REVISION OF MANUSCRIPT: JoVE60002
Rebuttal Letter

Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response: As requested, several native speakers did proofreading of the manuscript.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
Response: We uploaded the copyright permission and corrected the figure legends.

3. Please revise lines 81-82, 340-342, and 376-381 to avoid previously published text.
Response: We thank for the comment and revised the text accordingly.

4. Authors and affiliations: Please provide an email address for each author.
Response: We added the email addresses of the co-authors on the title page.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Zivic Instruments, Agilent Dako, Tween, Vector Laboratories, Dako, Merck, etc.
Response: As requested, we removed commercial language from our manuscripts.

6. 1.5: Please specify the age, gender and type of the animal used. Please also specify the dose of ketamine and xylazine.
Response: We added additional information in the protocol section (1.5.).

7. Please specify all surgical tools used throughout the protocol.
Response: As requested, all surgical tools were specified in the manuscript and table of materials.

8. Lines 143, 150: How large is the incision?
Response: We added additional information in the protocol section.

9. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: 2.75 pages or less of the protocol was highlighted in yellow for the video.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We highlighted completed sentences and made sure that at least one action is written in imperative tense.

11. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

Response: As requested, all relevant details that are required to perform the step were highlighted.

12. References: Please do not abbreviate journal titles.

Response: We corrected the references accordingly.

13. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Response: We sorted the items in alphabetical order.

Reviewer #1:

Manuscript Summary:

This protocol describes the optical clearing and fluorescent labelling of kidney slices using a solvent-based clearing method called ECi. The protocol is clearly stated and easy to follow for any scientist with some experience with handling animals. However, I have a few comments regarding the introduction, results and discussion.

Minor Concerns:

1. It is mentioned in the introduction that 2D analysis with thin slices is time-consuming. At the same time, the presented protocol is 14 days long (which is pretty long for clearing and staining 1 mm thick tissue slices, other protocols would perform faster, like FACT). This should be mentioned somewhere in the paper.

Response: As suggested by Reviewer #1, we highlighted other protocols in the discussion which might perform better antibody penetration of the tissue. See lines 427-429.

2. When grouping different clearing protocols in the introduction, solvent-based and aqueous-based clearing protocols are mentioned, and hydrogel-based (or SDS-based)

clearing techniques are omitted. Of course, one could argue that hydrogel-based clearing is an aqueous-based technique, but the SDS-delipidation is such a crucial step of these protocols in terms of clearing efficiency and antibody penetration that I think they deserve to be mentioned separately. Also, hyper-hydrating protocols like CUBIC and Scale should maybe be mentioned on their own.

Response: We thank Reviewer #1 for his comment. We mentioned hydrogel-based embedding and hyper-hydrating methods on their own in the introduction. See lines 73-74.

3. The authors mention mild tissue expansion as a problem of aqueous-based techniques. I think this should be more clearly explained to the general reader, why is this a problem? Citation?

Response: We added citations and explained in the introduction that tissue deformation through shrinkage or expansion will affect absolute morphometrics. See lines 85-90.

4. I think the authors have to elaborate (or cite a paper) on what they mean with "protein modification" as a problem with aqueous techniques. Most people would argue that the antigen retrieval step (at 92-98°C) of the protocol presented in this manuscript is also modifying (denaturing) proteins.

Response: We absolutely agree with Reviewer #2. We detailed “protein modification” in the introduction and provided citations (see lines 84-85). We further mentioned in the discussion section that our protocol will denature proteins (see lines 396-398).

5. Our lab has experience with most clearing techniques. We also tried the ECi-protocol, and in our hands it caused a quite high background in kidney tissue. This is of course fine for very abundant targets where the staining is strong, but we found it to be a problem for less abundant targets where other techniques (like hydrogel-based or SDS-based techniques, or other aqueous immersion-based techniques) performed better in our experience. I think this could be mentioned in the discussion (if the authors have the same experience).

Response: In our hands, we did not experience a problematic signal-to-noise ratio when we used the ECi protocol. In fact, we obtained similar results when we compared the ECi-protocol with other protocols (see Puelles et al, Kidney International, Article in Press, 2019). However, we found that good perfusion (removal of red blood cells) and high-grade 100% ethanol at the dehydration step aids to lower autofluorescence and to achieve high penetration depth. In addition, we used antibodies against abundant proteins and had access to strong fluorescent reporter mice, thus we do not have enough experience to state whether this protocol might not be well suited for less abundant targets.

6. The "opening" of the tubules is mentioned as a crucial positive thing in the protocol. It should also be mentioned as a possible artifact when studying tubular proteins or structures.

Response: As requested, we mentioned in the discussion section (see lines 402-406) that artificially opened tubules may have a negative effect when studying tubule

structure. However, we also stated that the opening of tubules would also allow to better distinguish proteins expressed in the apical membrane from other proteins located in the apical membrane on the contralateral side.

7. In our hands (and showed by other groups, like in the PACT publication by Yang et al. 2014) SDS-based clearing like the PACT or FACT protocols are the single best way to increase antibody penetration. This could be mentioned in the "troubleshooting" as a possible solution.

Response: We thank Reviewer #2 for his suggestion, and mentioned SDS-based clearing methods as alternative approaches to increase antibody penetration (see lines 428-429).

8. Finally, it might be of interest for the reader to know why the authors used CLARITY for the publication cited in the discussion. When is this protocol favourable (apart from when fluorescent proteins need to be preserved)?

Response: As requested, we explained why we used CLARITY in our recent publication (see lines 451-452). In addition, we highlighted that aqueous-based protocols including CLARITY is compatible with RNA analysis while no studies have tested solvent-based methods to visualize RNA yet (see lines 386-389).

Reviewer #2:

Manuscript Summary:

The authors describe a simple method to combine immunolabeling of thick kidney slices, optical clearing with ethyl cinnamate and confocal imaging that enables visualization and quantification of three-dimensional kidney structures. This method is very useful for the 3D image analysis of tissue structure.

Major Concerns:

1. Protocol 1.2., lines 123-124:

The sentence that "Transfer 1x PBS and 3% PFA in 1x PBS to 50 mL plastic syringes." should be changed to "Transfer 1x PBS containing Heparin and 3% PFA in 1x PBS to separate 50 mL plastic syringes."

Response: As requested, we reworded this for clarity in 1.2 (protocol section).

2. Protocol 1.9., line 151:

The sentence that "----- perfuse with 50 mL PBS, then switch and perfuse with 50 mL PFA." should be changed to "----- perfuse with 50 mL PBS containing Heparin, then switch and perfuse with 50 mL 3% PFA."

Response: We changed this sentence in 1.9 (protocol section).

3. Protocol 4.1. and 4.2., lines 228 and 236:

"ethyl cinnamate" should be changed to "ECi".

Response: We changed "ethyl cinnamate" to "ECi" in 4.1 and 4.2 (protocol section)

4. Representative Results, lines 261:
Please discuss on the merits of this method compared with the aqueous-based methods such as Scale S method in the Discussion.

Response: As suggested, we highlighted the advantage of aqueous-based methods including ScaleS in the manuscript (see lines 80-90, lines 301-302, lines 386-389).

5. Table 1. Table of materials and equipment.
The sentence "Citrate-based antigen retrieval soluVector Laboratories Cat#H-3300" should be corrected to "Citrate-based antigen retrieval solution Vector Laboratories Cat#H-3300 or H-3301".

Response: Thank you. We updated the table of materials.

6. Table 1. Table of materials and equipment.
Please add the Vibratome (Protocol 1.10. Note, line 158).

Response: As requested by Editor and Reviewer #2, the vibratome was listed in the table of materials.