

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 conducive 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 10th stained paraffin section. The labor-intensive analysis led the investigators to compare morphometric analyses of every 10th, 20th, and 40th stained paraffin sections. The authors found that the same data were obtained within 5% margin error when analyzing every 40th stained paraffin section (or every 8th 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.