

**Quantitative Assessment of Lesion Progression Phenotypes**  
**Final 8/09/05**

**I. Introduction:**

The document is an attempt by Core A (The Lesion Analysis Core) to provide a standard protocol for the assessment of lesion progression phenotypes for various mouse atherosclerosis models. The protocol is an amalgam of the methods currently in use in the Breslow and Tabas laboratories. In general the methods agree and will be presented as one. Occasionally, minor differences persist and both methods will be presented.

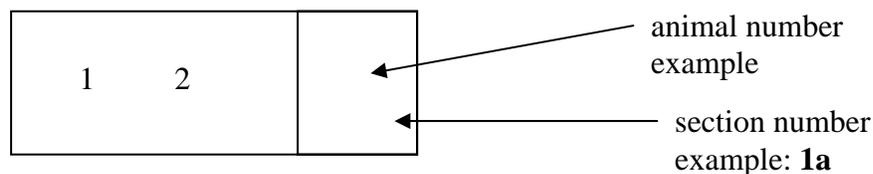
**II. Preparation of Hearts for Aortic Root Analysis:**

1. To obtain blood from the heart, draw up ~15 $\mu$ l of 0.5 M EDTA ph 8.0 into a 1 ml syringe with a 23 gauge needle attached (don't forget to recap). Tabas alternative: place 20  $\mu$ l of 100mM EDTA in PBS into an Eppendorf tube and prepare an empty 1 ml syringe with a 26 gauge needle.
2. To flush blood from the heart, draw up ~50-100  $\mu$ l of heparin (1000 units per ml) into a 10 ml syringe with a 30 gauge needle attached; remove the needle and then fill the syringe with 10 ml of PBS; remove all the air from the syringe; reattach the 30 gauge needle.
3. Anesthetize the mouse.
4. Weight the mouse (for metabolic studies measure the length of the mouse).
5. Tape the mouse's arms to 3-4 layers of paper towels and place under a light source (optional: swab the ventral area of the mouse with 70% ethanol).
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. To draw blood from the heart, stick the needle of the 1 ml syringe into the apex of the left ventricle. Slowly pull back on the syringe while rotating the needle. Keep extraction time to 1 minute to avoid blood clots. Empty the syringe into an Eppendorf tube. Invert the tube a few times for mixing. Spin for 10 minutes in a microfuge in the cold room, and carefully withdraw the clear plasma and place in a fresh Eppendorf tube.
10. To flush blood from the heart (and other organs), make a small incision in the right atrium for drainage.
11. Stick the needle of the 10 ml syringe into the apex of the left ventricle and flush the blood from the mouse using all 10 ml of PBS.
12. If the liver is to be saved, divide it into several sections with a scalpel and freeze them immediately at  $-80^{\circ}\text{C}$ .
13. Turn the mouse over and press it against paper towels for drying; place the mouse on clean, dry paper towels and re-tape the arms.
14. Cut away the remaining ventral part of the rib cage and place the mouse under a dissecting microscope.
15. Cut away the pulmonary artery and veins as well as the fat surrounding the heart.
16. Refill the 10 ml syringe with 5 ml of PBS and flush the heart again through the right ventricle, left atrium, and left ventricle to clean out any residual blood.

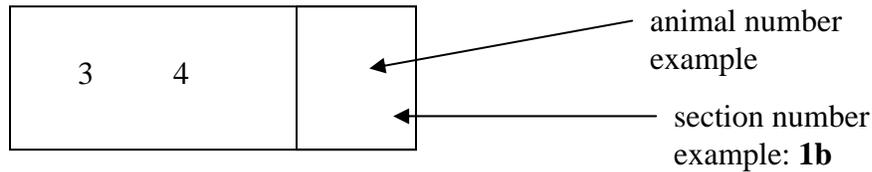
17. Remove the heart and the aorta; refill the 10 ml syringe with 2 ml of PBS and use it to flush the “heart-aorta” preparation through the aorta.
18. Locate the left and right atria. Using a sharp razor-blade, cut off and discard the bottom half of the heart in a plane parallel to the atria. NOTE: It 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.

### III. Sectioning of hearts embedded in OCT for aortic root analysis:

1. Place the upper half of the heart and aorta in Tissue Tek OCT and let it sit for 5 min at RT to allow the chambers inside the heart to be equilibrated with OCT.
2. Freeze the specimen on dry ice and place in  $-80^{\circ}\text{C}$  freezer until ready to section.
3. Set cutting temperature of microtome to  $-30^{\circ}\text{C}$ .
4. Mount hearts embedded in OCT on the cutting pedestal in the cryostat with the aortic side of the block facing the pedestal.
5. Set section thickness to  $30\mu\text{m}$  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.
6. Once a complete heart section is visible, place it on a slide to check the anatomic location of the section. (Note: to flatten the section for better viewing under the microscope, dip the slide in water).
7. Cut  $\sim 10$ - $20$  of these  $30\mu\text{m}$  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 will not be thick yet, and the valves will not 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.)
8. The Breslow Lab alternates cutting  $12\mu\text{m}$  thick sections that are saved and  $6\mu\text{m}$  thick sections that are discarded. It is felt that the  $6\mu\text{m}$  sections serve two purposes. After the  $12\mu\text{m}$  section is taken and looked at under the microscope, the  $6\mu\text{m}$  section can be used to reposition the pedestal (to have all 3 leaflets of the valve in one plane). It is also felt that the  $6\mu\text{m}$  section allows the cutter to take a better next  $12\mu\text{m}$  section. Although unstained and frozen  $6\mu\text{m}$  or  $12\mu\text{m}$  sections can be used for LCM, if an aorta is being sectioned solely for LCM  $8\mu\text{m}$  sections are taken and every one is saved. In the standard procedure put 2  $12\mu\text{m}$  sections on one slide in the order shown and label with mouse and slide numbers. Use Fisher Colorfrost Plus slides to save sections.
9. Save the first 2  $12\mu\text{m}$  sections on one slide (slide **1a**) and store in a covered glass dish with formalin vapors (put some formalin on bottom of dish) at  $4^{\circ}\text{C}$  for at least 12 hours prior to staining.



10. Save the next 2 12  $\mu\text{m}$  sections on another slide (slide **1b**) and freeze at  $-80^{\circ}\text{C}$ .



11. Cut until the aorta is rounded and all of the valve leaflets are gone. Usually, this is after 20 to 24 12  $\mu\text{m}$  sections (5-6 times slide **a**, and 5-6 times slide **b**). Save the a slides for oil-red O staining and the b slides for Movat's stain or immunohistochemistry.
12. You should end up with 12 slides as follows:
- Slide **1a**: 2 sections for oil-red O staining, stored in formalin vapor at  $4^{\circ}\text{C}$   
 Slide **1b**: 2 sections for Movat's stain or immunohistochemistry, stored at  $-80^{\circ}\text{C}$   
 Slide **2a**: 2 sections for oil-red O staining, stored in formalin vapor at  $4^{\circ}\text{C}$   
 Slide **2b**: 2 sections for Movat's stain or immunohistochemistry, stored at  $-80^{\circ}\text{C}$   
 Slide **3a**: 2 sections for oil-red O staining, stored in formalin vapor at  $4^{\circ}\text{C}$   
 Slide **3b**: 2 sections for Movat's stain or immunohistochemistry, stored at  $-80^{\circ}\text{C}$   
 Slide **4a**: 2 sections for oil-red O staining, stored in formalin vapor at  $4^{\circ}\text{C}$   
 Slide **4b**: 2 sections for Movat's stain or immunohistochemistry, stored at  $-80^{\circ}\text{C}$   
 Slide **5a**: 2 sections for oil-red O staining, stored in formalin vapor at  $4^{\circ}\text{C}$   
 Slide **5b**: 2 sections for Movat's stain or immunohistochemistry, stored at  $-80^{\circ}\text{C}$   
 Slide **6a**: 2 sections for oil-red O staining, stored in formalin vapor at  $4^{\circ}\text{C}$   
 Slide **6b**: 2 sections for Movat's stain or immunohistochemistry, stored at  $-80^{\circ}\text{C}$

**Tabas Lab Modifications: After section III subsection 7 “when the valve leaflets become clearly visible” proceed as follows:**

1. Change thickness of sections to 10  $\mu\text{m}$  (or 6  $\mu\text{m}$  for use with LCM) and save every section on Fisher Superfrost Plus slides.
2. Four sections are placed on a slide:
  - Slide 1a: sections 1, 3, 5, 7
  - Slide 1b: sections 2, 4, 6, 8
  - Slide 2a: sections 9, 11, 13, 15
  - Slide 2b: sections 10, 12, 14, 16
  - Slide 3a: sections 17, 19, 21, 23
  - Slide 3b: sections 18, 20, 22, 24
  - Slide 4a: sections 25, 27, 29, 31
  - Slide 4b: sections 26, 28, 30, 32
  - Slide 5a: sections 33, 35, 37, 39
  - Slide 5b: sections 34, 36, 38, 40
  - Slide 6a: sections 41, 43, 45, 47
  - Slide 6b: sections 42, 44, 46, 48
  - Slide 7a: sections 49, 51, 53, 55
  - Slide 7b: sections 50, 52, 54, 56
  - Slide 8a: sections 57, 59, 61, 63
  - Slide 8b: sections 58, 60, 62, 64

Slide 9a: sections 65, 67, 69, 71

Slide 9b: sections 66, 68, 70, 72

Slide 10a: sections 73, 75, 77, 79

Slide 10b: sections 74, 76, 78, 80

Slides 1a through 10a are stored in formalin vapors at 4<sup>0</sup>C and as in section IV below stained with ORO and counterstained with Hematoxylin. Slides 1b and 10b are stored at -80<sup>0</sup>C and can be used for other stains, immunohistochemistry or LCM.

**Note: The aortic root, as defined by the presence of all 3 aortic valve leaflets, is ~500 µm in length. The Breslow lab takes 24x 12µm plus 23x 6µm sections at the aortic root for a total length of 426 µm. Lesion area is determined by averaging the area on one section from each of 5 slides (slides 1a through 5a or 2a through 6a (depending on section quality). All of these sections fall within the aortic root. The Tabas lab takes 80x 10µm sections for a total length of 800 µm. This includes the aortic root and ascending aorta. Lesion area is determined by averaging the area on one section from each of 6 slides (1a through 6a). This encompasses a total length of ~480 µm corresponding to the aortic root. Slides 7a through 10a are examined for lesion characteristics of the ascending aortic arch but not used to quantify aortic root lesion area.**

#### **IV. Stain with ORO and counterstain with Hematoxylin for nuclei in aortic root sections of hearts embedded in OCT:**

1. This applies to the Breslow Lab slides 1a thru 6a and the Tabas lab slides 1a thru 10a, all slides fixed in formalin vapors as described in Section III.
2. Wash slides twice in H<sub>2</sub>O for 1 min each time
3. Expose slides to 60% isopropanol for 30 sec
4. Expose slides to filtered oil red-O (0.24% in 62.5% 2-propanol) for 18 min
5. Rinse slides in 60% isopropanol for 30 sec
6. Wash slides twice in H<sub>2</sub>O for 1 min each time.
7. Place slides in Harris Hematoxylin for 2 min
8. Place slides in Bluing Solution (H<sub>2</sub>O with a few drops of ammonia) for 1 min
9. Wash slides in H<sub>2</sub>O for 1 min.
10. Expose slides to Light Green for 30 sec
11. Dip slides in H<sub>2</sub>O and keep them there until ready to put on cover slip
12. Fix cover slips with glycerol gelatin
13. Wash slides to remove excess glycerol gelatin

#### **V. Quantification of cross-sectional lesion area in aortic root sections stained with ORO from hearts embedded in OCT**

1. Use a microscope equipped with a video imaging system attached to a computer with Image Pro software.
2. The area to be quantified begins where 3 distinct valve leaflets first appear at the start of the aorta. It ends at the section where the valve leaflets have completely disappeared.
3. There are 6 ORO stained slides from each aortic root. The Breslow lab quantifies 5 consecutive slides (either slide series 1a to 5a or 2a to 6a, depending on the presence of 3 distinct valve leaflets in the sections). They preferentially use the first section on each

slide, however, if this section is unreadable they use the second section on the same slide. The Tabas lab quantifies all 6 ORO stained slides from each aortic root.

4. Measure the area of the lesion on top of the internal elastic lamina, including oil red-O stained areas as well as unstained regions (acellular cores and the fibrous cap). 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.
5. In the Breslow lab the atherosclerotic lesion area is the mean of the lesion areas of one section per slide from 5 consecutive slides from one aortic root and in the Tabas lab the mean from 6 slides is used.

#### **VI. Sectioning of hearts embedded in paraffin for aortic root analysis:**

1. After section II subsection 18 place the specimen, which consists of the top half of the heart-aorta preparation, in a vial containing 5 ml of 10% buffered formalin over night.
2. The next morning place the formalinized specimen in a biopsy cassette and then into the Leica tissue processing machine (TP-1020) over night.
3. The next morning embed the processed tissue in paraffin blocks using the Leica EG 1160 machine.
4. Mount hearts embedded in paraffin on the cutting pedestal in the microtome with the aortic side of the block facing the pedestal.
5. Set section thickness to 30 $\mu$ m 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.
6. Once a complete heart section is visible, place it on a slide to check the anatomic location of the section.
7. Cut ~10-20 of these 30 $\mu$ m 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 will not be thick yet, and the valves will not be distinct.
8. When the valve leaflets become clearly visible reposition the pedestal (if necessary) to have all 3 leaflets of the valve in one plane.
9. Adjust the thickness of the sections to 6  $\mu$ m and start saving the sections
10. Put 2 sections on one slide and label with mouse and slide numbers. Use Fisher Superfrost Plus slides to save sections. Collect 50 sections on 25 slides.

#### **VII. Staining with H&E of aortic root sections of hearts embedded in paraffin:**

1. Pick up every fifth slide (Slide numbers 1, 5, 10, 15, 20 and 25) for H&E staining.
2. Heat the paraffin sections on the slides at 60C in an oven for 20 min to ensure the proper adhering of the tissue section to the glass slide.
3. Deparaffinise the sections by 3 changes in xylene-15 dips at each change or leave for 3 minutes each. Blot excess xylene before next step.
4. Transfer the sections to 100% ethanol, 2 changes-10 dips at each change or leave for 2 min. each.
5. Next 2 changes in 95% ethanol-10 dips or leave for 2 min. each.
6. Transfer to 80% ethanol-10 dips or leave for 2 min.

7. Wash in tap water (leave the slides in running water in plastic container) for about 1 min. Rinse in deionized water. Blot excess water.
8. Transfer to Harris Hematoxylin solution for 7 min.
9. Wash in tap water to remove excess hematoxylin. Leave in tap water for 2 minutes to allow the stains to develop. Rinse in deionized water.
10. Acid alcohol-one quick dip (acid alcohol is prepared by mixing 500 ml of 100% ethyl alcohol and 2.5 ml of concentrated HCl).
11. Rinse in tap water for 1 minute- 2 changes. Rinse in deionized water.
12. Ammonia water-one dip (gives blue color to the nuclei). Ammonia water is prepared by mixing 500 ml deionized water and 2.5 ml of ammonium hydroxide).
13. Rinse in tap water.
14. 95% ethanol-10 dips or leave for 2 minutes each.
15. Eosin-1 minute (30 seconds is enough in fresh eosin solution)
16. Two changes in 95% ethanol -15 dips or leave for 2 minutes each.
17. Two changes in 100% ethanol-15 dips or leave for 2 minutes each.
18. Three changes in xylenes-15 dips of leave for 3 minutes each (do not use the same set of xylenes used for deparaffinize the tissue earlier). The slides can also leave overnight in xylenes.
19. Wipe the slides for excess xylenes.
20. Place a drop of permount on the tissue using a pipette avoiding air bubbles. Angle the cover slip and let fall gently on the slide.
21. Allow the slides to dry in air for over night.

#### **VIII. Quantification of cross-sectional lesion area in aortic root sections stained with H&E from hearts embedded in paraffin:**

1. Quantify 6 consecutive slides from one aortic root.
2. Preferentially use the first section on each slide. If this is not readable, go to the next section on the same slide.
3. Quantify lesion area as well as the acellular (non-nuclear stained) area. It is also possible to divide the acellular area into fibrotic and necrotic areas and these can be quantified as well.
4. Average the 6 numbers for each mouse.

#### **IX. Other types of staining such as Movat's Pentachrome and immunohistochemistry:**

See Section III, subsection 12: The general plan for the six frozen slides containing aortic root sections from hearts embedded in OCT is that 2 be stained with Movat's Pentachrome stain to provide a general picture of lesion composition and the other 4 be stained for various purposes depending on the mouse model under study and to clarify lesion characteristics suggested by the initial oil red-O and Movat's stains.

See Section VI: The slides 2-4, 6-9, 11-14, 16-19, and 21-24 containing aortic root sections from hearts embedded in paraffin can also be used for Movat's and other stains.

These special stains can be used to obtain quantitative measures of lesion composition, expressed as absolute or relative area and cell number. For example, stained areas of interest in the Movat's Pentachrome stain quantified with the Image Pro software can be expressed as the mean of the areas of one section per slide from the 2 slides. The relative area can then be expressed as

the percentage of stained area of the total lesion area. The nuclei of a particular cell type can also be counted and expressed as a percentage of the total number of nuclei in the lesion.

#### **X. Movat's Pentachrome Stain:**

1. Take slides containing aortic root sections from hearts embedded in OCT from -80°C freezer and put them through hydration as follows (see beginning of Section VII): xylene (3 x 2 min), 100% EtOH (3 x 2 min), 95% EtOH (3 x 2 min), 70% EtOH (1 x 2 min), water (1 x 2 min). For aortic root sections of hearts embedded in paraffin take slides from Section VII subsection 7).
2. Preheat Alkaline Alcohol in 60°C oven
3. Mordant in pre-heated Bouin's solution in 50°C water bath for 15 minutes
4. Wash in running water for 10 minutes.
5. 5% sodium thiosulfate for 5 minutes.
6. Wash in running water for 5 minutes.
7. Stain in 1% Alcian Blue for 20 minutes.
8. Running water for 2-5 minutes.
9. Pre-heated Alkaline Alcohol (60°) for 10 minutes.
10. Running water for 2-5 minutes.
11. Stain in Weigerts Hematoxylin for 40 minutes
12. Wash briefly in running water, rinse well in distilled water.
13. Stain in Crocein Scarlet/Acid Fuchsin for 1 minute.
14. Rinse in distilled water.
15. 5% Phosphotungstic acid for 5 minutes.
16. Transfer directly to 1% acetic acid for 5 minutes.
17. Rinse well in distilled water.
18. Dehydrate – 1 x 95% EtOH x 1 minute  
2 x 100% EtOH x 1 minute
19. Stain in alcoholic saffron for 10 minutes.
20. Rinse – 2 x 100% EtOH x 1 minute.
21. Clear in xylene or histoclear 3 x 5 minutes.
22. Coverslip with VectaMount medium

#### **Reagents and Stains**

##### **Alkaline Alcohol**

95% EtOH	45 ml
conc. Ammonium hydroxide	5 ml

##### **1% Alcian Blue**

Alcian blue	1 g
Distilled water	100 ml

##### **Weigert's Iron Hematoxylin Set (Sigma)**

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##### **WORKING Weigert's solution**

Part A	25 ml
Part B	25 ml

##### **STOCK Crocein Scarlett**

Crocein scarlett MOO 3B	1 g
Distilled water	99.5 ml
Conc. glacial acetic acid	0.5 ml

**STOCK Acid Fuchsin**

Acid fuchsin	0.1 g
Distilled water	99.5 ml
Conc. Glacial acetic acid	0.5 ml

*Filter and let stand 24 - 48 hours.*

**WORKING Crocein Scarlett / Acid Fuchsin**

Stock Crocein Scarlett	40 ml
Stock Acid Fuchsin	10 ml

**5% Phosphotungstic Acid**

Phosphotungstic acid	5 g
Distilled water	100 ml

**1% Glacial Acetic Acid**

Conc. Glacial acetic acid	1 ml
Distilled water	99 ml

**Alcoholic Saffron**

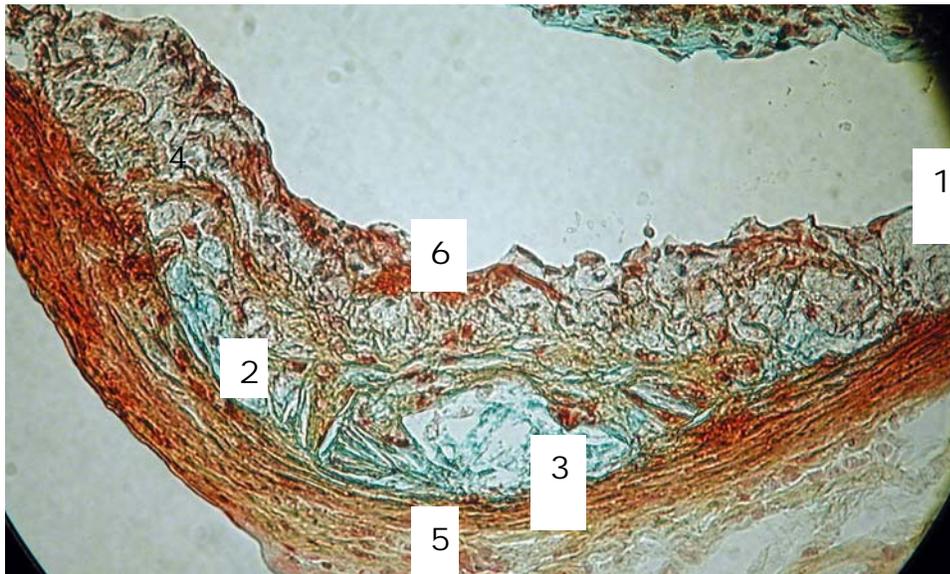
Saffron, spanish	6 g
100% EtOH	100 ml

*Place in air tight bottle in 55 – 60° oven for 48 hours to extract saffron. Cool the extract to room temperature. Filter through coarse filter paper in a covered funnel. Store in a dark, tightly-stoppered container.*

**5% Sodium Thiosulfate**

Sodium thiosulfate	5 g
Distilled water	100 ml

**Figure 1.** Movat's Stain advanced aortic root lesion



1. Lateral Xanthoma (foam cells)
2. Cholesterol Cleft
3. Necrotic Core
4. Fibrous Cap

5. Thinning Media and Discontinuous elastic lamina
6. Mixed Plaque (SMCs, foam cells, collagen fragments)

### **XI: Immunohistochemistry and other special stains:**

As summarized in table I, many special stains can be used to reveal features of lesions:

**Table I.**

<b>Feature</b>	<b>Stain</b>	<b>1°Ab/2°Ab (NovaRed substrate)</b>	<b>Appearance</b>
Monocyte-derived foam cells	CD68/macrosialin (immunochemistry)	MAC1957GA(Serotec) / HRP STAR72(Serotec)	Red
Cholesterol clefts	Movat's Pentachrome / Oil red-O		Blue with streaks / Red
Acellular core	Movat's Pentachrome / TUNEL or activated caspase-3		Blue or colorless / Fluorescence
Fibrous cap	Movat's Pentachrome		Yellow (for collagen)
Calcification*	Goldner's Modified Masson-Trichrome Stain		Green (Hydroxyapatite)
IEL	Movat's Pentachrome		Black (for elastin)
Smooth muscle cells	$\alpha$ -Actin (immunochemistry)	Anti-Actin IgG (Biomed Tech) / HRP STAR54 (Serotec)	Red
T-Lymphocytes*	CD3 (immunochemistry)		Red
Endothelial cells	CD31 (immunochemistry)	Anti-mCD31(BD Pharm) / HRP STAR72(Serotec)	Red
Lipids	Oil red-O		Red
Extracellular matrix components	Movat's Pentachrome		Yellow (collagen) Black (elastin) Blue (proteoglycans)

\* Protocol not yet implemented

### **XII: Immunohistochemical stain for macrophages-CD68**

1. Prepare the acetone with the dry ice (use the freezer at -20°C)
2. Remove slides from -80°C. Air dry sections for 10 minutes at room temperature
3. Fix them in ice cold acetone (-20°C) for 10 minutes
4. Let them dry.
5. Prepare the quench peroxidase solution (60ml PBS + 2ml of 30% Peroxidase), final concentration 1%.
6. Wash 3x in PBS for 5 minutes
7. Quench peroxidase activity with 1% H2O2 in PBS (final concentration) for 10 minutes
8. Wash 2x in PBS for 5 minutes
9. Prepare fresh mixture of 5% goat serum in PBS on -- 1.9ml PBS +0.1ml goat serum
10. Use the Glue Pen to circle the two sections in each slide
11. Do not let them dry out
12. Block with 5% goat serum in PBS (final concentration) for 20 minutes -- 35ul drop
13. Put the slides in the humid plastic box
14. Prepare dilutions :
  - 1<sup>st</sup> set: Antibody CD68
    - 1:100 = 1.8ul CD68 (Ab) + 178.2ul (5% Goat serum in PBS)
  - 2<sup>nd</sup> set: Negative control IgG2a

1:100 = 1.8ul IgG2a Negative control (Nc) + 178.2ul 5% Goat serum in PBS

15. Remove blocking solution
16. Apply first antibody (rat anti mouse CD68, MAC1957GA, Serotec) on the left of the two on the slide sections 1:100 (dilute in PBS containing 5% goat serum)
17. Stain other sections with control antibody (rat IgG2a negative control, MCA1212, Serotec) with the same dilutions respectively 25-60 minutes -- 35ul to each section
18. Remove the 1<sup>st</sup> antibody
19. Wash 3x with PBS for 5 minutes
20. Preparation of 2<sup>nd</sup> antibody (goat anti rat IgG:HRP (Mouse adsorbed) STAR72) in a 1:50 dilution with 5% goat serum ( 8ul of Ab + 392ul 5% Goat serum in PBS)
21. Apply secondary antibody for 30 minutes (30ul)
22. Wash 3 x in PBS for 5 minutes
23. Preparation of Nova Red substrate from the kit for peroxidase (Vector labs SK-4800) :  
Add 5ml of dH2O  
Add 3 drops of Reagent 1 → mix well  
Add 2 drops of Reagent 2 → mix well  
Add 2 drops of Reagent 3 → mix well  
Add 2 drops of H2O2 Solution → mix well
24. Stain with Nova Red, incubate for 15 minutes
25. Wash sections for 5 minutes in 3 different glasses of water
26. Wash in tap water
27. Counterstain with Vector Hematoxylin QS Nuclear counterstain H-3404  
- incubate with Hematoxylin QS for 20 seconds  
- rinse sections with running tap water, until colorless
28. Wash them in 95% ethanol 2X 1min each time
29. Wash them in a 100% ethanol 2X 1 min each time
30. Wash them with xylene 3X 3 min each time
31. Mount using *Vecta Mount* permanent mounting medium (H-5000 P0505 -Vector Lab. Inc)
32. Let them dry under aluminum foil (no light)

### **XIII: Categorization of lesions by developmental stage:**

As summarized in Table II, the ORO, H&E, and Movat's stains as well as immunohistochemistry can be used to categorize lesions by developmental stage using morphological criteria (modified from Reddick *et al.*, 1994 and de Winther, AHA 2004).

**Table II.**

	<b>Early Lesions (Type I)</b>	<b>Moderate Lesions (Type II)</b>	<b>Advanced Lesions (Type III)</b>	<b>Most Advanced Lesions (Type IV)*</b>
Foam cell characteristics	Small groups of foam cells	Multilayered or diffuse	Multilayered (lateral xanthoma) and diffuse	Multilayered (lateral xanthoma) and diffuse
Cholesterol clefts	None	Occasional	May have cholesterol clefts	Cholesterol clefts common
Necrotic core	None	Few or no acellular areas	May have acellular cores superficially covered by SMCs	Consistent acellular cores

Fibrous cap	None	May have fibrous cap	Fibrous cap	Consistent fibrous cap
Calcification	None	Little or none	May have calcification	Consistent calcification
Media/Intima characteristics	No media infiltration	No media infiltration	IEL/EEL infiltration	IEL/EEL rupture
Plaque characteristics	Foam cell-predominant		Mixed plaque (SMCs, collagen, elastic fragments)	Mixed plaque (SMCs, collagen, elastic fragments)

\*Type IV lesions are differentiated from Type III lesions based on presence of all (rather than some) advanced plaque characteristics.

Note: Should models of plaque instability (Type V lesions) become available, the following features should be assessed: rupture at fibrous cap or shoulder region of lesion, hemorrhage (visualization of RBCs), and thrombosis (apparent accumulation of platelets at rupture site).