

Response to JoVE editorial and peer review comment for: *A Standardized Approach to Histomorphometric Evaluation of Osteoarthritis in A Surgical Mouse Model*

First, we would like to thank the editor and reviewers for taking the time to review our work and provide us with their valuable comments. Below is a numerical list of the editor's comments addressed one at a time.

1. Document has been proofread and spelling and grammar issues have been addressed.
2. Email addresses have been provided for each author (lines 21-27).
3. Abbreviations missing definitions have been corrected, specifically OARSI on line 49 in the introduction section.
4. Commercial language has been removed throughout the protocol.
5. Animal ethics statement has been moved to the beginning of the Protocol section (lines 97-101).
6. Protocol has been modified to include action items with the intent of directing readers through the described protocol. Supplemental information originally included throughout the protocol section has been moved to the discussion section.
7. Protocol numbering format has been modified in accordance with JoVE instructions. Bullets and dashes have been removed throughout.
8. A single line space between each step, substep, and note has been included in the newly revised protocol section.
9. Protocol section has been modified to use the imperative tense and modified with more direct action words and steps to lead through the protocol.
10. Protocol has been simplified to more discrete action steps to direct readers through each distinct step within the protocol.
11. Previous significance subsections within the protocol sections have been moved to the discussion section.
12. "How" questions for each step were answered/addressed to the best of our ability without providing excessive extraneous details.
13. The volume and concentration of ketamine/xylazine drug combination was described in greater detail. We have also provided greater detail regarding secondary step for euthanasia by perfusion fixation. Detailed actions of knee joint harvesting and decalcification steps have been provided.
14. OARSI scoring information has been modified. Please see identified references and OARSI guidelines for further details regarding the scoring system. Information on the staining process we used has been modified. Details regarding slide selection, staining, and imaging have been included. It is difficult to provide full details on software clicks without including commercial language. More detailed demonstration will occur during the video process.
15. Some details moved to introduction and discussion sections. Again, some details regarding software clicks are difficult to describe in full details without using some commercial language of the software product.

16. We have highlighted approximately 2.75 pages of the protocol to be used for filmable content. We will be focusing on the histomorphometric measurements that we describe within the protocol section.
17. We have not reused any figure from any previous publications and thus have not had to obtain explicit copyright permission within our manuscript.
18. Embedded table has been removed from the manuscript and uploaded to the submission portal as an excel file.
19. Discussion section has been revised to include a) critical protocol steps, b) modifications and troubleshooting, c) limitations of the technique, d) significance compared to other current available methods, and e) future applications and developments of the technique.
20. Format for the references has been modified using the JoVE formatting for EndNote.

Replies to Reviewers' Comments:

Reviewer #1:

Major Concerns:

Discussion Lines 331-336: Here you introduce the concept of score repeatability and interobserver reliability, yet no data supporting these statements is presented anywhere in the manuscript. Calculation of relevant statistics is not described in the statistical methods. Can you compare the repeatability and reliability of this evaluation method compared to the OARSI scoring system?

Response: Figure 4 was added to address the topic of inter-observer variability. Additional information was included in the discussion section. Calculation of relevant statistics was included (line 358-366).

Minor Concerns:

Title

As this system was only evaluated in a single preclinical model, the title seems too generalized. Consider altering to more accurately reflect what was done.

Response: Title was modified to reflect specific mouse model used for this protocol. Potential application to other protocols is discussed at the end of the discussion section (line 541).

Protocol

Lines 78-91: The mice used need to be described according to standard reporting protocols, including age, sex, strain, housing, and number used. Why was there not randomization of the treated/sham joints?

Response: Detailed description of mice used for DMM surgery was added to the beginning of the protocol section including all appropriate reporting protocols, lines 97-101.

Lines 103, 114, 117: Please be more specific about the "levels" that were selected. A figure would be most useful to illustrate this, but a more thorough description of the selected anatomical locations would suffice. Were these same levels selected for every mouse? If not,

why not?

Response: Description of level selection was added to protocol section (lines 211-219).

Lines 201-202: This sentence is redundant and does not make sense as written, please revise.

Response: Sentence was modified, no longer the same number line.

Reviewer #2:

Manuscript Summary:

In protocol section there was not information about the anaesthetic protocol used as well as the use of anti-inflammatory, analgesic and antibiotic drugs. In my opinion should be provided information about this because the proper use of an anaesthetic protocol and post-surgery medication would improve the animal protocol as well as the animal welfare.

Response: Detailed description of animal protocol was added and mentioned following NIH and institutional animal care guidelines (lines 97-107).

Sample collection:

- Slides were selected from 3 representative levels of each sample. Please clarify in the text which were considered representative levels.

Response: Description of level selection was added to protocol section (lines 211-219).

OARSI scoring:

- Again it is necessary information about the three representative levels.

Response: Description of level selection was added to protocol section (lines 211-219).

Histomorphometry using OsteoMeasure:

- The lines for cartilage and subchondral bone area measurement were manually drawn or automatically detected by the program?

Response: This was described in the revised manuscript throughout the protocol section.

4.3 Please, explain how the counting function Works, or the parameters to have into account, to differentiate producing and non-producing cells. Describe how you made these discriminations.

Response: Description was added to the protocol section of the manuscript (lines 297-302).

Discussion:

In introduction you explain that other authors tried to make an histomorphometric evaluation of cartilage and subchondral bone parameters. In discussion you may explain why the use of OsteoMeasure software would improve this evaluation respect to other methods.

Response: This was addressed at the end of the discussion section (lines 527-546).

Major concern:

Sample collection:

- Authors used only ketamine for euthanasia? Please provide information about the euthanic

method. If only ketamine for immobilization of animals and perfusion of formaldehyde were used it was a non-valid method for mice euthanasia. In the AVMA Guidelines for the Euthanasia of animals: 2013 Edition (ISBN: 978-1-882691-21-0) explain accepted method for euthanasia. For ketamine says: " These agents are not FDA approved for use as agents of euthanasia. (2) Doses that consistently produce rapid death have not been established for most drugs and species. (3) The cost of the higher doses of agents required to cause death may substantially exceed that of an approved euthanasia agent. " and also "In species for which effective euthanasia doses and routes have been established, overdose of dissociative agent-a-2-adrenergic combinations is an acceptable method of euthanasia" so a combination with xylazine or medetomidine should be done. Also with formaldehyde says "Formaldehyde is an acceptable method of euthanasia for Porifera species. Formaldehyde is acceptable as an adjunctive method of euthanasia for Coelenterates (comb jellies, corals, anemones) and Gastropod molluscs (snails, slugs) only after these animals have been rendered nonresponsive by other methods (eg, magnesium chloride³³⁰). Formaldehyde is unacceptable as a first step or adjunctive method of euthanasia for other animal species". Having this in concern, please provide the euthanic protocol used or provide an explanation and the ethical committee approval for the use of this method.

Response: Detailed description of animal protocol was added and includes greater information regarding the euthanic method for this protocol. Ethical committee approval was also noted at the beginning of the revised protocol section.

Thank you again for taking the time to review and edit our work. We look forward to working with the JoVE team for filming the protocol.