En face preparation and quantification of aortic surface area covered by atherosclerotic lesions

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Summary: This protocol describes how the AMDCC members prepare the En Face staining for atherosclerotic lesions quantification.

Reagents and Materials:

Reagent/Material	Quantity Required	Vendor	Stock Number
Dissecting microscope			
Microdissection kit			
Phosphate Buffered Saline			
Glass Microscope slides			
Large glass cover slips			
Buffered Formalin			
Parafilm			
Dish with dark blue bottom			
Digital Camera			
Image Pro Plus software			

Protocol:

Aortic Surface Lesion Area Quantitation by the En Face Method:

1. Mice are fasted prior to sacrifice.
2. The mice are weighed prior to euthanasia.
3. The mice are euthanized with an overdose of isoflurane (death is noted by the lack of respiration).
4. The abdominal cavity is opened using a midline incision and transverse cuts are made in the abdominal wall to improve drainage of the perfusate.
5. Blood is collected from the abdominal vena cava using a heparinized 1 ml syringe and a 1.5 inch 22 gauge needle. (The needle is bent away from the bevel to ease the

insertion of the needle). The needle is pushed up through the vessel after collection of the blood and the intervening section of the vena cava scrapped with a scalpel to provide drainage.

6. Blood is placed into a chilled heparinized tube and the sample is centrifuged for 5 minutes at 10,000g.
7. The thoracic cavity is opened by cutting the ribs laterally to the sternum, with the sternum being retracted toward the head. The diaphragm is cut next to the ribs to improve drainage.
8. A 22-gauge needle attached to a gravity perfusion set up is inserted into the left ventricle and PBS containing 0.5 mM EDTA is started. The PBS flush is continued until the blood is removed (perfusate coming from the vena cava is clear).
9. The perfusion is now changed to a 4% paraformaldehyde, 7.5% sucrose, and 0.5 mM EDTA solution in PBS for 15 minutes.
10. The animal then flushed for a short time with PBS/EDTA solution to remove the paraformaldehyde from the lumen for the vessel.
11. The ribs, lungs, gastrointestinal and reproductive systems are removed, leaving the heart and kidneys *in situ*. The liver and spleen are weighed and along with a piece of the pancreas are saved in the fixative solution.
12. The mouse is taped to the dissection pad over the front and hind limbs allowing access to the body cavity.
13. The aorta is dissected out under the dissection microscope using mini-Vanna scissors and forceps. The aorta is dissected out from the heart until 3-5 mm after the iliac bifurcation. All the major arteries are branching off the aorta are left in place until later, excepting the gastric and hepatic arteries that were previously severed when the GI tract was removed. All adventitial tissue is removed by careful dissection.
14. The vessel is split *in situ* by making a small transverse cut partially through the iliac artery to allow scissors to be inserted into the lumen of the artery. The cut proceeds anteriorly until the kidneys are reached and then the other iliac artery is split. The cut is then continued anteriorly being angled at the aortic arch to the inside curve of the arch until the heart is reached. The heart is now removed and weighed, being saved with the other organs. The right and common carotid arteries are then severed close to the aorta and the scissors are placed into the right carotid towards the common carotid to begin a second cut, this cut allows the arch to lay flat the pinned. This second cut through the carotids and the innominant (brachiocephalic) artery until the aorta is split. At this point the aortic arch will resemble a Y. The renal arteries are now severed close to the aorta and the kidneys are removed from their capsules, weighed and saved. The iliac arteries are severed 3 mm past the bifurcation and the aorta removed.
15. The arteries can now be stored in PBS in the refrigerator or pinned and imaged.
16. The aortae are pinned onto a standard black wax dissection pan using 0.15 mm-black anodized pins (Fine Science Tools).
17. The pan is filled with PBS to prevent the vessels dehydrating and the vessels are pinned flat.
18. Sufficient pins will be used in order to make the vessel lay flat, typically requiring more pins in the arch than the thoracic and abdominal portions of the vessel. A slight tension is placed on the vessel when pinning to allow the vessel to lay flat.

Additionally, at this point any remaining adventitia must be removed from the vessels using forceps and scissors.

19. The pan containing the pinned vessels is stained using the following procedure. The PBS is drained and replaced by 70% ethanol for 5 minutes. The 70% ethanol is then drained and the Sudan IV solution is placed into the pan for 15 minutes. The Sudan IV is then drained and the vessels are destained using 80% ethanol for 3 minutes. After 3 minutes the 80% ethanol is drained and the stained vessels are rinsed under running water to remove all ethanol. The drained pan is then filled with filtered PBS (this removes particles that could interfere with the images) until the liquid level covers the pins completely.
20. A marker bar containing three 15mm segments with separate colors is pinned next to the vessel prior to obtaining the image to provide a delineation of the vessel into segments. The aortic arch thoracic segments are proximal to the diaphragm (above the take off of the hepatic artery). The abdominal aorta is distal to the diaphragm.

21. Images of the vessels are then obtained and the extent of atherosclerosis is determined using ImagePro software.