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Imaging and analysis of Oil Red O stained whole aorta lesions in an aneurysm hyperlipidemia mouse model

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Phillip Steindel, Ph.D.
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Dear Phillip,

Please find attached the latest revision of our manuscript.
We hope this revision address all outstanding issues.

Sincerely yours,

Professor Michael Simons

TITLE:

Imaging and Analysis of Oil Red O-Stained Whole Aorta Lesions in an Aneurysm Hyperlipidemia Mouse Model

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KEYWORDS:

ascending aortic aneurysm, abdominal aortic aneurysm, atherosclerosis, apolipoprotein E-deficient hyperlipidemic mice, low density lipoprotein receptor-deficient hyperlipidemic mice, Oil Red O staining, en face aorta

SUMMARY:

This protocol provides a step-by-step procedure to analyze atherosclerotic burden in mice. Investigators can use this protocol to compare the abundance, location, and size of atherosclerotic lesions in different animals.

ABSTRACT:

Apolipoprotein E (*Apoe*)- or low density lipoprotein receptor (*Ldlr*)-deficient hyperlipidemic mice are the two most commonly used models for atherosclerosis research. They are used to study the impact of a various genetic factors and different cell types on atherosclerotic lesion formation and as well as test the development of new therapies. Isolation, excision of the whole aorta, and quantification of Oil Red O-stained atherosclerotic lesions are basic morphometric methods used to evaluate atherosclerotic burden. The goal of this protocol is to describe an optimized, step-by-step surgical method to dissect, perfuse-fix, isolate, stain, image and analyze atherosclerotic lesions in mouse aortas with Oil Red O. Because atherosclerotic lesions can form anywhere in the entire aortic tree, this whole aorta Oil Red O staining method has the advantage of evaluating lipid-laden plaques in the entire aorta and all branches in a single mouse. In addition to Oil Red O staining, fresh isolated whole aortas can be used for variety of in vitro and in vivo experiments and cell isolations.

INTRODUCTION:

Coronary artery disease, a leading cause of mortality in the US, is usually caused by atherosclerosis, a process that leads to the buildup of plaque inside arterial walls[1]. Hyperlipidemia-prone *Apoe*- and *Ldlr*-deficient mice are central to investigations of atherosclerosis and its complications and development of therapies[2-5]. Quantification of atherosclerotic lesions from an en face aorta is an important endpoint analysis for evaluating the impact of genetic manipulation in different cell types. It also helps to study novel therapies designed to affect atherosclerotic disease initiation, progression, and regression. Atherosclerotic lesions can form anywhere in the aorta and its branches (i.e., brachiocephalic, carotid and subclavian arteries in the chest, as well as renal, common iliac and femoral arteries below the diaphragm)[6]. A comprehensive evaluation of atherosclerosis burden and appropriate therapy requires assessment of disease burden in different locations, a challenge that is often overlooked.

This protocol describes how to perform a comprehensive analysis of atherosclerotic lesions, starting with an unopened whole aorta and proceeding to en face preparation, in a single mouse. Unopened whole aorta Oil Red O staining allows rapid, qualitative assessment of lipid-laden plaques in the entire aorta and its branches, while en face preparation provides a quantitative assessment of atherosclerotic lesion distribution in the mouse aorta.

The technique uses 8 week-old mice with a smooth muscle cell-specific TGFβR2 deletion on the Apoe$^{-/-}$ hyperlipidemic background (*MYH11-CreER^{T2};Tgfbr2$^{f/f}$;mT/mG$^{f/f}$;Apoe$^{-/-}$*; hereafter referred to as TGFβR2$^{iSMC\text{-}Apoe}$ mice) and littermate Apoe$^{-/-}$ controls (*MYH11-CreER^{T2};mT/mG$^{f/f}$;Apoe$^{-/-}$*; hereafter referred to as Apoe$^{-/-}$ mice). The animals are kept for 16 weeks on a high cholesterol, high fat diet (HCHFD) as study materials[7]. At study termination, the unopened whole aortas are stained and imaged (including all major branches) with Oil Red O for qualitative assessment of lipid-laden plaques. The aortas are cut open via en face preparation, and all atherosclerotic lesions are imaged and quantified. This protocol can be used to study atherosclerotic lesion development in Apoe$^{-/-}$ or Ldlr$^{-/-}$ hyperlipidemia mice models and extended to general aorta-related vascular biology applications.

PROTOCOL:

mT/mG (stock no. 007676), and Apoe$^{-/-}$ (stock no. 002052) mice were purchased from the Jackson Laboratory. Myh11-CreER^{T2} mice were a gift from Stefan Offermanns (available from the Jackson Laboratory as stock no. 019079). *Tgfbr2*$^{fl/fl}$ mice were obtained from Harold L. Moses (Vanderbilt University). All animal procedures were performed using protocols approved by the Yale University Institutional Animal Care and Use Committee.

1. Mice

1.1. Produce *MYH11-CreER^{T2};mT/mG$^{f/f}$;Apoe$^{-/-}$* and *MYH11-CreER^{T2};Tgfbr2$^{f/f}$;mT/mG$^{f/f}$;Apoe$^{-/-}$* mice as previously described[7]. Breed mutant strains to the C57BL/6J background for more than six generations.

NOTE: The Myh11-CreER^{T2} Cre mouse line provides a powerful tool for studying the role of smooth muscle cells in vascular homeostasis and vascular pathology. The Cre allele is inserted into the Y chromosome; thus, female mice do not express this construct.

2. Mouse genotyping, tamoxifen induction, and high cholesterol/high fat diet

2.1. Perform mouse genotyping using mouse ear DNA and PCR analysis. Mouse ear DNA should be isolated using the blood and tissue DNA isolation kit (**Table of Materials**) according to the manufacturer's instructions. PCR primers are listed in **Table 1**.

2.2. Induce Cre-Lox recombination by tamoxifen injection at 1 mg/day i.p. for 5 days in 6 week-old *MYH11-CreER^{T2};mT/mG$^{f/f}$;Apoe$^{-/-}$* and *MYH11-CreER^{T2};Tgfbr2$^{f/f}$;mT/mG$^{f/f}$;Apoe$^{-/-}$* male mice.

2.3. Induce atherosclerosis by placing 8 week-old male mice (2 weeks after tamoxifen treatment) on a HCHFD (40% kcal fat, 1.25% cholesterol, 0% cholic acid) for 16 weeks.

3. Reagent and dissection tool preparation

3.1. Stock Oil Red O solution preparation: dissolve 1 g of Oil Red O in 100 mL of isopropyl alcohol.

3.2. Working Oil Red O solution preparation: mix 24 mL of stock Oil Red O solution with 16 mL of dH_2O. Filter the diluted Oil Red O with 0.45 μm sterile syringe filters (the solution is only good for 1–2 h).

3.3. 60% isopropyl alcohol preparation: mix 60 mL of isopropyl alcohol with 40 mL of dH_2O.

3.4. 4% formaldehyde in 1x DPBS preparation: dilute 10 mL of 16% formaldehyde in 30 mL of 1x DPBS.

3.5. Clean all dissection tools with 70% ethanol (**Figure 1**).

4. Euthanasia (Figure 2A)

4.1. Measure the mouse's weight prior to euthanasia.

4.2. Euthanize the mouse by intraperitoneal injection of ketamine and xylazine (each milliliter contains 10 mg/mL ketamine and 2 mg/mL xylazine).

4.3. Place the mouse in supine position (belly side face-up).

5. Opening of chest and abdominal cavity and heart perfusion (Figure 2B)

5.1. Prepare a 10 mL syringe with 10 mL of 1x DPBS. Cap with a 25 G needle. The syringe will be used to flush the heart.

5.2. Hold up the skin with tweezers (Style 5) and cut with fine scissors from the base of the abdomen to the top of the neck.

5.3. Open the abdominal wall below the ribcage.

5.4. Lift the sternum with tweezers (Style 5) and cut the diaphragm, then cut away the ribcage to expose the thoracic cavity.

5.5. Make a small incision in the right atrium of the heart.

5.6. Perfuse through the apical left ventricular puncture by slowly injecting 10 mL of 1x DPBS. Once thoroughly perfused, the liver and kidney become light brown in color.

5.7. Clean the chest cavity of extraneous blood and fluid by using a non-woven sponge to absorb the material.

6. Isolation of aorta and branches (Figure 2C)

6.1. Remove organs (i.e., lung, liver, spleen, and gastrointestinal and reproductive organs) and cut the clavicle using tweezers (Style 5) and fine scissors while leaving the heart, kidney, and aorta intact in situ.

NOTE: Make sure not to lacerate the heart or any major blood vessels.

6.2. Place the mouse under a stereomicroscope.

6.3. Dissect aorta and aorta branches including brachiocephalic artery, carotid arteries, subclavian arteries, renal arteries, common iliac arteries, and femoral arteries using tweezers (Style 4) and spring scissors.

NOTE: Cover the aorta with a wet, non-woven sponge to avoid dehydration while dissecting the aorta branches.

6.4. Carefully dissect and remove adventitial adipose and connective tissue around the aorta and aorta branches using tweezers (Style 4) and spring scissors.

NOTE: Since inflammation is prominent in the aneurysm hyperlipidemia mouse, it is difficult to remove adventitia. Be careful not to tear or nick the aorta and aorta branches. This step requires practice and patience.

7. Fixing of heart and aorta (Figure 2D,E)

7.1. Prepare a 10 mL syringe with 10 mL of 4% formaldehyde in 1x of DPBS. Cap with a 25 G needle.

CAUTION: Formaldehyde is hazardous. Read the MSDS before working with this chemical. Wear gloves and safety glasses and produce the dilution solutions inside a fume hood.

NOTE: 4% formaldehyde solution degrades over time. It is important to use freshly made 4% formaldehyde for fixation.

7.2. Fix the vascular tree through apical left ventricular puncture by slowly injecting 10 mL of 4% formaldehyde.

NOTE: Formaldehyde fixation interferes with several downstream applications, such as cell culture, FACS analysis, and single-cell RNA sequencing analysis. Skip this step if the aorta will be used for any of these applications.

7.3. Clean the chest cavity of any extraneous fluid with a non-woven sponge to absorb the material.

7.4. Separate the heart from the aorta by holding the heart with tweezers (Style 4) and using micro-dissecting spring scissors.

NOTE: To perform en face Oil Red O staining after this step, it is recommended to cut the aorta open in situ instead of ex vivo and proceed to section 8. This makes it easy for the en face aorta to lay flat.

7.5. Isolate and excise the aorta and its major from 1 mm above the carotid artery to the end of femoral artery using tweezers (Style 4) and spring scissors.

7.6. Transfer the vessel into a wax Petri dish or 1.5 mL microcentrifuge tube and fill with 1x DPBS until it covers the aorta.

NOTE: The protocol can be paused here.

8. Oil Red O staining and imaging of unopened whole aorta (Figure 3)

8.1. Pin the vessel onto a wax Petri dish using minutien pins (**Figure 3A**).

8.2. Rinse the vessel once with 1x DPBS.

8.3. Pour 25 mL of fresh Oil Red O solution into the Petri dish (**Figure 3B**).

NOTE: (1) Isopropanol is hazardous and a flammable liquid. Use proper personal protective equipment. (2) Oil Red O solution can easily precipitate. The precipitated particles can interfere with subsequent staining. It is important to remove the precipitate by filtering the Oil Red O solution through a 0.45 µm filter before use. (3) It is best to prepare fresh Oil Red O solution and discard any unused solution. (4) In addition to Oil Red O, Sudan IV is another chemical compound used for staining of lipids, triglycerides, and lipoproteins. However, Oil Red O has gradually replaced Sudan IV because the red color produced by Oil Red O is more intense and can thus make fat much easier to see.

8.4. Stain the aorta for 60 min at room temperature (RT). Oil Red O will stain lipid-rich plaque red, leaving other non-plaque containing areas pale in color.

8.5. Wash once for 20 min with 60% isopropanol at RT.

NOTE: Over-rinsing can destain the plaque.

8.6. Rinse the aorta 3x with dH_2O for 5 min to remove isopropanol.

8.7. Under a stereomicroscope, gently clean all perivascular adipose tissue around the aorta using tweezers (Style 4) and spring scissors (**Figure 3C,D**).

NOTE: It is important to clean all perivascular adipose tissue around the aorta and its branches after staining, because Oil Red O-stained perivascular adipose tissue can yield false background and interfere with plaque morphometry and plaque area quantification. Make sure not to remove a portion of the aortic wall. Fill the wax dish with dH_2O until it covers the stained aorta during cleaning. This step requires practice and patience.

8.8. Transfer the vessel to a clean, glass microscope slide.

8.9. Acquire digital micrographs using a camera connected to a light microscope. Save high resolution images, preferably in tagged image file format (TIFF) (**Figure 3E**).

NOTE: The protocol can be paused here. To prevent the aorta from drying, transfer the vessel into a 1.5 mL microcentrifuge tube and fill with 1x DPBS until it covers the aorta. Store at 4 °C.

9. En face aorta mounting (Figure 4, Figure 5)

9.1. Transfer the vessel to a wax Petri dish and fill with 1x DPBS until it covers the aorta.

9.2. Sever the carotid, subclavian arteries of the aortic arch and iliac arteries in the abdominal aorta 1–2 mm after bifurcations. Sever the renal arteries. (**Figure 4A**)

9.3. Longitudinally cut open the aorta preparation along the inner curvature (**Figure 4B1**) and alone iliac arteries (**Figure 4B2**) with micro-dissecting spring scissors.

9.4. Cut open the three branches of the aortic arch (i.e., innominate, left common carotid, left subclavian artery) along the greater curvature until the base level of inner curvature (x-mark) (**Figure 4B3–B8**) with micro-dissecting spring scissors.

9.5. Pin the aorta flat (lumen side face-up) in a wax dish with minutien pins and apply 1x DPBS until it covers the aorta to prevent it from drying (**Figure 4C**).

NOTE: (1) It is important to make the rolled-up aorta flat and pin it en face without stretching. This step will take a few days depending on the severity of atherosclerosis. (2) For aortas from $Apoe^{-/-}$ or $Ldlr^{-/-}$ animals, it is recommended to pin the aorta flat for 24 h. (3) The protocol can be paused here.

9.6. Clean the glass microscope slides with 70% ethanol and delicate task wipers (**Figure 5A**).

9.7. Transfer the aorta into a clean glass microscope slide and put 15 drops of optimal cutting temperature (OCT) compound onto another clean glass microscope slide (**Figure 5B**).

9.8. Carefully place the glass microscope slide with OCT compound over the aorta and avoid trapping air bubbles on the slide (**Figure 5C**).

9.9. Label the slides with sample names (**Figure 5D**).

NOTE: The mounted en face aorta slides can be stored in the moisture chamber at 4 °C for several months.

10. Imaging and lesion quantification of en face aorta (Figure 6)

10.1. Acquire digital micrographs using a camera connected to a light microscope. Save high resolution images, preferably in tagged image file format (TIFF) (**Figure 6A**).

10.2. Transfer images of the en face stained whole aorta to a computer equipped with ImageJ software.

10.3. In ImageJ, select the “Freehand selection” tool and circle all Oil Red O-stained plaque manually (intense red spots) while pressing the “Alt” key (for PC) or “Shift” key (for Mac). Then, click “Measure” in the “Analyze” menu to display lesion areas in the result window (**Figure 6B left**).

NOTE: There are several pitfalls of the quantification of atherosclerotic lesions: (1) any small pieces of stained adventitial fat that remained attached to the aorta from step 8.7 can yield false background and interfere with plaque quantification; (2) removing a portion of the aortic wall or damaging the aorta from steps 6.4 and 8.7 can interfere with plaque quantification; (3) bubbles and folds formed in the aorta after mounting (step 9.8) can interfere with plaque

quantification; and (4) atherosclerotic plaque is a 3D phenomenon, and measurements performed in a 2D plane may not reflect the true extent of the plaque. In addition to analysis of the en face aorta plaque area, it is recommended to analyze plaque size in the aortic root, brachiocephalic artery, ascending aorta, and abdominal aorta separately[8].

10.4. Circle the outer border line of the aorta and click "Measure" in the "Analyze" menu to display the aorta area in the result window (**Figure 6B right**).

10.5. Export all measurements to an Excel file.

10.6. Calculate the ratio of plaque area from the total aorta area and normalize the value as the percentage of total Oil Red O surface area.

10.7. Calculate the ratio of plaque area in 8–10 $Apoe^{-/-}$ and 8–10 $TGF\beta R2^{iSMC\text{-}Apoe}$ mice. Present the data as mean ± SEM (**Figure 6C**).

10.8. Perform an unpaired Student's t-test for statistical analysis of the ratio of plaque area data compared to another mouse group. Consider the differences in mean values as significant at $p < 0.05$.

REPRESENTATIVE RESULTS:

In this protocol, atherosclerotic lesions in $TGF\beta R2^{iSMC\text{-}Apoe}$ mice were analyzed after 4 months on a HCHF diet[7]. In addition to extensive atherosclerosis, these mice developed both thoracic and abdominal aortic aneurysms, as previously reported. Compared to $Apoe^{-/-}$ mice, $TGF\beta R2^{iSMC\text{-}Apoe}$ mice aortic walls showed severe atherosclerosis, making it difficult to dissect the lesions (**Figure 2C,D,E**). In addition, the aneurysms wer particularly extensive below the suprarenal aorta, highly reminiscent of advanced human aortic aneurysms.

A representative unopened aorta Oil Red O staining image from HCHFD-fed $TGF\beta R2^{iSMC\text{-}Apoe}$ mouse is shown in **Figure 3E**. The image shows a $TGF\beta R2^{iSMC\text{-}Apoe}$ mouse that developed both ascending and abdominal aortic aneurysm, and it shows accelerated atherosclerotic lesion formation in aorta branches (here, the brachiocephalic artery, carotid artery, subclavian arteries, iliac arteries, femoral arteries, and renal arteries).

Figure 6A shows the en face Oil Red O staining image of $Apoe^{-/-}$ and $TGF\beta R2^{iSMC\text{-}Apoe}$ mice. Compared to the $Apoe^{-/-}$ group, $TGF\beta R2^{iSMC\text{-}Apoe}$ mice exhibited severe aneurysmal enlargement and marked elongation of the entire aorta.

FIGURE AND TABLE LEGENDS:

Figure 1: Dissection tools used in the protocol.

Figure 2: Step-by-step protocol for excision of aorta from mouse on HCHF diet. This is from a 24-week old TGFβR2$^{iSMC\text{-}Apoe}$ mouse fed for 4 months on a high cholesterol, high fat (HCHF) diet**.** (**A**) Mouse under ketamine/xylene anesthesia. Dashed lines indicate where to cut the skin. (**B**) Dissection of the mouse to expose the thoracic and abdominal cavities. (**C**) Careful removal of the internal organs (i.e., lung, liver, spleen, and gastrointestinal and reproductive organs) followed by exposure of the mouse aorta under a dissection microscope. (**D**) Careful removal of the connective tissues along the aorta as cleanly as possible. (**E**) Image of the isolated whole aorta with branches.

Figure 3: Step-by-step protocol for unopened aorta Oil Red O staining and imaging. (**A**) Pinning of the whole aorta with branches on a wax Petri dish. (**B**) Covering of the aorta with Oil Red O staining solution. (**C**) Illustration of the whole aorta after Oil Red O staining. (**D**) Illustration of Oil Red O-stained whole aorta after cleaning. (E) Representative photomicrographs of Oil Red O-stained whole aorta of TGFβR2$^{iSMC\text{-}Apoe}$ mice after 4 months on a HCHF diet. (**A'**) High magnification image of ascending aorta from (A), and (**B'**) high magnification image of abdominal aorta from (B).

Figure 4: Step-by-step protocol for en face aorta preparation. (**A**,**B**) The arterial tree stained with Oil Red O is opened longitudinally to flatten the aorta for imaging. Dotted lines along the vessel wall and numbers indicate sequential cuts that are made to open up the vessels. (**C**) Longitudinally split and pinned whole aorta on a wax Petri dish in a Y-shape.

Figure 5: Step-by-step protocol for en face aorta mounting. (**A**) Gentle cleaning of the glass microscope slides with 70% ethanol and drying with clean laboratory wipes. (**B**) Application of OCT compound onto the surface of one glass microscope slide, then spreading of the en face aorta flat on the other glass microscope slide. (**C**) Gentle placement of the glass microscope slide with OCT compound on top of the en face aorta sample. (**D**) Labeling of the slide with the sample name.

Figure 6: Step-by-step protocol for en face aorta imaging and atherosclerotic lesion quantification. (**A**) Microphotographs of en face aortas from *Apoe*$^{-/-}$ and TGFβR2$^{iSMC\text{-}Apoe}$ mice after 4 months on a HCHF diet and stained with Oil Red O. (**B**) Images illustrating the process for computer-assisted quantification of atherosclerotic lesions. (**C**) Lesion area quantification: % lesion area refers to Oil Red O-stained as a % of the total aortic surface. All data shown as mean ± SEM (***$p < 0.001$; unpaired two-tailed Student's t-test; for each timepoint, n = 9 for *Apoe*$^{-/-}$ mice and n = 9 for TGFβR2$^{iSMC\text{-}Apoe}$ mice).

Table 1: Genotyping primers.

DISCUSSION:

Apolipoprotein E (*Apoe*) and low density lipoprotein receptor (*Ldlr*) deficient mice are useful for studying development and treatment of atherosclerosis. Investigators can evaluate the impact of genetics and therapeutic manipulations on atherosclerosis-related diseases initiation,

progression, and regression using Oil Red O staining of the whole aorta[9]. Aorta Oil Red O staining and lesion quantification is the gold standard endpoint for atherosclerosis research. This technique is inexpensive and does not require special equipment[10]. However, it is not easy to obtain high quality Oil Red O-stained tissue. Based on prior experience, there are three critical steps in this protocol, and the whole procedure requires practice and patience. The first critical step is the ability to dissect, remove, and clean all perivascular adipose tissue around the aorta and its branches before and after Oil Red O staining (**Figure 2D, Figure 3C,D**). The second key step is the preparation of freshly made and filtered Oil Red O solution. Finally, it is important that the en face aorta lies flat on a wax dish before mounting onto the glass microscope slides (**Figure 4C, Figure 5B,C**).

In comparison with other Oil Red O staining protocols, this method provides qualitative and quantitative assessments of lipid-laden plaques in the unopened aorta and en face aorta from a single mouse. The initial qualitative assessment of the unopened Oil Red O staining provides a general idea about the plaque distribution and plaque size in aorta, as well as all the branches before quantification of the en face aorta. Limitations of the study are that (1) 2D comparison and analysis of 3D atherosclerotic plaques does not reflect the true extent of atherosclerotic plaque volumes, (2) atherosclerotic lesion quantification is time-consuming, and (3) it requires animal sacrifice.

After the aorta is successfully isolated, it can be used for a wide variety of assays for molecular studies. For example, it can be used for biomechanical studies and histological analysis to characterize reginal aorta morphology[11]. Additionally, users can isolate endothelial cells and smooth muscle cells from freshly isolated whole aorta for cell culture, FACS analysis, and single-cell RNA sequencing analysis. In summary, this protocol provides a step-by-step procedure to analyze atherosclerotic burden in mice. Investigators can use this protocol to compare atherosclerotic lesion abundance, location, and size between animals.

DISCLOSURES:

The authors declare no competing financial interests.

ACKNOWLEDGMENTS:

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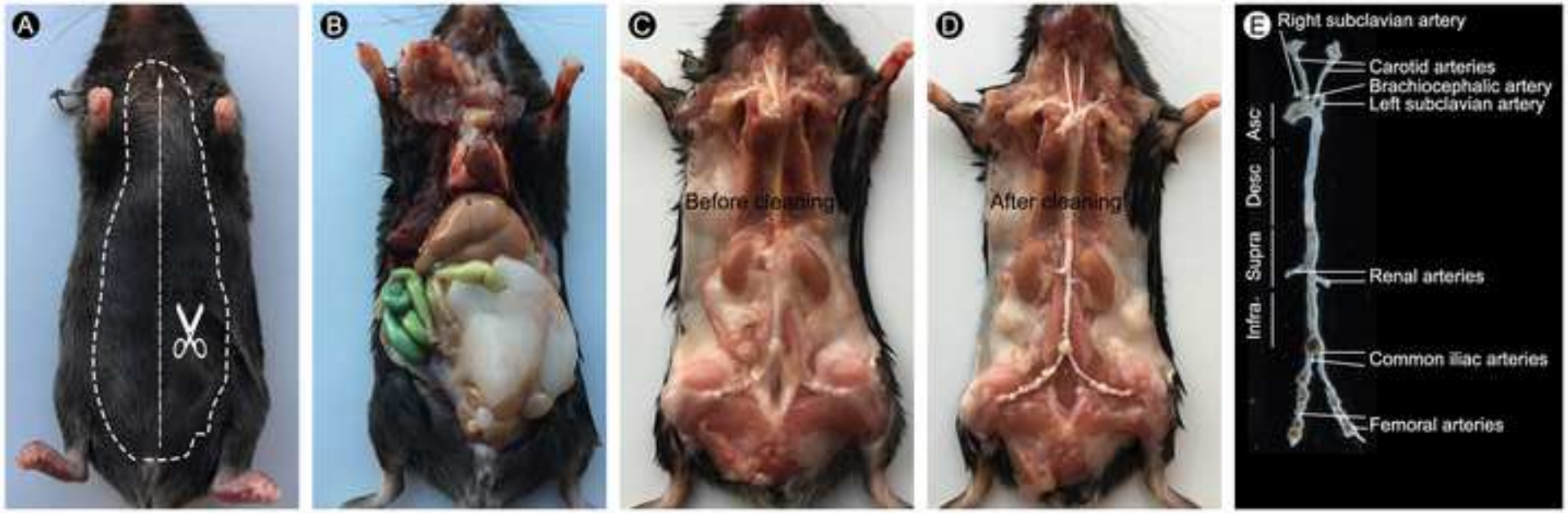
Figure

Figure



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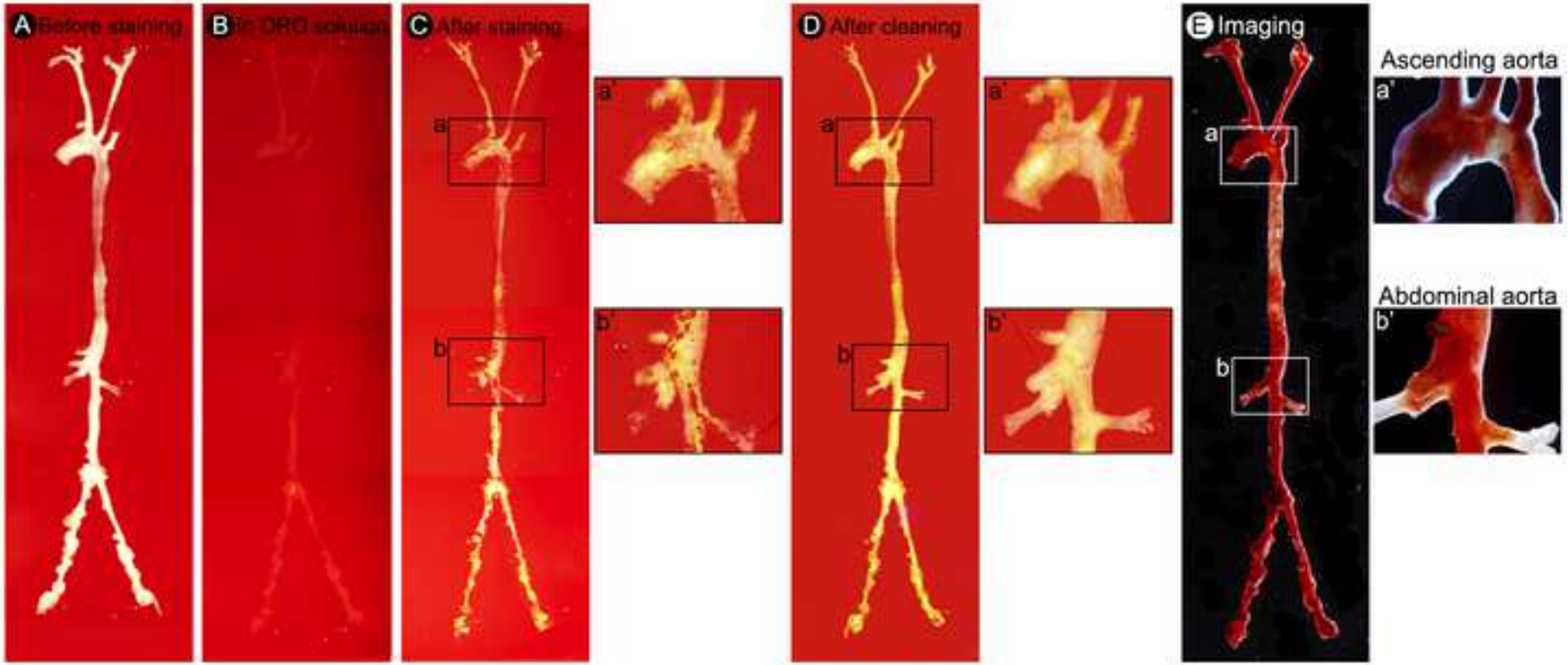

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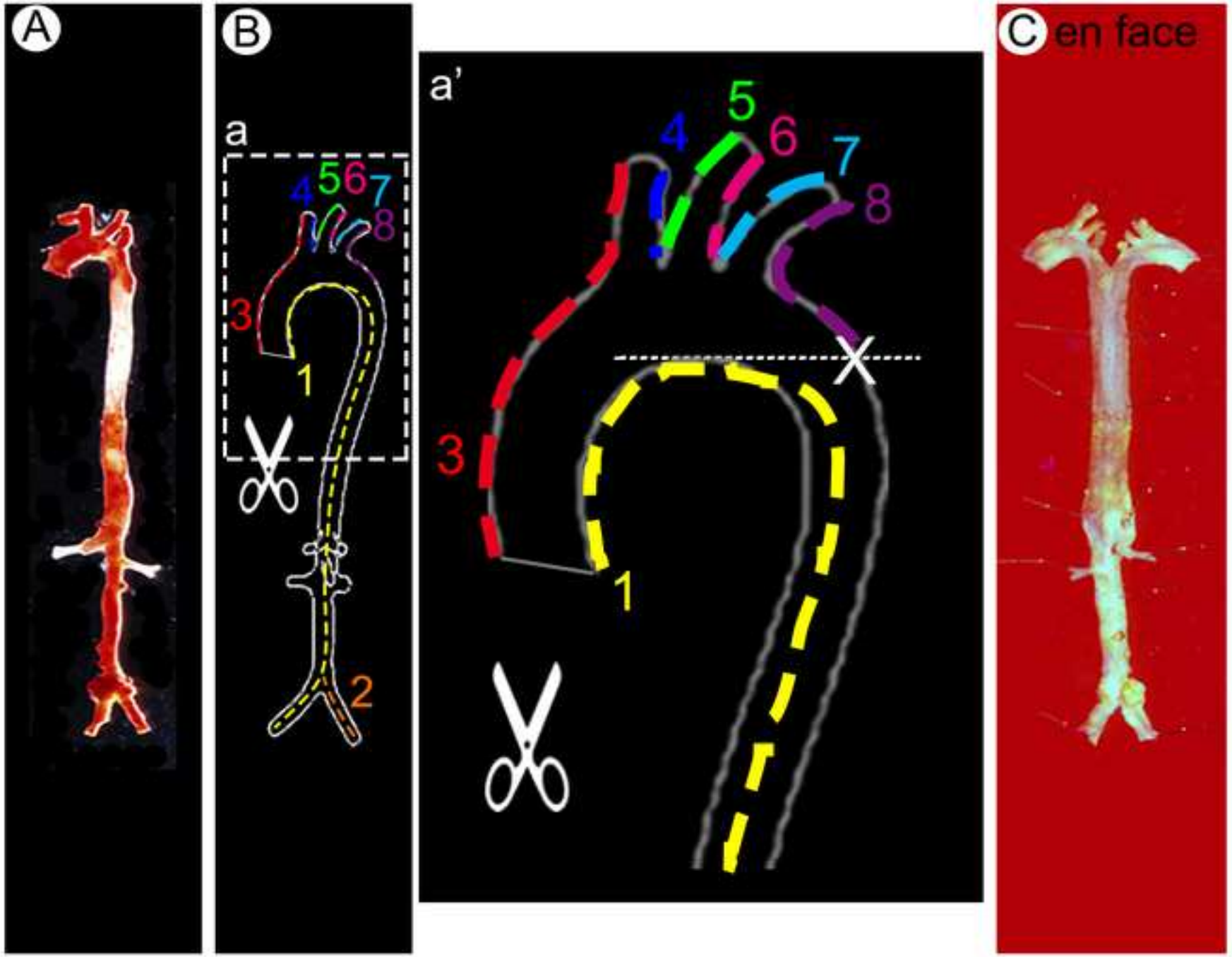

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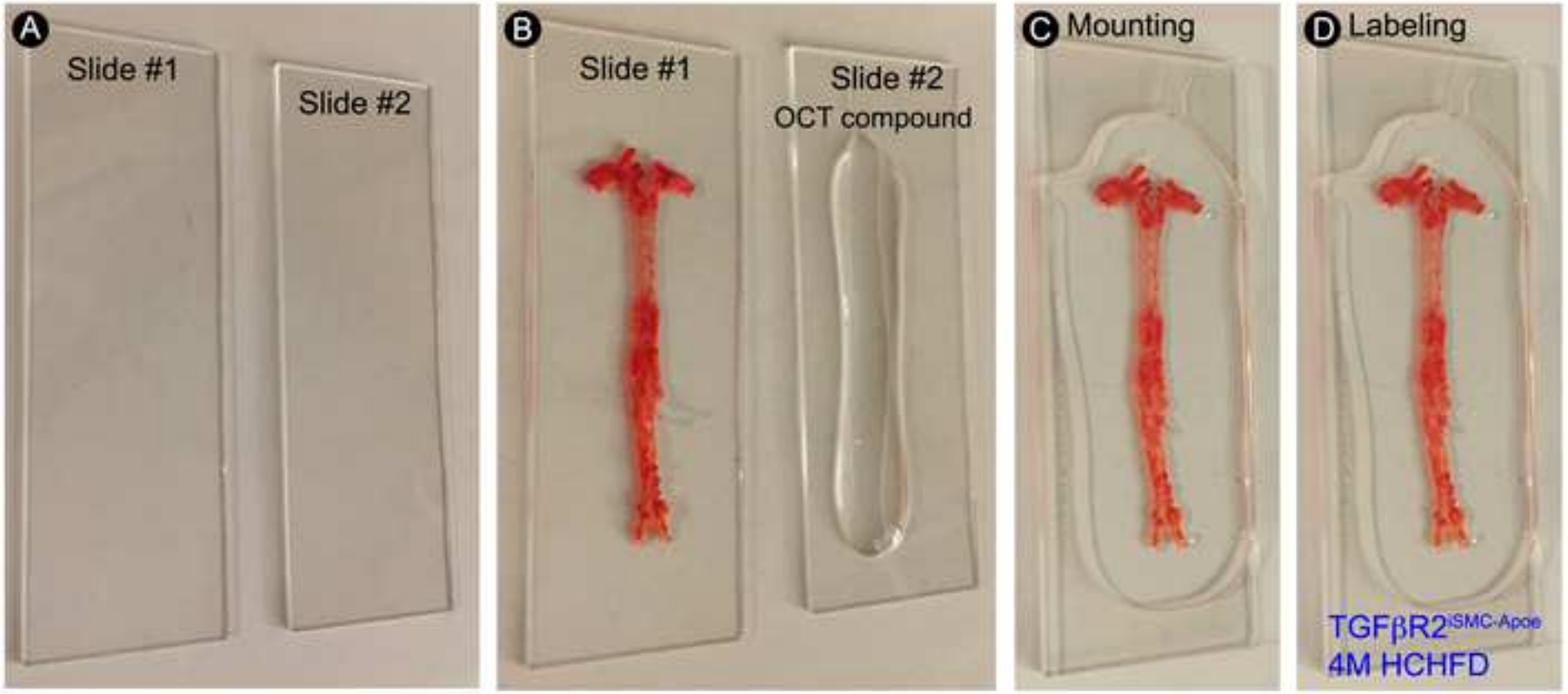

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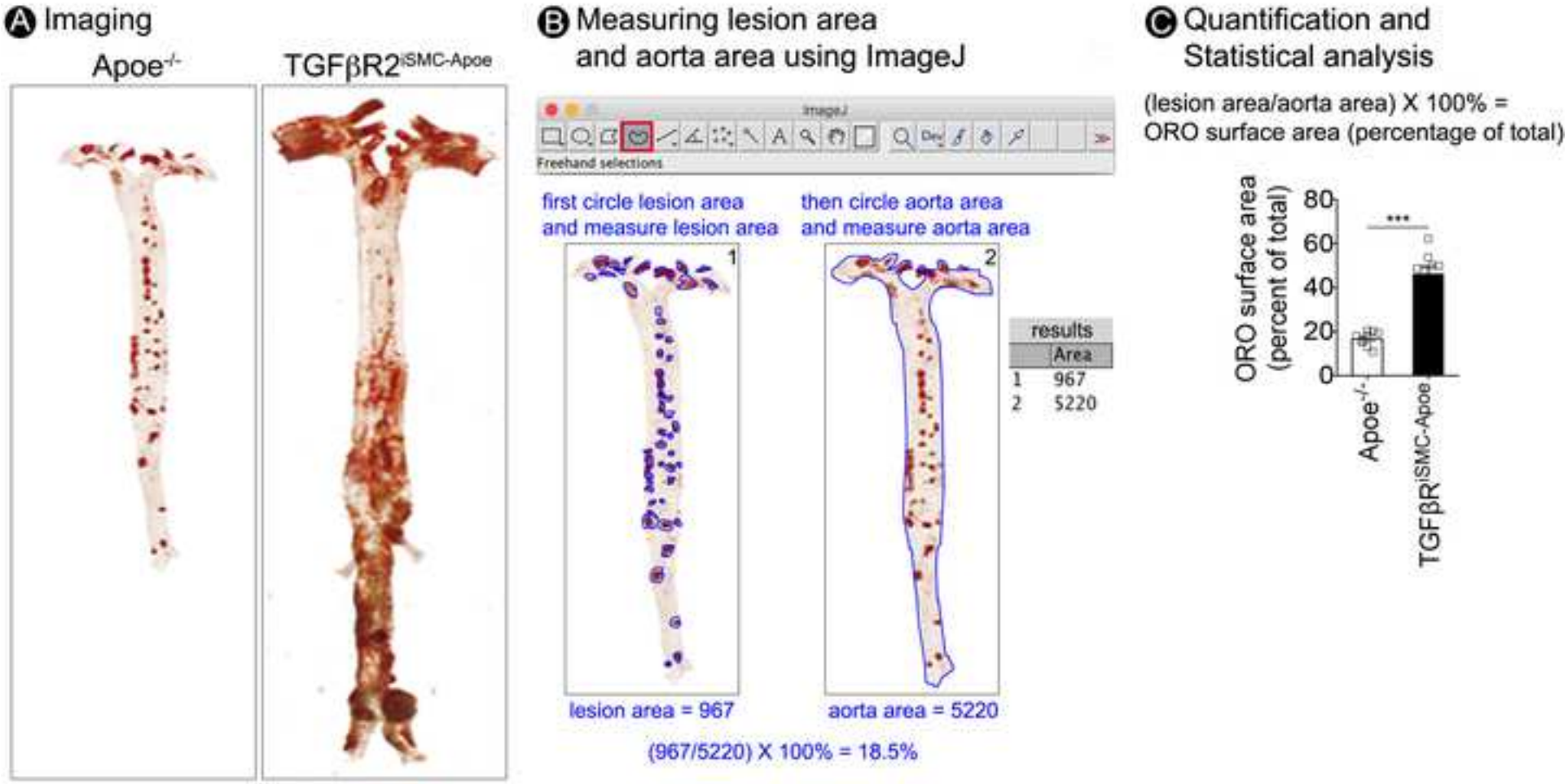

Table 1

MYH11-CreER^{T2}	5’-TGA CCC CAT CTC TTC ACT CC-3’
	5’-AAC TCC ACG ACC ACC TCA TC-3’
	5’-AGT CCC TCA CAT CCT CAG GTT-3’
Tgfbr2 $^{fl/fl}$	5’-TAA ACA AGG TCC GGA GCC CA-3’
	5’-ACT TCT GCA AGA GGT CCC CT-3’
Apoe	5’-GCC TAG CCG AGG GAG AGC CG-3’
	5’-TGT GAC TTG GGA GCT CTG CAG C-3’
	5’-GCC GCC CCG ACT GCA TCT-3’

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL Eppendorf tube	DENVILLE	C2170	
10 mL syringe	BD	302995	
16% Formaldehyde	Polysciences	18814-10	
70% ethanol	VWR	RC2546.70-5	To clean the dissection tools
Black dissection wax	CR Scientific	C3541	
Corn oil	Sigma	C8267	Solvent for Tamoxifen
DNeasy Blood & Tissue kit	QIAGEN	69506	To isolate DNA from mouse ear
Dulbecco's Phosphate-buffered saline (1X DPBS), pH 7.4	Gibco	14190-144	
Fine scissors	Fine Science Tools	14059-11	To cut the mouse skin and open the ribcage
Fisherbrand Economy Plain Glass Microscope Slides	Fisher Scientific	12-550-A3	
High cholesterol high fat diet	Research Diets	D12108	To induce atherosclerosis
Imaging software	National Institutes of Health	Image J	Aortic lesion quantification
Isopropanol	VWR	JT9079-5	
Kimwipes	Fisher Scientific	06-666A	To clean the glass microscope slides
McPherson-Vannas Micro Dissecting Spring Scissors	ROBOZ	RS-5602	To separate the heart and the aorta and to cut open the aorta and aorta branches
Microscope control software	Olympus	DP Controller	For aorta imaging
Minutien pins	Fine Science Tools	26002-10	
Needle-25G	BD	305124	
NonWoven Sponge	McKesson	94442000	
Oil Red O	Sigma	O-0625	To stain the atherosclerosis lesions
Pall Acrodisc Sterile Syringe Filters with Super Membrane	VWR	28143-312	To filter working Oil Red O solution
Spring Scissors	Fine Science Tools	15021-15	To dissect and clean the aorta
Statistical software	GraphPad	Prism 8	Statical analysis
Stereomicroscope	Nikon	SMZ1000	For aorta dissection
Stereomicroscope	Olympus	SZX16	For aorta imaging
Tamoxifen	Sigma	T5648	To induce Cre-loxP recombination
Tissue-Tek O.C.T Compound, Sakura Finetek	VWR	25608-930	
Tweezer Style 4	Electron Microscopy Sciences	0302-4-PO	To cut the mouse skin and open the ribcage
Tweezer Style 5	Electron Microscopy Sciences	0302-5-PO	To dissect and clean the aorta

We appreciate the Editor and reviewers' constructive comments on our manuscript ms. JoVE61277. The specific points raised by the Editor and reviewers were addressed as follows (The original reviewer comments are in italics and the replies are in a regular font):

Editorial comments:

General:

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

Reply: We have gone through our revised manuscript and corrected minor typing mistakes and grammatical errors.

2. Please include at least 6 key words or phrases.

Reply: We added 3 additional key words on page 1 of our revised manuscript.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: DNeasy, Eppendorf

Reply: We checked our revised manuscript and there are no trademark symbols (™) or registered symbols (®), and company names before an instrument or reagent in our manuscript.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Reply: We highlighted the filmable content in yellow.

2. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Reply: Yes. We answered all "how" questions in each step.

Specific Protocol steps:
1. 1.1: Where do the mice come from? Also, please provide more detail about genotyping or a citation.

Reply: We added mouse strain information, genotyping primer sequences and a reference on page 4 step 1&2 and Table of Materials.

2. 8.10: Please provide more detail about lesion quantification.

Reply: We added lesion quantification details on page 7 step 10 and Figure 6.

Figures:
1. Figures 2-5: Please provide descriptions of the panels (A, B, etc.) in the legends for these figures.

Reply: We added detailed information on page 10 Figure legends 2-6 in our revised manuscript.

*2. Figure 5D: Please explain what the error bars measure, as well as what '***' indicates (including the statistical test used).*

Reply: In the revised manuscript, Figure 5D become Figure 6C. We explained the error bars measure and added *'***'* information on page 10 Figure 6C Figure legend.

References:
1. Please do not abbreviate journal titles.

Reply: We used full journal name in our revised manuscript.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Reply: We included all materials and equipment used in the revised Table of Materials.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The manuscript describes a method for the dissection and staining of mouse aortas with Oil Red O for the visualization of atherosclerotic plaques. The unique aspect of this method is the wholemount staining and visualization of the aorta as well as the en face preparation which is required for quantification of plaque area.

Reply: We appreciate that the reviewer found our method unique.

Major Concerns:
Image quality should be better.

Reply: We apologize the low-resolution of the figures. We have uploaded all high-resolution figures (300 dpi) in our revised manuscript.

Minor Concerns:
The protocol needs more detail on methods of image capture and quantification as well any pitfalls to the quantification process. In addition, there should be a discussion of the use of Oil Red O over Sudan IV and which one is better and why in this particular protocol.

Reply: We added imaging and lesion quantification details on page 7 step 10 and Figure 6. We also discussed the pitfalls in the atherosclerotic lesion quantification process on page 8 step 10.3.
As the reviewer suggested, we added a description comparing Oil Red O vs. Sudan IV on page 6 step 8.3.

Reviewer #2:

Manuscript Summary:
Chen et al. describe a widely used protocol for the quantification of the atherosclerotic burden in mice using Oil Red O stained whole aortas.
The authors demonstrate the use of this protocol in conditional knock out mice with a specific deletion of the TGFβR2 gene in smooth muscle cells on a hyperlipidemic Apoe knock out background, which they have fed a high-cholesterol, high-fat diet for 16 weeks. Under these conditions, the mice develop atherosclerotic lesions throughout the vascular tree and, in addition, they develop aneurysms at different vascular locations. An initial and common approach in the field of experimental atherosclerosis is to quantify the atherosclerotic burden in

hypercholesterolemic mice using the en face Oil Red O staining procedure.

Reply: We appreciate the reviewer's helpful comments on our manuscript.

Major Concerns:

1. The scientific experiment in which they demonstrate the protocol is not thoroughly described and could potentially be flawed:
- What was the sex of the experimental mice? This is important since the Myh11-Cre gene is most often located on the Y-chromosome.

Reply: We appreciate the concern. We added the mouse strain information, genotyping primer sequences and a reference on page 4 step 1&2 and Table of Materials.

- Why were the TGFβR2iSMC-Apoe mice administered tamoxifen while the control Apoe-/- littermates were administered vehicle (corn oil) [point 1.2 in the protocol and line 246-248 in Representative results]? This makes two different manipulations between the intervention and the control group. TGFβR2(I/I) vs TGFβR2(Wt/Wt) genotypes AND tamoxifen vs vehicle injections. This is a crucial point in the performed experiment.

Reply: We apologize for the typo. Both $Apoe^{-/-}$ and $TGF\beta R2^{iSMC\text{-}Apoe}$ mice were received tamoxifen treatment. We corrected this information on page 4 step 2.2 and page 9 Results section.

2. The authors claim in the title that this protocol can be used for "imaging and analysis" of Oil Red O stained lesions. However, no protocol for obtaining images nor for the following quantification of the Oil Red O positive area is provided. This is a critical limitation for the protocol.

Reply: We added imaging and quantification details on page 7 step 10 and Figure 6.

3. The potential caution, if any, in the vessel-isolation procedure and assessment of plaque burden in aneurysmal and hypercholesterolaemic mice is not addressed.

Reply: We added critical steps on page 5 step 6.4, page 6 step 8.7, page 7 step 9.5, and page 8 step 10.3.

4. The resolution of the figures, in particular figure 3, is too low to show the details described and needed for this kind of protocol. However, figure 4 and 5 are well executed and give a good

overview of the main steps they cover.

Reply: We apologize the low-resolution of the figures. We have uploaded all high-resolution figures (300 dpi) in our revised manuscript.

5. What is the intention and purpose of the qualitative assessment of the unopened Oil Red O staining?

Reply: The initial qualitative assessment of the unopened Oil Red O staining gives us a general idea about the plaque distribution and plaque size in aorta and all the branches before quantification of the en face aorta. We added this information on page 11.

6. Figure legends are too brief and the different steps should be explained in more detail.

Reply: We added detailed information on page 10 Figure legends 2-6 in our revised manuscript.

7. The authors state that this protocol can be used in other species than mice [discussion, line 278], however, they show no data to support this. I am concerned that e.g. the incubation time for the Oil Red O staining could potentially differ in different animal species due to the anatomical variance in artery wall thickness and atherosclerotic plaque composition. At least this should be acknowledged in the discussion.

Reply: We appreciate the concern. We deleted this sentence.

8. Compared with my own personal experience, this is a quite time-consuming protocol since incubation time alone accounts for 80 min and then afterwards step 8.5 in the protocol can last up to "a few days", line 219. Why is that? Perhaps it has something to do with the fact that the Oil Red O staining is done on unopened vessels and therefore might require a longer incubation period compared to opened en face prepared vessels?

Reply: This is because aneurysmal and atherosclerotic vessels are prone to coil and fold. This process flattens the aorta tissues further and make it easier to mount the aorta. But for aortas from $Apoe^{-/-}$ or $Ldlr^{-/-}$ animals, we suggest the investigators to pin the aorta flat for 24 hrs. We added this information on page 7 step 9.5.

9. Are the aneurysmal and atherosclerotic vessels in combination more fragile and prone to coil than atherosclerotic vessels alone? If so, what steps are implemented to prevent this in this protocol? This could be pointed out in the protocol and later on discussed.

Reply: Aneurysmal and atherosclerotic vessels are not very fragile but they are prone to fold and coil. We added critical steps on page 5 step 6.4, page 6 step 8.7, page 8 step 10.3, and page 11 Discussion section second paragraph.

10. Another potential application of the Oil Red O staining is quantification of the atherosclerotic lesion area in cross-sections of OCT-embedded plaques. This could also be described or discussed in the protocol and would significantly increase its usefulness.

Reply: We added this information on page 8 step 10.3.

Minor Concerns:
The above-mentioned major concerns need to be clarified first.

Reply: We understood. We clarified all major concerns.

Reviewer #3:

Manuscript Summary:
In this manuscript, Chen and coworkers describe a protocol for mouse atherosclerotic plaque size quantification based on en face Oil Red O staining of aortae.

Major Concerns:

- *The manuscript lacks a description of the devices and settings used to image en face aortae. It also lacks a description of the analytical procedure for plaque size quantification and representative images of this analysis.*

Reply: We added imaging and quantification details on page 7 step 10 and Figure 6.

- *The manuscript would benefit from a more thorough discussion of limitations and alternative approaches. For instance, one of the limitations of this technique is the lack of assessment of plaque volumes, plaques are obviously 3D, but this is a 2D analysis; this should be mentioned. Similarly, there are several ways of dissecting the aorta and analyzing en face atherosclerosis. The one described here is OK, but It would help the reader to cite other alternatives (e.g. Andres-Manzano et al, https://doi.org/10.1007/978-1-4939-2929-0_5)*

Reply: We added the plaque volume limitation in our revised manuscript on page 8 step 10.3 and page 11 Discussion section third paragraph. As the reviewer suggested, we added the reference on page 8 step 10.3.

Minor Concerns:

- It would be useful to mention that atherosclerosis develops at very different rates in the different aortic segments, and, therefore, it is sometimes advisable to analyze plaque size separately in the aortic arch, the descending thoracis aorta, the abdominal aorta, etc.

Reply: We added this information on page 8 step 10.3.

Reviewer #4:

This is a nicely written article from Simons Lab describing the imaging and analysis of Oil Red O stained whole aorta lesions in an aneurysmal/hyperlipidemia mouse model.

Reply: We appreciate the positive assessment of the study.

The reviewer has no major concerns.

1. However, there are some grammatical errors, which need to be fixed. E.g.
Commonly used mouse models
Because a therosclerotic lesions
aortic tree, our whole aorta
freshly isolated whole aorta……etc.

Reply: We apologize for the grammar mistakes and corrected all the grammatical errors.

2. What is the significance if oil red O staining in AAA?
Does oil red O stains only foam cells? Are foam cells prevalent in AAA too? Is there a better way to differentiate between atherosclerosis and AAA.

Reply: Oil Red O not only stains foam cells but also smooth muscle cells. AAA have foam cells but also have lots of smooth muscle cells in the intima. HE and EVG staining can distinguish the differences between atherosclerosis and aneurysm. Atherosclerotic arteries have neointima formation. Aneurysmic arteries have media layer elastin broken down.