Response to the reviewer and editorial comments - JoVE59139

We want to thank the Reviewers for the constructive comments and suggestions for revisions. We feel that we have addressed virtually all of the reviewers' points and that the revised manuscript is much improved. For ease of review, our responses are in blue.

Reviewer #1:

Manuscript Summary:

Mahan et. al. described a standard protocol to characterize the cellular composition of murine atherosclerotic lesions with paraffin-embedded sections, immunofluorescent staining and confocal microscopy with focus on the brachiocephalic arteries. The effect of Interleukin-1β inhibition on SMC phenotype is shown as an example.

Major Concerns:

Figure 4C is not clear and rather confusing, should be removed.

We thank the reviewer for this comment and agree on the lack of clarity of Figure 4. Other reviewers raised a concern about the broad application of our single-cell counting method that we initially performed using the Nikon NIS-Elements software and possible inter-software variations. To address this concern, we have modified our protocol and described single-cell counting and phenotyping using ImageJ. Key parameter setting and representative pictures of single cell counting using ImageJ are included in Figure 5.

Minor Concerns:

Smooth muscle cell lineage-tracing mouse diet and treatment:

- Temperature of PBS and PFA solutions for perfusion should be indicated

PBS and 4% PFA solutions are used at room temperature. We added this information in the text.

- Source of high fat diet should be indicated

We indicated the source (vendor, catalog number) of high-fat and chow diet in the Materials list associated with the manuscript.

Harvesting the Brachiocephalic artery

- In the protocol point #5 "Exposing the BCA." BCA abbreviation used should be indicated before in the text.

We modified the text accordingly. We defined BCA the title of section 2: “Harvesting of the Brachiocephalic Artery (BCA).”

- Protocol point #6 "Removing the BCA" Reference to figure 2C does not correspond to the figure 2C but to figure2B.

We have modified Figure 2 and changed the references to Figure 2 subpanels accordingly.

Tissue Processing and sectioning

- The use of foam pads in the cassette to ensure the tissues retain correct orientation and remain in the cassette is difficult to imagine, to overcome this point a real picture of an embedding cassette with the artery should be included. In addition, a short description of the type and source of the embedding cassette used.

We added a picture of the BCA and right carotid artery positioned in the cassette and added cassettes (Figure 3) and foam pads to the Materials list.

- For how long where the cassettes immersed in 70% ethanol should be mentioned

Cassettes are immersed in 70% ethanol solution for 24 to 72 hours. Cassettes remain in 70% ethanol solution until tissues processing. Although the time of immersion in 70% ethanol solution can vary based on laboratory or histology core facility schedules, we did not experience variation in immunofluorescent staining for the incubation times mentioned above.

- Reference of Figure 2D on the text does not correspond with figure

We have modified Figure 2 and changed the references to Figure 2 subpanels accordingly.

- Description and source of microtome blade used should be included

The reference of the microtome blades was added to the Materials list.

- Reference of Figure 2E on the text does not correspond with figure

We have modified Figure 2 and changed the references to Figure 2 subpanels accordingly.

Immunofluorescence staining

- Point #4, temperature to incubate the slides in PBS for 5 min should be indicated

All incubations in PBS are performed at room temperature. We added the information in the text.

Representative results

Figure 2A

- "and Apoe-/- mouse fed a high fat diet for 26 weeks (right picture)" must be revised. Figure 2A right actually shows a 26-week-old Apoe-/- fed an 18 weeks high fat diet.

We revised Figure 2A (now Figure 2C) accordingly.

Figure 4

- Figure 4A: A proper delineation of the atherosclerotic lesion image is not shown and should be including.

We agree with the reviewer that the delineation of the regions selected for single-cell counting is critical. We decided to present in Figure 4 representative images illustrating how delineation of the lesion and fibrous cap areas are performed. We also included delineation of the areas of interest for single cell counting on each representative image in Figures 6 and 7.

- Figure 4B: Figure legend does not correspond to actual figure and labels

- Figure 4C: not essential for the methodology of this manuscript.

We have modified the content of the figures, and we hope that the Reviewer will find that these revised versions provide more clarity to illustrate key steps of single cell counting (Figures 4 and 5) and examples of staining and counting of the fibrous cap area (Figure 6) and lesion area (Figure 7).

Discussion

- Conclusion and other sentences such as: "This method allows for a meticulous assessment of lesion morphology and cellular composition" should be revised since the method described here does not provided steps to evaluate lesion morphology which includes lumen diameter but only provide a standard protocol to characterize the cellular composition as stated in the tittle.

We agree with the Reviewer that although we routinely perform lesion morphology analysis based on Movat staining, our manuscript does not describe this protocol and focus on immunofluorescent staining based single cell counting. Consequently, we have carefully edited the discussion and removed any mention to lesion morphometric analysis.

Reviewer #2:

Manuscript Summary:

This manuscript describes a protocol to standardize the evaluation of advanced atherosclerotic lesions in the brachiocephalic artery of ApoE-deficient lineage-tracing mice. The standardized analysis of advanced plaques, in particular their cellular composition, could provide important information for future intervention studies. There are several issues that should be addressed by the authors.

Major Concerns:

1. A major problem is the low quality of the IF pictures shown in Figs. 3 and 4, making it impossible to evaluate the immunofluorescence data. In Fig. 3, the DAPI staining is not visible at all. Please provide higher resolution images for Figs. 3 and 4. In addition, show a better DAPI staining in Fig. 3 and scale bars in Fig. 4.

We are sorry for the lack of quality of the pictures presented in the initial version of the manuscript. We carefully edited the figures, and we used images with a minimal resolution of 300 dpi. Based on the reviewer comment, we also change the color of DAPI staining from blue to gray to increase the visibility. We added scale bars to all images (Figure 4, 6 and 7) except for representative images in Figure 5. These pictures are screen captures illustrating the key parameter setting and single cell counting using ImageJ. We also systematically added the delineation of the region subjected to single cell counting (i.e., fibrous cap area in Figure 6, and lesion area in Figure 7).

2. Fig. 3 and Fig. 4: Compared to the staining of RUNX2 and YFP, the LGALS3 staining pattern looks very much different. Where are these proteins localized in the cells? Are the respective IF-positive structures single cells? It is sometimes difficult to match DAPI and IF signal. How was the specificity of the antibodies validated? How reliable is single-cell counting performed by the image software (line 328)? Have authors validated automatic counting by comparing with manual counting?

We thank the Reviewer for these pertinent questions:

1. RUNX2 is a transcription factor localized in the nucleus. YFP and LGALS3 are located in the cytoplasm.

2. Manual single cell counting allows the assessment of the association of cytoplasmic staining like YFP and LGALS3 with single nuclei (DAPI). We increase the brightness of DAPI for clarity.

3. Every staining is validated by the used of an isotype-matched IgG control. Confocal settings are based on the IgG control picture to assure that non-specific signal, if any, is excluded from the acquisition.

4. All single cell counting data presented in the manuscript has been acquired by manual counting. We have not, thus far, found automated software to perform cell counting reliably.

5. For broader applicability of our protocol and manuscript, we have replaced the use of a brand-specific microscope software by ImageJ, free software provided by the NIH. Of note, cell counting is still performed manually. ImageJ is used to visualize images and perform manual population annotations.

3. It appears that some of the IF pictures contain "black boxes" that cover distinct parts of the IF stainings, e.g. in Fig. 3 upper row (IgG control) "LGALS3" or in Fig. 4B, "3. YFP+ counting". Please explain why.

The pictures do not include any black box, modification, or alteration. We are sorry that the low quality of the picture or a mistake during the figure preparation leads the reviewer to this impression. We modify the figures to include high quality (300 to 600 dpi) images. We are happy to provide raw images upon request.

4. It should be indicated that injection of tamoxifen and high fat feeding are animal experiments and require approval of animal protocols. Moreover, tamoxifen is a biohazard and must be handled with care.

We have modified the manuscript accordingly.

5. Lineage tracing should be explained in more detail. Please describe the used mouse lines (correct names with references) and how the experimental animals were generated by breeding and identified by genotyping.

We extended the description of the lineage tracing system and the breeding strategy used to generate the experimental mice. We provided the reference of the three strains obtained from Jackson Laboratory and used to generate the atheroprone SMC lineage tracing mice (*Myh11* cre/ERT2^{+} *R26R*-EYFP$^{+/+}$ ApoE$^{-/-}$). We and others employed the generic strain names used in previous studies and listed as official nomenclature by Jackson Laboratory (mouse provider).

6. Authors make a strong point about the importance of perfusing the mouse with a "gravity perfusion system" that mimics physiological blood pressure (120/70 mmHg). Please explain how this pressure can be achieved.

We added a schematic of the gravity-driven perfusion station that we use in our laboratory and the equation to determine the pressure of perfusion (Figure 2).

The pressure is driven by the height of the column of liquid independently of the shape, total mass, or surface area of the liquid. The pressure is calculated as followed:

$P = \rho \times g \times h$ with:

P: Pressure in kg/ms^2 or Pascal (Pa) 1 mmHg = 133.322 Pa

ρ: Density of fluid in kg/m^3. The density of 4% PFA solution in PBS is 1016.8 kg/m^3

g: Acceleration of Gravity in m/s^2 = 9.8 m/s^2

h: Height of fluid in m.

With our system, the height of the fluid varies between h_1 = 1.5 m and h_2 = 1.35 m

Thus, the pressure of perfusion is comprised between:

P_1 = 1016.8 * 9.8 * 1.5 = 14946.96 Pa = 112.11 mmHg and,

P_2 = 1016.8 * 9.8 * 1.35 = 13452.264 Pa = 100.9 mmHg

In Figure 3, we included this equation and indicated the heights to reach the average systolic blood pressure in mice (120 mmHg).

We also mentioned in the text that a perfusion pump can also control the pressure of perfusion. It allows a finer control and consistency in the perfusion pressure. However, this option is certainly more expensive and maybe less broadly available to in contrast with the gravity perfusion system that can be built by investigators.

7. It is understand able that authors cite their own work on lineage tracing (e.g. refs 13 in line 110; refs 15/16 in line 112 and line 426). However, they should present a more balanced introduction and discussion of the topic and also acknowledge the many other laboratories that have developed and used inducible lineage tracing of SMCs in atherosclerosis. In fact, this technique has been described already at the beginning of the 2000s.

We revised the fourth paragraph of the introduction to not only more precisely describe how SMC lineage tracing works but also acknowledge studies that have been used SMC lineage tracing systems. We mentioned seminal studies by Speer et al. utilizing SM22 Cre LacZ mice to identify SMC transdifferentiating into chondrogenic cells [1]. However, we also highlighted the major limitations of non-inducible Cre systems. We also cited more exhaustive studies employing inducible SMC-specific Cre (Myh11 [2-5]; Acta2 [2, 6]) and other types of reporters (mTmG [2], Confetti or rainbow mice [3-6], and LacZ [7]).

Please discuss the following issues in the section on "limitations of the methodology presented here" (beginning at line 466):

8. The analysis of a single vessel per mouse (brachiocephalic artery) might not be sufficient to get a realistic picture and should be combined with the analysis of additional atherosclerotic regions such as the aortic arch and abdominal bifurcations of the aorta. Similar results in different regions of the aorta should strengthen the relevance of the study. Also, considering the 3Rs principle of animal experimentation, it would be welcome to obtain as many data as possible per mouse.

We thank the reviewer for this pertinent comment. While the present protocol focuses on brachiocephalic artery analysis, similar studies can be done on other vascular beds using the same standardized procedures. Additional vessels of tissues of interest can be harvested at the same time (aortic root, aortic arch, abdominal aorta). Analysis of the aortic root region has been the focus of previous publications [8] including a JOVE manuscript [9]. We revised the discussion of the manuscript to include a discussion on the reviewer's concern.

9. Feeding ApoE-deficient mice a high fat diet for 26 weeks (18 weeks plus additional 8 weeks with/without intervention) will typically cause extensive atherosclerosis with large lesions and almost closed vessels or even death of the animals. In this model it will be difficult to detect pro-atherosclerotic effects of genetic or pharmacological interventions.

We respectfully disagree with the reviewer regarding the relevance of long-term intervention studies. We feel that a model in which the therapeutic intervention is performed on advanced atherosclerotic lesions recapitulates therapeutic interventions in patients with coronary artery disease. Indeed, atherosclerosis is an asymptomatic and silent disease in its first stage, and patients often experience symptoms with advanced atherosclerotic lesions. Besides, the mortality rate in mice between 18 and 26 weeks of high-fat diet does not exceed 5%. Using this experimental design, we were able to identify key modifications of atherosclerotic lesions after treatment with an anti-IL-1β antibody that are classically described as indices of plaque rupture.

That said, by no mean, we suggest that the protocol described in our manuscript can only be used for long-term intervention studies. The standardized tissue processing, sectioning, staining, and analysis described here can be implemented for virtually all types of atherosclerosis studies (short and long-term, prevention and intervention). We included a statement reflecting this point in the discussion.

10. Authors mention in the introduction (line 99-100) and discussion (line 436-437) that en face staining is unable to distinguish between fatty streaks and advanced lesion. It is, however, not clear how the present protocol can achieve this, if only advanced lesions are being analysed.

Once again, we give as an example the implementation of a standardized protocol for atherosclerotic tissue harvesting, processing, sectioning, staining, and analysis using our recent study consisting in long-term high fat diet associated with a pharmacological intervention. This analysis of atherosclerotic vessel cross-section by immunofluorescent staining allows a rigorous investigation of the lesion size (based on DIC images; see figure 4) and cell composition and the determination of the stage of atherosclerotic lesions (fatty streaks vs. advanced lesions). In contrast, *en face* Sudan IV staining, while informative regarding atherosclerosis burden, cannot be used to assess the disease stage, lesion morphology, or cell composition.

11. The Myh11CreERT2 transgene is integrated on the Y chromosome, so only male mice can be studied.

This is correct; the *Myh11* Cre/ERT2 transgene is located on the Y chromosome precluding the use of female mice for these studies. This is a limitation for sex difference studies that we added to the list of limitations detailed in the last paragraph of the discussion. However, this is to date the most rigorous and reliable SMC lineage tracing system.

Minor Concerns:

12. Line 3: Revise title: "…composition in ADVANCED atherosclerotic lesions of…

We agreed with this suggestion and modified the title accordingly.

13. Line 133: How is the Tam solution prepared?

The description of tamoxifen preparation has been added.

14. Line 199: Fig. 2C should red Fig. 2B

15. Line 225: Fig. 2D should read Fig 2C

16. Line 238: Fig. 2E should read Fig 2D

We fixed Figure 2 and its reference in the text.

17. Line 246-248: "Lesion morphology, collagen content, and intraplaque hemorrhage can be analyzed by Movat, PicroSirius Red, Ter119 staining, respectively." Please include references to aid readers finding the corresponding protocols.

References were added to the section mentioned above.

18. Line 305: Give an example for "mounting media suitable for fluorescence".

Reference of the mounting media is listed in the Materials List associated with the manuscript.

Reviewer #3:

Manuscript Summary:

This manuscript details a procedure for systematic characterization of morphology, and cell populations present in brachiocephalic arteries in murine models of atherosclerosis. The proposed protocol is intended to standardize assessment of lesions specifically in an intervention setting, but would also be applicable to prevention studies. This is a useful framework for anyone working in pre-clinical atherosclerosis models.

Major Concerns:

-Delineating fibrous cap area and/or necrotic core is an extremely important and non-trivial step in quantifying such sections. Please describe exactly how one would differentiate fibrous cap and necrotic core from the rest of the lesion.

We agree with the reviewer that delineation of the lesion or sub-compartments of the lesions (i.e., fibrous cap, necrotic core) should be standardized to accurately compare the effects of two experimental conditions on the cellular composition and distribution. We added in the manuscript the description and representative images (Figure 4) of the delineation of the lesion and the fibrous cap area based on z-stack confocal images and published papers [10, 11].

- It is probably important to quantitate staining for various cell populations at some regular interval over the course of the whole BCA. Please suggest what you believe to be the optimum number of sections, and interval between them, that should be assessed.

We thank the reviewer for this important remark. The standardized protocol that we present here allows the unambiguous analysis by IF staining at precise locations along the brachiocephalic artery. For cell composition analysis by IF staining, we suggest to stain and analyze the lesions at two distances from the aortic arch. We suggest in the manuscript to analyze sections at 480 μm and 780 μm from the aortic arch. Additional locations can be investigated, but there should be consistency in the number of sections and their locations.

We included in the revised version of the manuscript an example of single cell counting at two different locations of cell populations within the fibrous cap area (Figure 6).

Minor Concerns:

- Good fixation is not indicated by livery discoloration. Liver discoloration indicates sufficient removal of blood, but does not necessarily indicate proper fixation.

We agree with the reviewer. We removed the inaccurate sentence from the text.

- Gravity-driven perfusion does not guarantee constant flow speed and pressure. It is highly prone to variability due to bubbles in the lines, user error when it comes to placement of the needle in the heart, duration of time the mouse is in the CO2 (i.e. quality of the heart to pump the fluid through the circulation), etc. Perhaps describe how you set up your system such that you achieve 70-120mmHg pressure.

Although the use of a gravity perfusion system does not guarantee a lack of variation of flow speed and pressure of PBS or PFA solutions, by far this method permits a much higher consistency than manual syringe perfusion. Also, perfusion of PBS and PFA solutions at a pressure close to physiological blood pressure avoids major alteration of the vessel and atherosclerotic lesion morphology. As indicated in the protocol, bubbles in the tubing system are flushed out before insertion of the needle into the apex of the left ventricle. All parameters including the time of euthanasia into the CO2 chamber, the location of insertion of the needle in the apex of the left ventricle, the volume of solutions perfused are kept as consistent as possible. We added in Figure 2 a schematic of the gravity perfusion system. We also included calculation of the height range for the gravity perfusion system based on the hydrostatic pressure equation: Pressure = density of fluid * acceleration of gravity * height.

- Line 227: I don't know what you mean by "face the blocks." Do you mean section the blocks?

Facing a block consists in removing the top section of the block where there is only paraffin, but not the tissue. Once sections include the tissue, they should be collected for careful assessment of the location and orientation. We rephrased the sentence in the manuscript.

- line 239: perhaps explain why less than or more than 10um sections would not be desirable.

Unlike thinner sections, 10 μm thick sections allow the quantification and unambiguous characterization of a larger number of cells. The acquisition of 8 to 10 μm thickness by stacks of 1 μm using a confocal microscope permits to associate a given staining (e.g., YFP, ACTA2, and LGALS3) to a single nucleus more accurately. Assessing the colocalization of the lineage tracing reporter and other phenotypic markers with single nuclei along several consecutive stacks is critical to avoid inaccuracy due to the proximity of several cells. This point has already been discussed [12, 13] and justify the use of 10 μm-thick sections and z-stack confocal microscopy.

- Line 268: perhaps list how many watts that ends up being in your microwave?

For antigen retrieval treatment in the microwave, we set up the machine at 50% power. Considering that our microwave has a maximal power of 1350W, the wattage during the antigen retrieval incubation is 675. Higher wattage can be used, but it will require optimization of the time of incubation.

- The description of single-cell counting seems fairly specific to your software. Hopefully the video associated with this manuscript will show how this is done in such a way that it is

generalizeable across software platforms. Is there open-source software that could be used for this?

We thank the reviewer for this highly pertinent comment and the great idea to adapt the protocol to open-source software. We elected to develop a single cell counting protocol using Image J, NIH supplied free software. The text has been modified accordingly, and Figure 5 illustrates the key steps of the procedure using Image J as well as representative pictures of counting.

- The figures seem to be in a low-quality format and the IFs are difficult to see in the reviewer version. I cannot see the annotations in figure 4.

We apologize for the low quality of the figures in the previous version of the manuscript. We increase the quality of the images.

Reviewer #4:

Manuscript Summary:

The manuscript describes a protocol for quantification of smooth muscle cell-derived cells in atherosclerotic lesions using genetic lineage tracing. Detailed protocols covering the entire experiment from genetic labelling, diet, dissection, tissue processing to immunostaining, imaging and quantification are included. While protocols for individual steps are readily available elsewhere, this presents a comprehensive collection that would be very useful to newcomers to the field. However, the protocol is very biased as detailed below, which limits it's quality. This could be accounted for by discussing alternative analyses.

Major comments:

Atherosclerotic lesions are very heterogeneous and the region considered for quantification has immense impact on the result. In the present manuscript this is very poorly described (line 330: " Delineate the region to analyze "). It is crucial that this delineation is more clearly described: how are the regions selected for quantification, and how are the appropriate regions identified in a confocal image (ie w/o H&E staining - even if this is provided for an adjacent section).

We thank the Reviewer for this comment highlighting the lack of information in the initial version of our manuscript.

First, we agree with the reviewer that if possible, the analysis should not be limited to one vascular bed and that vascular territory-specific differences can appear after pharmacological or genetic interventions. We focused on analyzing the cell composition and the SMC phenotype in the brachiocephalic artery, but similar analyses can be done on cross-sections of the aortic sinus or abdominal aorta. We mentioned this point in the discussion section of the revised manuscript.

Second, we provided more details regarding the delineation of regions of interest, namely the lesion area and the fibrous cap area, based on confocal images (see new Figure 3). We use Differential Interference Image (DIC) to localize precisely the intima and the internal elastic lamina. We trace along these borders to delimit the lesion area. The fibrous cap is classically defined the accumulation of Acta2+ cells and extracellular matrix components (including collagen) under the endothelial layer. Previous studies have shown that the average thickness of the fibrous cap in Apoe-/- mice fed with a WD for 18 to 26 weeks was 30 µm [10, 11, 14]. However, as highlighted by the Reviewer, plaques may not have a fibrous cap with a constant thickness or a continuous coverage of the endothelial layer. That said, we believe that the detailed analysis of the cellular composition in the 30µm-thick area localized under the endothelial layer (30µm fibrous cap area) is an objective and unbiased method to ascertain the changes in this critical region. The combination of DIC and ACTA2 channels allows measuring the 30 µm thick area under the intima. Examples of delineation of the lesion and the fibrous cap areas are shown in Figure 3.

The protocol describes analysis of lesions in the BCA of Myh11-CreERt2/EYFP/ApoE-null animals using FFPE, which appears unjustly restricted. In my opinion this should be corrected. For example,

* The advantages and disadvantages of FFPE are discussed, but it would be beneficial to mention alternatives - such as analysis of frozen samples to allow for direct detection of fluorescent protein expression, eliminating potential issues with antibody specificity

* Atherosclerosis development in different vascular beds appear to be differentially controlled (e.g. Newman et al JCI insight 2018), hence the focus on a single vascular bed might obscure important effects

* Other reporters have been used successfully for smooth muscle cell lineage tracing

We agreed with the reviewer and mentioned that similar immunofluorescence analysis of frozen sections in the discussion section. This protocol focuses on the analysis of BCA lesions by immunofluorescent staining on PFFE tissue sections. By no mean, this analysis excludes the use of alternative tissues, vascular beds and the implementation of complementary techniques. We have developed further this point in the discussion section.

Other lineage-tracing strategies have been reported including the use of alternative inducible Cre [1, 2, 6], and labeling (tomato/GFP [2], rainbow or confetti for clonal expansion analysis [3-6]). We have expanded the introduction section on SMC lineage tracing to reflect these alternatives as well as to mention the first generation of lineage tracing systems used in the 2000's by Speer et al. [1]. However, we emphasize that an inducible system be necessary for rigorous tracing of the SMC. Indeed, a caveat of the use of lineage tracing models employing non-inducible Cre systems, such as the widely used SM22 Cre LacZ is that any cell not coming originally from the SMC lineage would be positive for the tracing system upon activation of the SMC marker gene.

The manuscript includes data on how IL1beta neutralising antibody treatment affect Runx2 expression in the plaque. This data is derived from animals used for a recently published study

(Gomez et al Nat Med 2018). This point is not a concern - however, the data collection (region of analysis, number of cells quantified etc) is poorly described and the data is only compared for all cells. The conclusions made appears to be based on very preliminary analysis and in my opinion this data should not be included.

We think that it provides an example of analysis that can be performed with this protocol. However, we agree with the reviewer that the description of the quantification parameters was missing. In the new version of the manuscript, we provided two examples of single cell counting in the fibrous cap area (Figure 6) or the lesion area (Figure 7). We have provided more detail about these analyses in the representative results sections.

Minor comments:

References to figure 2 are wrong

We have modified Figure 2 and revised the references in the text.

The genotype annotation (line 131: Myh11 ERt2 Cre YFP Apoe-/-, sometimes R26R is used with YFP) is unconventional.

We revised the manuscript to employ consistent genotype annotations: Myh11 Cre/ERT2 and R26R eYFP. These annotations have been used before and are listed as official nomenclature by Jackson Laboratory.

Tissue processing conditions:

* Appear to lack an ethanol gradient - is this a typo?

We thank the reviewer for noticing our mistake. We corrected the tissue processing protocol.

* Conditions appear harsh (length of incubation times) for mouse BCA, with the risk of drying out the tissue and introducing artifacts

We corrected the tissue processing protocol. We routinely use this protocol, and we have not found that it introduces artifacts or alterations of the tissue.

* It would be worth mentioning that Xylene can be substituted with other clarifying agents that are more friendly to the environment and the operator (e.g. Histoclear)

Although we agree that environment-friendly reagents should be suggested when possible, we have reservations stating in the article that Histoclear is a good substitute to Xylene since we have never tested and compared the use of Xylene and Histoclear.

Details lacking:

Details of the tamoxifen solution makeup to ensure bioavailability should be included (line 154)

Tamoxifen is dissolved in preheated peanut oil and incubated at 55 °C on a rotator until the full dissolution of Tamoxifen. A more detailed protocol has been included in the revised version of the manuscript.

Reference for EDTA vacuum tube needed (line 155)

The reference and catalog number of the EDTA vacuum tube have been added to the Materials list associated with the manuscript.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was carefully proofread.

2. Please define all abbreviations before use.

All abbreviations are defined before use.

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We added the ethics statement.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We edited the manuscript accordingly.

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We edited the manuscript accordingly.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

7. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

We edited the manuscript accordingly.

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We edited the manuscript accordingly.

9. Line 154: Please describe how to perform cardiac puncture. What volume of blood is collected?

We provided a more detailed description of the cardiac puncture.

10. Line 157: What is used to withdraw the top plasma phase?

Pipette and tips. We edited the manuscript accordingly.

11. Lines 162-167: Please specify all surgical instruments used. How large is the incision?

The incision is made with scissors and is approximatively ~ 2cm large. We edited the manuscript accordingly.

12. Lines 179: How large is the incision?

The incision is approximatively ~ 2mm large. We edited the manuscript accordingly.

13. Line 259: What does diH2O refer to?

diH2O refers to deionized H2O. The abbreviation is now defined after its first use.

14. Line 262: Please add more specific details (or move the details in lines 263-271 to the actual step).

We edited the manuscript accordingly.

15. Line 284: Please specify incubation temperature.

We edited the manuscript accordingly.

16. Line 315-316: Please provide specific values (ranges).

We edited the manuscript accordingly.

17. Lines 330-337: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.

We edited the manuscript accordingly.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

We edited the manuscript accordingly.

19. After you have made all the recommended changes to your protocol (listed above), 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.

We highlighted the steps that should be included in the video.

20. 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.

Done.

21. 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.

Done.

22. Please remove commercial language and use generic terms instead: Shandon Excelsior, Eppendorf, etc.

We edited the manuscript accordingly.

23. Figure 2D: Please include a space between all numbers and their corresponding units (i.e., 10 µm, 130 µm, 280 µm, etc.).

We edited the figures accordingly.

24. Figure 3: Please also explain the DAPI and Merge images in the figure legend.

We edited the figure legends accordingly.

25. Figure 5: Please define error bars in the figure legend.

Results are expressed as mean ± SEM. We added this information in figure legends.

26. References: Please do not abbreviate journal titles.

We used the JoVE style output in Endnote for citation and bibliography formatting.

REFERENCES

1. Speer, M.Y., et al., *Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries.* Circ Res, 2009. **104**(6): p. 733-41.
2. Herring, B.P., et al., *Previously differentiated medial vascular smooth muscle cells contribute to neointima formation following vascular injury.* Vasc Cell, 2014. **6**: p. 21.
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