit detection of defects/changes with a small effect size that would otherwise be missed with a single random section approach. There is another way of looking at this (important in the context of reduction, refinement and replacement of animals) - if this analysis provides greater sensitivity and reduced variability then it should be possible to use FEWER animals to obtain the same experimental sensitivity. This point should be emphasized in the discussion.
The authors thank the reviewer for this comment. Although this concept was already discussed in the original first paragraph of the discussion, the authors added a sentence to bring the point home at the end of the discussion.

Supplemental Table 1

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Section number	Wound length (measured, in mm)	Wound area (calculated, in mm2)	Wound area (measured, in mm2)	Wound volume (calculated, in mm3)	Epidermal length (measured, in mm)	Epidermal area (calculated, in mm2)	Epidermal area (measured, in mm2)	Epidermal volume (calculated, in mm3)
71	3.63	1016.40	1.86	520.8	3.63	1016.40	0.22	61.60
111	4.35	1218.00	2.24	627.2	4.35	1218.00	0.39	109.20
151	5.49	1537.20	2.48	694.4	5.49	1537.20	0.42	117.60
191	6.44	1803.20	2.29	641.2	3.82	1069.60	0.28	78.40
231	6.3	1764.00	2.65	742	3.55	994.00	0.23	64.40
271	5.91	1654.80	2.55	714	3.35	938.00	0.25	70.00
311	5.27	1475.60	2.86	800.8	4.71	1318.80	0.42	117.60
351	4.3	1204.00	1.98	554.4	4.3	1204.00	0.35	98.00
391	1.81	506.80	0.41	114.8	1.81	506.80	0.2	56.00
		Wound area of the entire wound		**Wound volume of the entire wound**		**Epidermal area of the entire wound**		**Epidermal volume of the entire wound**
		12180.00		**5409.60**		**9802.80**		**772.80**

Percentage of epidermal volume in the wound
14.29

Percentage of epidermal area among the wound area
80.48