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Quantification of atherosclerosis at the aortic sinus

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We use this protocol and it's working

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Abstract

Summary:

This protocol describes the procedures for fixing, sectioning and staining the hearts of mice for signs of atherosclerosis. The following protocol covers each aspect of the procedure.

Diabetic Complication:

Materials

Reagents and Materials:

- Buffered Formalin
- Embedding capsules
- Gelatin
- OCT
- Razor blades
- Cryosection microtome
- Microscope slides
- Water
- Isopropanol
- oil red-O stain
- Harris Hematoxylin
- Bluing Solution
- Light Green Solution
- Glycerol gelatin
- Image Pro software
- Microscope with video imaging system

1 **Preparation of Aortic Root and Brachiocephalic Artery for Lesion Quantitation:**

1. Prepare a 1 ml syringe by filling with 15µl 0.5 M EDTA ph 8.0 and then capping with a 23g needle. The syringe will be used for drawing blood from the heart.

2. Prepare a 10 ml syringe with 50-100 µl of heparin (1000 units per ml), then fill the syringe to 10 ml with PBS. Remove all the air from the syringe. Cap with a 30g needle. The syringe will be used to flush the heart.

3. Anesthetize the mouse.

4. Determine weight and length of the mouse.

5. Tape the mouse's arms to 3-4 layers of paper towels and place under a light source.

6. Cut the skin of the mouse from the abdomen to the top of the thorax.

7. Open the abdominal wall below the ribcage.

8. Lift the sternum with tweezers and cut the diaphragm. Then cut away the lower part of the ribcage to partially expose the heart

9. Draw blood from the heart by sticking the needle of the 1 ml syringe (see 1) into the apex of the left ventricle. Draw blood by slowly pulling the plunger, twisting the needle. Do not draw for over a minute to avoid blood clots. Transfer the blood into an Eppendorf tube. Mix the tube by inverting it a few times. Centrifuge the tube of blood and transfer the plasma into a fresh tube.

10. Make a small incision in the right atrium for drainage.

11. Stick the 10 ml syringe into the apex of the left ventricle and flush the blood from the mouse with 10 ml of PBS.

12. If the liver is to be saved take several sections and immediately freeze at –80°C.

13. Dry the mouse by turning it over and pressing it against paper towels. Transfer to clean, dry paper towels and re-tape the arms.

14. Place the mouse under a dissecting microscope.

15. Remove the remaining ventral part of the ribcage, carefully cut the right clavicle leaving the brachiocephalc artery and its branches intact. Using microdissection scissors, cut out all of the fat around the ascending aorta and brachiocephalic artery.

16. Cut all the fat and tissue surrounding the heart, including the pulmonary artery, and veins.

17. Flush the heart again through the right ventricle, left atrium, and left ventricle using a 10 ml syringe containing a total of 3 ml of PBS to clean out residual blood.

18. Cut the brachiocephalic artery distal to where it branches into the right subclavian and right carotid and then cut it at its branching site from the aorta so you end up with a Y-shaped piece. Put it in a base mold (Fisher cat no 22-038217) and using a 10 ml syringe perfuse it with 1 ml of PBS to get it clean. It is important while handling the brachiocephalic artery to touch it only at its ends and not in the middle.

19. Cut the aorta proximal to the branching site of the brachiocephalic artery.

20. Take out the heart with the aorta. The atria must remain intact as they serve as landmarks when cutting the heart in the atherosclerosis assay.

21. Flush the heart-aorta preparation through the aorta with a 10 ml syringe containing 2 ml of PBS and place the heart in a 20 ml scintillation vial containing 10 ml of formaldehyde solution (10% v/v in aqueous phosphate buffer, Mallinckrodt).

22. Take the cleaned piece of the brachiocephalic artery and place it in a new base mold, fill it up to the first lip of the base mold with Tissue Tek O.C.T. compound. Move the brachiocephalic artery around carefully to make sure it fills with OCT.

23. Turn the Y-shaped piece of brachiocephaic artery piece so the prongs of the fork face the bottom of the base mold and freeze it in that position on a block of dry ice.

2 **Embedding Hearts in Gelatin for Aortic Root Analysis:**

1. Fix hearts in buffered formalin for 2 weeks at 4°C.

2. Place each heart in an embedding capsule; and place up to 40 capsules in a 1000 ml beaker. Wash under cold running tap water for 1 hour.

3. Make gelatin solutions containing 5%, 10% and 25% gelatin in H_20 and dissolve at 50°C in a water bath.

4. Place washed heart capsules in a shallow plastic container, float container in a 42°C water bath and cover heart capsules with 5% gelatin for 2 hours.

5. Pour back gelatin into a bottle (can be stored at 4°C for future use) and cover hearts with 10% gelatin for 2 hours.

6. Pour back gelatin into a bottle (can be stored at 4°C for future use) and cover hearts with 25% gelatin. Incubate in water bath over night.

7. Place container with heart capsules in refrigerator for 3 hours.

8. Remove heart capsules from solidified gelatin, discard gelatin.

9. Remove embedded hearts from capsules, trim gelatin around the heart to a cube and place trimmed hearts back into buffered formalin.

10. Store at 4°C for a minimum of 24 hours before sectioning the hearts.

3 **Sectioning Hearts for Aortic Root Analysis:**

1. Locate the left and right atria. Using a sharp razor-blade cut the bottom half of the heart off in a plane parallel to the atria. **NOTE:** It is essential that the gross cut is parallel to the atria so that a cross section of all three aortic valves is in the same geometric plane. Discard lower half of the heart.

2. Make a second cut above the top of the heart parallel to the first cut on the bottom.

3. Store the heart at 4°C in buffered formalin until ready to section.

4. Mount the trimmed heart on a sample pedestal in the cryostat using OCT compound, the aortic side of the block facing the pedestal.

5. Cover the rest of the gelatin block with OCT and let it freeze.

6. Set section thickness to 30µm and start sectioning until the part of the block that contains the heart tissue is visible. If a complete heart section is not visible, reposition the pedestal.

7. Once a complete heart section is visible, place it on a slide and to check the anatomic location of the section (initially you will see just a thick muscular wall).
(**note**: to flatten the section for better viewing under the microscope dip the slide in water).

8. Cut approx. 20 of these 30µm sections and check under the microscope to see if the aortic sinus approaches. The presence of the left and right atria suggests that the sinus is close. The aortic sinus will usually appear rounded, the aortic wall won't be thick yet and the valves won't be distinct. Start saving the sections when the valve leaflets become clearly visible. (Reposition the pedestal if necessary to have all 3 leaflets of the valve in one plane)

9. Change thickness of sections to 12 µm and save every other section. Put 4 sections on one slide in this order, and label mouse and slide no:

1	2	mouse no.
3	4	slide no.

10. Cut and save sections until the aorta is rounded and all of the valve leaflets are gone. Usually, this is after 20 to 24 sections (5 to 6 slides).

11. Place slides in a covered glass dish with formalin vapor (put some formalin on bottom of dish) and store slides at 4°C for at least 12 hours prior to staining.

4 **Staining Heart Sections for Aortic Root Analysis:**

1. 1 min. H_2O
2. 1 min. H_2O
3. 30 sec. 60% isopropanol
4. 18 min filtered oil red-O (0.24% in 62.5% 2-propanol)
5. 30 sec. 60% isopropanol rinse
6. 1 min. H_2O
7. 1 min. H_2O
8. 2 min Harris Hematoxylin
9. 1 min Bluing Solution (H_2O with a few drops of ammonia)
10. 1 min. H_2O
11. 30 sec. Light Green
12. 1 dip H_2O
13. Keep in H_2O until ready to put cover slip on the slide
14. Fix cover slips with glycerol gelatin
15. Wash slides to remove excess glycerol gelatin

5 **Sectioning Brachiocephalic Artery for Quantitation of Cross Sectional Lesional Area:**

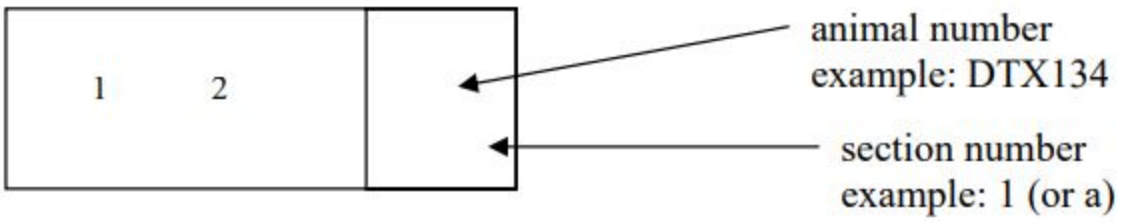
1. Set cutting temperature of microtome to −30°C. Mount specimens embedded in OCT on cutting pedestal, the opening of the Y shaped vessel should face towards the microtome cutting blade.

2. Set section thickness to 10µm. Start sectioning the right carotid and right subclavian arteries up to the point where they first combine into the brachiocephalic. A section of this point will not have the separation between the two vessels and will be considered the starting point for the following distances.

3. Once this point has been defined, cut 200µm discarding the sections.

4. Use Fisher Colorfrost plus slides to save sections

5. Save the following 2 sections (10µm each) on a the first slide (slide 1) in the following order:



6. Proceed sectioning and discard the following 80µm (8 sections)

7. Save the next 2 sections (10µm, each) → slide 2

8. Proceed sectioning and discard the following 80µm (8 sections)

9. Save the next 2 sections (10µm, each) → slide 3

10. Proceed sectioning and discard the following 80µm (8 sections)

11. Save the next 2 sections (10µm, each) → slide 4

12. Save 4 additional sections (10µm each), one per slide → slides a,b,c,d
The purpose is to have consecutive sections available for immunostaining. These sections are not relevant for

quantification and can be discarded if immunostaining will not be done.

13. Proceed sectioning and discard the following 40µm (4 sections)

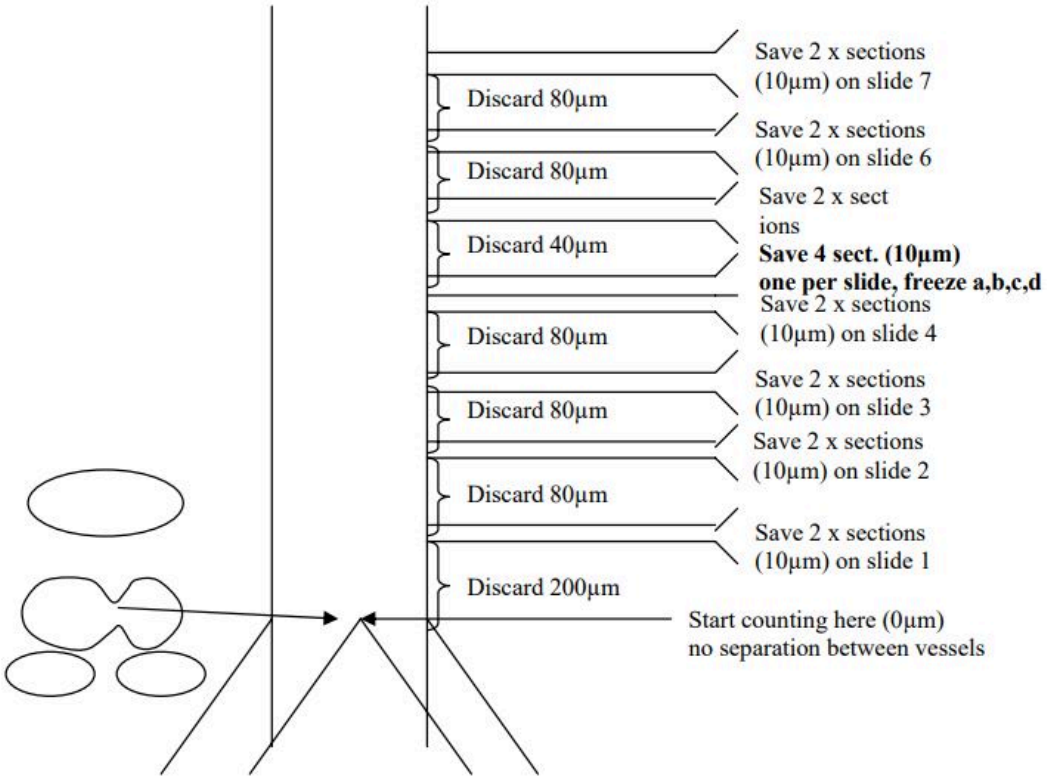
14. Save the next 2 sections (10µm, each) → slide 5

15. Proceed sectioning and discard the following 80µm (8 sections)

16. Save the next 2 sections (10µm, each) →slide 6

17. Proceed sectioning and discard the following 80µm (8 sections)

18. Save the next 2 sections (10µm, each) →slide 7



6 Staining Brachiocephalic Artery Sections for Lesion Quantitation:

a. 1 min. H_2O

b. 1 min. H_2O

c. 30 sec. 60% isopropanol

d. 18 min filtered oil red-O (0.24% in 62.5% 2-propanol)
e. 30 sec. 60% isopropanol rinse
f. 1 min. H_2O
g. 1 min. H_2O
h. 2 min Harris Hematoxylin
i. 1 min Bluing Solution (H_2O with a few drops of ammonia)
j. 1 min. H_2O
k. 30 sec. Light Green
l. 1 dip H_2O
m. Keep in H_2O until ready to put cover slip on the slide
n. Fix cover slips with glycerol gelatin
o. Wash slides to remove excess glycerol gelatin

7 **Quantification of Aortic Root and Brachiocephalic Artery Lesion Area by Image Pro Software:**

1. Use a microscope equipped with a video imaging system attached to a computer with Image Pro software

2. Quantify all 5 slides obtained from one aortic root and seven slides obtained from one brachiocephalic artery, preferentially use section number 1 on each slide. If section number 1 is not readable, go to the second section of the slide.

3. Measure only the area of oil red-O stained lesions on top of the internal elastic lamina, do not quantify any oil red-O stained material underneath the internal elastic lamina. In some cases, lesions may be sheared off the internal elastic lamina and are found in the lumen. Those lesions should also be counted.

4. The atherosclerotic lesion area is expressed as the mean of the lesion areas of one section per slide from the 5 aortic root slides or the 7 brachiocephalic artery slides.