# Mouse Models of Atherosclerosis



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## ABSTRACT

Genetically altered mice carrying mutations of genes encoding crucial components of the immune system and lipid metabolism have been widely used to study the role of immune responses and inflammation in atherosclerosis. These mice are often fed a diet, with a high content of cholesterol and saturated fat in order to induce hypercholesterolemia and arterial lesions. We review the different mouse models of atherosclerosis, type of diets, and techniques to measure lipid deposition and lesion size in the arterial walls. Moreover, the methods used to determine the presence of the immune cells in atherosclerotic lesions are also described here. *Curr. Protoc. Immunol.* 96:15.24.1-15.24.23. © 2012 by John Wiley & Sons, Inc.

Keywords: atherosclerosis • mice • diet • lipids • staining

## INTRODUCTION

Atherosclerosis is a ubiquitous disease of elastic and muscular arteries, which is the major cause for myocardial and cerebral infarctions and ischemia of the extremities (Fuster et al., 1996). Risk factors include family history, hypercholesterolemia, hypertension, obesity, and cigarette smoking. Histopathologic and clinical data, as well as an extensive body of experimental work, indicate that innate and adaptive immune responses and resulting chronic inflammation are integral to the pathogenesis of atherosclerosis. Immune responses modulate lesion initiation, progression, and potentially devastating thrombotic complications (Hansson and Libby, 2006; Packard et al., 2008). The disease is usually associated with dyslipedemia, such as elevated blood levels of low-density lipoprotein (LDL) and cholesterol, which become deposited in the intima of certain atherosclerosis-prone regions of the arterial tree. Lipoprotein oxidation and uptake by macrophages and dendritic cells within the arterial wall induces an inflammatory process that involves a large number of cellular and soluble mediators, resulting in smooth muscle cell migration and proliferation in the intima, lipid-laden macrophage foam cell formation and death, and extracellular matrix deposition (Galkina and Ley, 2009; Mallat et al., 2009). This vascular wall remodeling may gradually narrow the lumen, resulting in progressively impaired blood flow to end organs. Sudden exposure of lesion contents to the blood, due to inflammation-driven breakdown of the lesion matrix often results in catastrophic thrombosis and ischemic infarction of tissues. This unit will review methods to study atherosclerosis in mouse models, with an emphasis on studies interrogating the way inflammation and the immune system affect disease.

## CHOICE OF MOUSE MODELS TO STUDY ATHEROSCLEROSIS

Rodent models that have been used to study atherosclerosis include genetically hyperlipidemic rabbits, cholesterol- and cholate-fed C57Bl/6 mice, and others. Since the 1990s, however, the most popular models have been genetically modified mice with defects in lipid metabolism resulting in hypercholesterolemia. Mice do not normally eat cholesterol or saturated fats, and do not develop atherosclerosis. Feeding normal inbred strains of mice diets with cholesterol and saturated fat for extended periods of time only leads



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**Table 15.24.1** Genetically Altered Mouse Strains Used to Study Atherosclerosis

| Strain | Characteristics | Reference(s) |
|---|---|---|
| *ldlr*$^{-/-}$ | Mice are mildly hypercholesterolemic on normal diets, and markedly hypercholesterolemic on cholesterol/high-fat diet. Extent of disease correlates with degree of hypercholesterolemia. Most blood cholesterol is in LDL. | Ishibashi et al. (1994) |
| *apoE*$^{-/-}$ | Mice are markedly hypercholesterolemic and develop spontaneous atherosclerosis under normal diet, and are severely hypercholesterolemic and develop more disease on cholesterol/high fat diet. Most blood cholesterol is in VLDL. | Plump et al. (1992); van Ree et al. (1994) |
| *apoE*$^{*}$*3Leiden (E3L)* | Mice carry a mutated human *apoE3* transgene. *E3L* mice have a hyperlipidemic phenotype, develop atherosclerosis on cholesterol/high fat diet, and are more sensitive to lipid-lowering drugs than *apoE*$^{-/-}$ and *ldlr*$^{-/-}$ mice. | van Vlijmen et al. (1994) |
| Transgenic *apoB* | Mice carry a human *apoB* transgene and develop atherosclerosis when fed a cholesterol/high fat diet. | Purcell-Huynh et al. (1995) |
| *apoB100* × *ldlr*$^{-/-}$ | Mice are more severely hypercholesterolemic and develop more severe arterial disease on cholesterol/high fat than *ldlr*$^{-/-}$ mice. | Sanan et al. (1998) |
| *apoBEC-1*$^{-/-}$ × *ldlr*$^{-/-}$ | ApoBEC is an enzyme that controls the RNA editing of ApoB100. Deficiency of both this enzyme and Ldlr leads to severe hypercholesterolemia and atherosclerosis. | Powell-Braxton et al. (1998) |

to minimal development of early lesions (so called "fatty streaks") in restricted areas of the arterial tree. Interestingly, there are strain differences in susceptibility to these diet-induced fatty streaks, and C57Bl/6 mice are significantly more susceptible than C3H/HeJ or BALB/c mice (Paigen et al., 1985). In the 1990s, the first gene-knockout mice that develop severe hypercholesterolemia and advanced atherosclerotic disease were designed. The low-density lipoprotein receptor deficient mouse (*ldlr*$^{-/-}$) was derived by the Brown and Golstein laboratory (Ishibashi et al., 1993, 1994). The apolipoprotein-E deficient mouse (*apoE*$^{-/-}$) was developed in the Breslow laboratory in 1994 (Nakashima et al., 1994). These two lines are by far the most widely used in mouse atherosclerosis research. Table 15.24.1 lists the major features of these and other mouse models.

A common strategy for studying the role of the immune system in atherosclerotic disease has been to crossbreed either *ldlr*$^{-/-}$ or *apoE*$^{-/-}$ mice with mice carrying null mutations in genes encoding immune regulatory proteins, or with mice carrying cytokine transgenes (see Table 15.24.2 for selected examples). In addition, *ldlr*$^{-/-}$ mice can be lethally irradiated and reconstituted with bone marrow from *ldlr*$^{+/+}$; they remain susceptible to diet-induced hypercholesterolemia and atherosclerosis because the critical cell type where the LDLR must be expressed to regulate serum cholesterol is the hepatocyte (Schiller et al., 2001). Thus, one can study the role of hematopoietic cell immune defects on atherosclerosis in *ldlr*$^{-/-}$ bone marrow chimeras without the necessity of breeding a second null allele into the *ldlr*$^{-/-}$ line. This is not true for the *apoE*$^{-/-}$ mouse, because ApoE produced by transplanted hematopoietic cells is sufficient to normalize blood lipoproteins in an *apoE*$^{-/-}$ background.

**Table 15.24.2** Selected Atherosclerosis-Prone Crossbred Mouse Strains

| Strain | Characteristics | Reference |
|---|---|---|
| $ldlr^{-/-} \times ABCG1^{tg}$ | These hypercholesterolemic mice have an increase of cholesterol efflux from cells and increase atherosclerosis. | Basso et al. (2006) |
| $ldlr^{-/-} \times Tbet^{-/-}$ | These mice can not mount Th1 responses and develop less atherosclerosis than $ldlr^{-/-}$ mice | Buono et al. (2005) |
| $apoE^{-/-} \times Rag^{-/-}$ | These hypercholesterolemic mice lack an adaptive immune system and develop less atherosclerotic lesions than $apoE^{-/-}$ mice. They have been used as recipients for adoptive transfer of selected lymphocyte populations. | Dansky et al. (1997) |

## SEX AND AGE OF MICE

Both male and female hypercholesterolemic mice develop quantifiable atherosclerotic lesions, and many studies use both sexes, to avoid excessive breeding costs. Some sex differences in lesion development have been reported, but they are not consistent between different lines or between different locations in the aorta. For example, female $apoE^{-/-}$ mice on the C57BL/6 background develop more and larger descending aortic lesions than males, but this is not true of $ldlr^{-/-}$ mice, and does not model the decreased susceptibility of premenopausal women to atherosclerotic disease. In several cases, genetic or pharmacologic interventions affecting the immune system have more profound effects in one sex or the other (Whitman et al., 2002). There is currently no broadly applicable reason why exclusively male or female mice should be used to best model human disease or to study the influence of the immune system on atherosclerosis, but it is recommended that experimental groups be sex matched, and include enough mice so that statistical analyses of sex-dependent differences are sufficiently powered.

Comparison between experimental groups in mouse atherosclerosis studies must be age matched, since lesion size and composition constantly changes as mice age. In most studies of lesion development, mice are less than 6 months of age. Interventions, such as initiation of atherogenic diet feeding, injection of neutralizing antibodies, or induction of cre-recombinase-induced gene deletion are started with 4- to 6-week-old mice. For the study of the effect of interventions on established disease, the mice are usually 8 to 20 weeks of age, sometimes older.

*NOTE:* Before beginning any work with mice, proper training and protocol approval must be obtained from the Institutional Animal Care and Use Committee (IACUC) or equivalent, and the study must conform to government regulations.

## INDUCTION OF ATHEROSCLEROTIC DISEASE IN GENETICALLY SUSCEPTIBLE MICE

***BASIC PROTOCOL 1***

$ApoE^{-/-}$ mice are severely hypercholesterolemic from birth and will develop quantifiable aortic atherosclerosis by ~6 weeks of age if fed a standard chow diet. $ApoE^{-/-}$ fed a cholesterol-containing diet will become even more hypercholesterolemic than their chow-fed counterparts, and will develop more lesions at a faster pace. Chow-fed $ldlr^{-/-}$ mice are only moderately hypercholesterolemic, and do not develop significant quantifiable lesions before 6 months of age. Therefore, most studies with $ldlr^{-/-}$ mice, and many studies with $apoE^{-/-}$, are conducted with special cholesterol-containing atherogenic diets. It should be noted that feeding $apoE^{-/-}$ these diets results in such a high level of hypercholesterolemia, that the effects of modifying immune/inflammatory pathways may be obscured (Nakashima et al., 1994).

**Table 15.24.3** Atherogenic Mouse Diets

| Diet | Features | Reference |
|---|---|---|
| "Western" diet | This is a purified diet with 21% anhydrous milk fat (butterfat), 34% sucrose, and 0.2% cholesterol. It is widely used with *apoE*$^{-/-}$ and *ldlr*$^{-/-}$ models. There are a number of modifications of the Western diet with varying levels of added cholesterol. Commercially available from Harlan Laboratories, Research Diets, Purina Diets. | Merat et al. (1999) |
| "Paigen" diet | This is a 1:3 mixture of the Thomas-Hartroft diet (30% cocoa butter, 5% cholesterol, 2% sodium cholate, 30% casein, 5% alphacel, 4% vitamin mixture, 4% salt mixture, 6.5% sucrose, 6.5% dextrose, 6.5% dextrin, and 0.5% choline chloride) with a standard mouse breeder diet (17% protein, 11% fat, 3% fiber, 6.5% ash, and 2.5% minerals). The Paigen diet has 15% fat, 1.25% cholesterol, and 0.5% sodium cholate. It is used in C57Bl/6 mice and in genetically modified mice. Commercially available from Harlan Laboratories. | Paigen et al. (1985) |
| Purified diets | Semi-purified AIN76A-based diets have been altered by adding in fat calories (i.e., dairy butter, hydrogenated coconut oil) in place of purified carbohydrate calories only (i.e., sucrose, corn starch), thereby maintaining nutrient-to-calorie ratios. We have used a semi-purified AIN76A base diet containing 39% kcal lipid, 1.25% cholesterol, 20% protein, and 40% carbohydrates. It is used in genetically modified mice. Commercially available from Research Diets. | Lichtman et al. (1999) |

Many different diets have been successfully used to induce atherosclerotic disease in genetically susceptible mouse lines. In both *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mice, the dietary cholesterol rather than the amount of saturated fat is the main factor that promotes atherosclerosis. Normal chow is often supplemented with cholesterol (e.g., $\sim$ 0.15%) and butter fat (e.g., 20%), with good results, although this approach may lead to variability between studies due to changes in chow diet formulations. Commonly used specially formulated diets for mouse atherosclerosis studies are listed in Table 15.24.3. All have higher fat content than standard mouse chow, and unlike the chow, they all contain cholesterol, albeit at varying levels. These diets are all commercially available, and they can be customized to vary total cholesterol and fat content.

### *Materials*

Chow- or cholesterol-containing diets (see Table 15.24.3; e.g., Research Diets, Harlan Laboratories, or Purina Diets)
Mice genetically altered (6- to 8-week-old *ldlr*$^{-/-}$ mice)

Additional reagents and equipment for euthanizing the animal (*UNIT 1.8*)

1. Order a commercially prepared diet, which may vary, depending on the study. Companies will prepare customized diets (e.g., with drugs added).
2. Start feeding 6- to 8-week-old *ldlr*$^{-/-}$ mice with atherogenic diets [e.g., normal chow or chemically defined diets supplemented with cholesterol ($\sim$ 0.15%-.25%) and/or butter fat (20%)].
3. Continue feeding *ldlr*$^{-/-}$ mice atherogenic diets for a minimum of 4 weeks before sacrifice to generate small but quantifiable lesions, mainly in the aortic root, or up to 18 to 20 weeks to detect larger lesions throughout the aorta.

4a. If using *apoE*$^{-/-}$ mice fed a normal chow diet, sacrifice mice (see *UNIT 1.8*) at least 10 weeks of age to ensure quantifiable lesions.

4b. If using *apoE*$^{-/-}$ mice fed atherogenic diets, start feeding 4- to 6-week-old mice, and sacrifice after 4 to 20 weeks on the diet, for small to extensive lesion formation, respectively.

## MEASUREMENT OF BLOOD LIPIDS IN MICE

***SUPPORT PROTOCOL 1***

In any experiment designed to test the effects of a change in the immune status of mice on atherosclerotic lesion development or phenotype, it is essential to measure and compare blood cholesterol levels between experimental groups, since changes in blood cholesterol levels may be a confounding variable in the interpretation of results.

### *Materials*

Mice under control or cholesterol diet
Total cholesterol analysis kit (Raichem)
ELISA kits
EDTA (American Bioanalytical)
Isoflurane (Webster Veterinary)
Capillary blood collection tubes (Fisher)

Additional reagents and equipment for blood collection from mice (*UNIT 1.7*)

1. Fast mice overnight before blood collection.
2. Anesthetize the mice with isoflurane. Collect venous blood in capillary blood collection tubes (by tail vein nicking or from the retro-orbital sinus) at any time point during a study, and by cardiac puncture at the time of sacrifice (see *UNIT 1.7*), to determine cholesterol levels in serum or plasma samples.
3. Measure total cholesterol with a commercial kit.
4. Determine the lipoprotein distribution of cholesterol in HDL, LDL and VLDL particles by FPLC-GC, which ideally requires EDTA-anticoagulated plasma samples of at least 200 µl that are usually pooled from 50-µl samples per mouse.

   *A dedicated laboratory setup for this purpose may not be available to many investigators. An alternative is to use ELISA or enzymatic-based assays, which can be purchased as kits, or performed by commercial laboratories.*

## QUANTITATIVE ANALYSIS OF MOUSE AORTIC ATHEROSCLEROTIC LESIONS

***BASIC PROTOCOL 2***

The only vessels in the mouse that consistently develop quantifiable atherosclerotic lesions are the aorta and the proximal portions of its major branches. Although there are some excellent studies of lesions in branches, such as the carotid and iliac arteries, the vast majority of studies focus only on the aorta. Different sites of the aorta (aortic root/sinuses, ascending aorta, aortic arch, thoracic and abdominal descending aorta) may be differentially susceptible to lesion development under different experimental conditions (VanderLaan et al., 2004).

Mouse atherosclerotic lesions can be quantified as measured areas on histologic sections or on en face gross specimens of the luminal surface of the vessel. Frequently, neutral lipid stains (e.g., Oil Red O, or Sudan IV; see Basic Protocol 3 and Alternate Protocol 2) are used to highlight lesions, but the area of lipid staining may underestimate the size of complex lesions with significant lipid-poor smooth muscle cell and fibrous matrix content. There are ample histological landmarks in routine hematoxylin-stained

histological sections to permit an accurate assessment of lesional area, while the assessment of lesion area on en face gross sections can be difficult without lipid stains, especially with early lesions. In some studies, only aortic root analyses or only en face analyses are performed. It is possible to prepare aortic root sections for histologic analysis, and prepare the remaining aorta from the same animal for en face analysis. This protocol describes how to remove and prepare mouse aortas that can then be analyzed histologically. Multiple serial 5- to 7-μm frozen sections of the aortic root, which includes the sinuses behind the aortic valve leaflets, are prepared for subsequent lipid staining as well as for standard histological and immunohistochemical staining. The sections are then suitable for quantifying lesion area. The technique requires skill using a cryostat (Wilson, 1905; Prophet et al., 1992).

### *Materials*

Mice with atherosclerotic disease (Basic Protocol 1)
Phosphate-buffered saline (Invitrogen, cat. no. 10010-023)
Heparin (Sigma)
OCT (optimal cutting temperature compound; Tissue-Tek)
Isopentane

Mini Vanna scissors and forceps
10-ml syringes
27-G needles
Dissecting light microscope
Cryosection plastic mold
−80°C freezer
Cryostat microtome
Camel hair brush, N°3
Glass slides (FisherBrand)
Plastic wrap

Additional reagents and equipment for euthanizing the mouse (*UNIT 1.8*) and immunohistochemistry (Support Protocol 3)

### *Remove mouse aortas*

1. Euthanize the mouse using an IACUC-approved procedure (*UNIT 1.8*).
2. Using scissors and forceps, open the thoracic cavity by cutting the ribs laterally to the sternum, with the sternum being retracted toward the head.
3. Perfuse via the left ventricle with ice-cold PBS+heparin (2000 U/ml), with a 10-ml syringe equipped with a 27-G needle.
4. Remove the ribs, lungs, gastrointestinal and reproductive systems, leaving the heart and kidneys in situ.
5. Dissect out the aorta under the dissection microscope using mini-Vanna scissors and forceps, leaving the upper third of the left ventricle of the heart attached, down to 3 to 5 mm after the iliac bifurcation. Carefully transect the brachiocephalic, left common carotid left subclavian arteries, leaving 1 to 2 mm attached to the aorta (Fig. 15.24.1).

### *Prepare frozen sections of aortic root*

6. Cut the heart/aortic root tissue away from the rest of the aorta at the level of the superior half of the atria.
7. Embed the heart/aortic root tissue in OCT compound within a cryosection plastic mold, so that the axis of the aorta is perpendicular to the base of the rectangular mold.

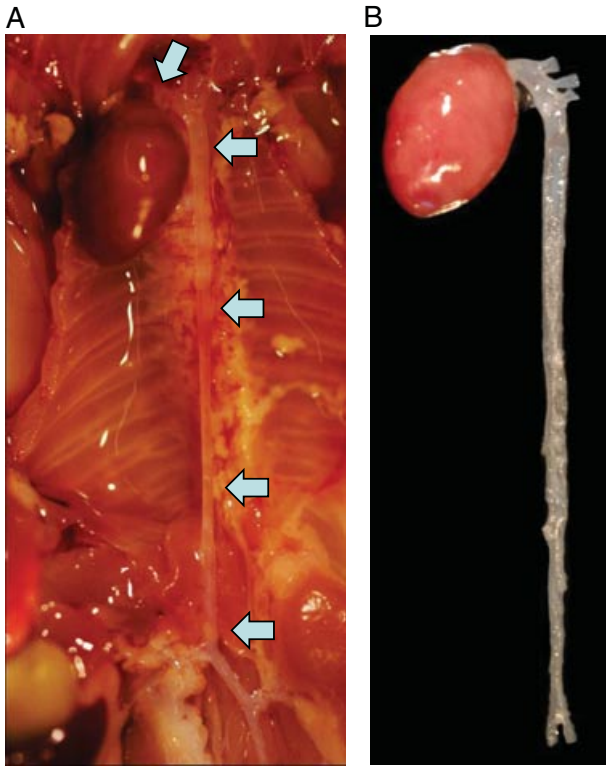


**Figure 15.24.1** Heart and aorta of an *ldlr*$^{-/-}$ mouse. (**A**) In situ after removal of lungs and abdominal viscera. (**B**) After dissection.

8. Freeze rapidly by placing the mold in isopentane to which dry ice has been added, and then store in a −80°C freezer until the tissue is cut.
9. Cut serial 5- to 7-µm sections with a cryostat microtome, using a thin brush to pull the curled section horizontally over the stage and then pick the section up onto a glass slide. Pick up three to four sections onto one glass slide and prepare up to ~12 slides per aortic root.
10. Air dry the slides for 30 min if stains for lipids or immunohistochemistry will be done immediately after cutting (see below), or wrap the slides in plastic wrap and freeze at −80°C for future staining. Do not fix the sections before freezing. Only fix the sections when proceeding immediately to staining, because each staining protocol may require a different fixation buffer.
11. Perform immunohistochemistry (see Support Protocol 3)

## PREPARATION OF AORTIC ARCH SECTIONS FOR HISTOLOGIC ANALYSIS

*ALTERNATE PROTOCOL 1*

Longitudinally oriented frozen sections of the aortic arch, including the highly lesion-prone lesser curvature, may also be used for analysis of lesion area in atherosclerotic lesions. This protocol describes a simple procedure for preparing aortic arches. For materials, see Basic Protocol 2.

1. After removing the entire aorta (see Basic Protocol 2, step 5), isolate the aortic arch by transecting the aorta several mm proximal to the take off of brachiocephalic artery and distal to the take off of left subclavian artery.
2. Embed the aortic root/aortic arch in OCT compound oriented so that the long axis is horizontal to the base of the mold.

3. Proceed to freeze, cut, and store the samples (see Basic Protocol 2, steps 8 to 10) with the aortic root preparation (Wilson, 1905; Prophet et al., 1992).

***BASIC PROTOCOL 3***

## LIPID STAINING OF AORTIC LESIONS

Quantification of atherosclerotic lesions is often performed by staining for lesional lipids (mainly cholesterol) and then quantifying the stained area. Lipid staining is most often performed on cryostat sections of mouse aortic roots and on the intact lumenal surface of the aortas (called "en face" analysis), and the area of staining is determined on photographic images. Lesion volume can also be estimated from the measured areas of multiple serial sections. The different methods used to perform the staining of frozen tissue sections and unsectioned tissue for en face analysis are described in this protocol, including light microscopic analysis, fluorescence microscopic analysis, and staining of whole aortas for en face analysis**.**

### *Materials*

Frozen sections (see Basic Protocol 2)
10% neutral buffered formalin (Fisher)
Distilled water
100% and 85% propylene glycol (Fisher)
Oil Red O (ORO; Solvent Red 27, Sudan Red 5B, C.I. 26125, $C_{26}H_{24}N_4O$) solution (see recipe)
Mayer's Hematoxylin solution (Newcomer Supply)
Glycerol/gelatin mounting medium (Sigma)
4% (v/v) paraformaldehyde/PBS (Fisher)
Phosphate-buffered saline (PBS; Invitrogen cat. no. 10010-023)
0.1% (v/v) Triton X-100/PBS (MP Biomedicals)
36% (v/v) triethylphosphate/$H_2O$ (Fisher)
Filipin (Sigma)
Dimethyl sulfoxide (DMSO)
Mounting medium with DAPI (Vectashield, Vector Laboratories)

37° or 60°C incubator
Coverslips (Fisherfinest)
Digital camera
Light microscope
Fluorescence microscope
4°C shaking incubator
Silicon-elastomere (Factor II) plates
60-mm dishes
Stainless steel minutien pins (Fine Science Tools)
25°C incubator

### *Stain the frozen sections with ORO for light microscopic analyses*

Oil Red O (ORO) is a lysochrome diazo dye (fat-soluble dye) used for staining of cholesterol esters and neutral triglycerides, which works on frozen sections, and, for some lipoproteins, on paraffin sections. This protocol below is for lipid staining on frozen sections. It may not be suitable for paraffin-embedded tissue sections.

1. Fix frozen sections in a container with 200 ml of 10% neutral buffered formalin for 10 min at room temperature.

   *If the tissue has been fixed before and then embedded in OCT and frozen, do not fix again (proceed to step 4).*

2. Rinse the sections in 200 ml distilled water extensively for 5 min at room temperature.

3. Place the sections in 200 ml of 100% propylene glycol for 2 min to dehydrate at room temperature.
4. Stain in 200 ml Oil Red O solution for 1 hr at room temperature or 30 min at 37°C or 20 min at 60°C.
5. Develop in 200 ml fresh 85% propylene glycol in distilled water for 1 min at room temperature
6. Rinse in two changes of 200 ml distilled water for 1 min each rinse.
7. Stain in 200 ml Mayer's hematoxylin or any aqueous hematoxylin for 2 min at room temperature (nuclei staining).
8. Wash in 200 ml warm tap water for 2 min.
9. Rinse in 200 ml distilled water two times.
10. Air dry the slides at room temperature.
11. Coverslip with prewarmed glycerol/gelatin or any aqueous mounting medium.
12. Digitally photograph the stained sections.

    *Lipids are stained in red color and nuclei in blue color (see Fig. 15.24.2).*

13. Alternatively, perform immunofluorescence analysis of samples (see Support Protocol 4).

***Stain fixed sections or cells with ORO and filipin for fluorescence microscopic analysis (optional)***

Oil Red O staining can be detected with the Texas Red excitation filter (540 to 580 nm; Koopman et al., 2001). Filipin is a polyene macrolide used in atherosclerosis studies to stain unsterified cholesterol and can be detected with the UV excitation filter (340 to 380 nm; Whitfield et al., 1955). Lipid staining can be combined with immunofluorescent staining for cell surface markers, which is done prior to Oil Red O or Filipin staining.

14. Fix sections (or cells cultured on coverslips) with 1 ml 4% paraformaldehyde/PBS per slide for 10 min at room temperature.

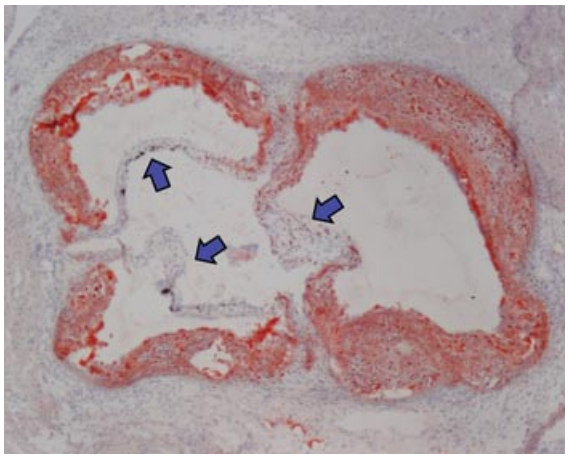

**Figure 15.24.2** Oil Red O staining for lipids in cryosections of aortic root from hypercholesterolemic *ldlr*$^{-/-}$. Lipids are identified by red color and delimit the area of the lesions within the sinus of the three leaflets of the aortic valve (arrows). For the color version of this figure go to *http://www.currentprotocols.com/protocol/im1524*.

15. Wash with PBS.

16. Permeabilize with 1 ml of 0.1% Triton X-100/PBS for 5 min at room temperature.

17. Wash three times, each time with 1 ml PBS for 10 min at room temperature

18. Stain the lipids for 45 min at room temperature with Oil Red O (ORO)/36% triethyl-phosphate or 50 μg/ml filipin/DMSO diluted in PBS.

19. Coverslip with aqueous mounting medium containing DAPI (nuclei staining).

20. Acquire images in a standard immunofluorescence microscope or a confocal microscope, using the filters/laser corresponding to each color (Texas Red and UV filters respectively). An example is shown in Figure 15.24.3 (Packard et al., 2008).

***Stain whole aortas with ORO for en face analysis***

Although the lesions can be discerned in open aortas based only on their increased opacity compared to uninvolved aorta, usually the aortas are stained for lipids using Oil Red O or Sudan IV, to permit easier detection of the lesions.

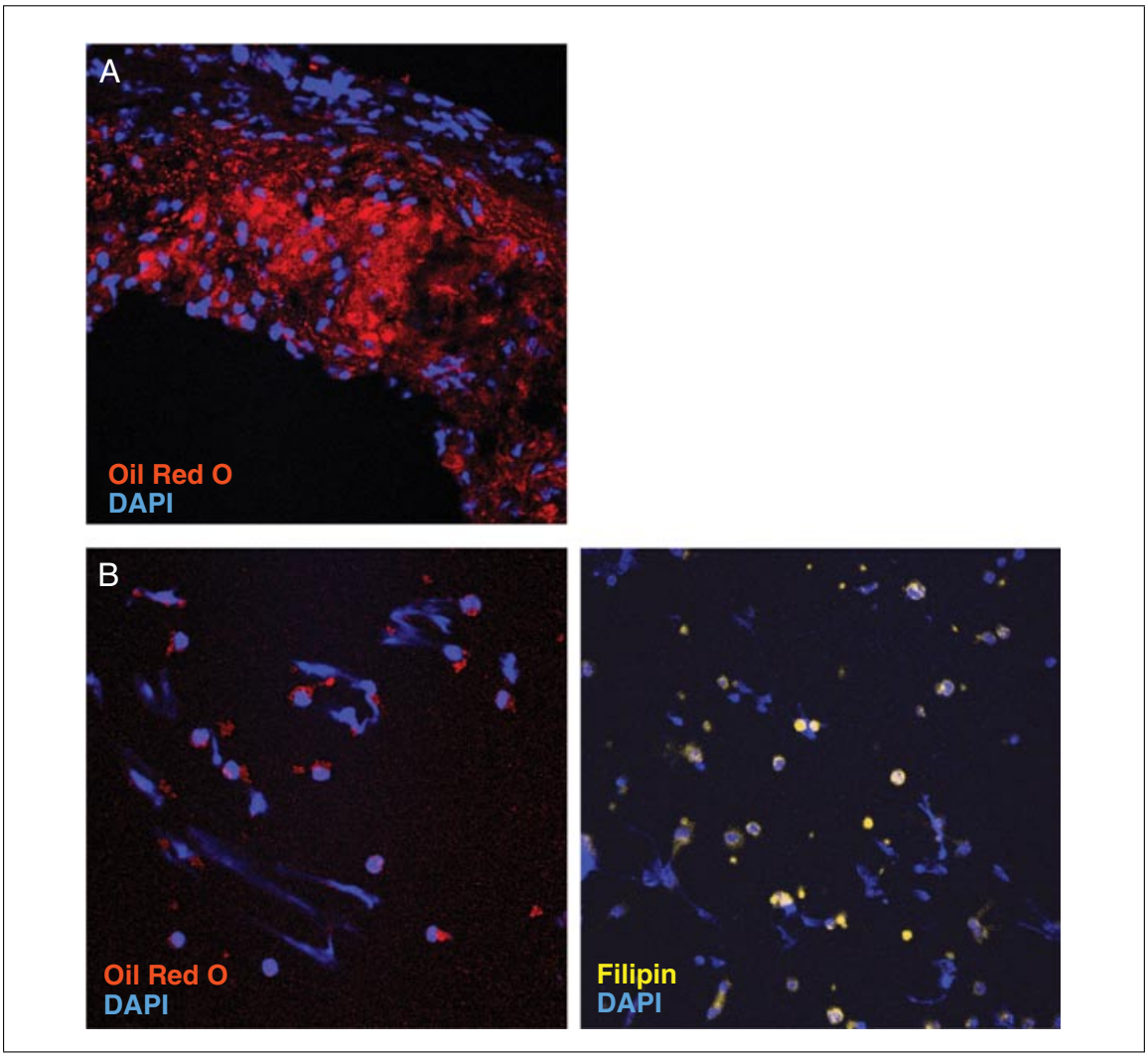


**Figure 15.24.3** Fluorescence images of lipids in cells in aortic arch of *ldlr*$^{-/-}$ mice fed a cholesterol diet for 10 weeks. Oil Red O staining for lipids in cryosections of aortic sinus (**A**). Oil Red O (cholesteryl esters) or filipin (unesterified cholesterol) staining of vivo generated lipid loaded dendritic cells (**B**). Nuclei are stained with DAPI. For the color version of this figure go to *http://www.currentprotocols.com/protocol/im1524*.

21. Thoroughly trim away adventitial fat from full-length dissected aorta before fixation.
22. Fix aortas in 5 ml 10% neutral buffered formalin solution at least overnight on a shaker or for indefinite time at 4°C.
23. Wash overnight in 5 ml PBS on a shaker at 4°C.
24. Place the aortas in a tube with 5 ml propylene glycol for 2 min (dehydration) at room temperature.
25. Transfer to a tube with 5 ml 0.5% Oil Red O for 2 to 4 hr at 25°C (longer staining times lead to increased background).
26. Wash by moving between a series of three to four dishes of 85% propylene glycol, 1 mm per dish.
27. Wash overnight in 5 ml PBS on a shaker at 4°C.
28. Pin out aortas on silicon-elastomere plates in 60-mm dishes using stainless steel minutien pins (see Fig. 15.24.4A).

    *Keep aortas covered in PBS during the entire procedure and subsequent storage.*

29. Drain the dish and add 5 ml propylene glycol for 2 min (dehydration) at room temperature.
30. Drain the dish and incubate with 5 ml 0.5% Oil Red O for 2 to 4 hr at 25°C (longer staining times lead to increased background).
31. Develop by washing through a series of three to four dishes of 85% propylene glycol, 1 mm per dish.
32. Wash three times, each time with 5 ml PBS and incubate overnight in PBS on a shaker at 4°C.

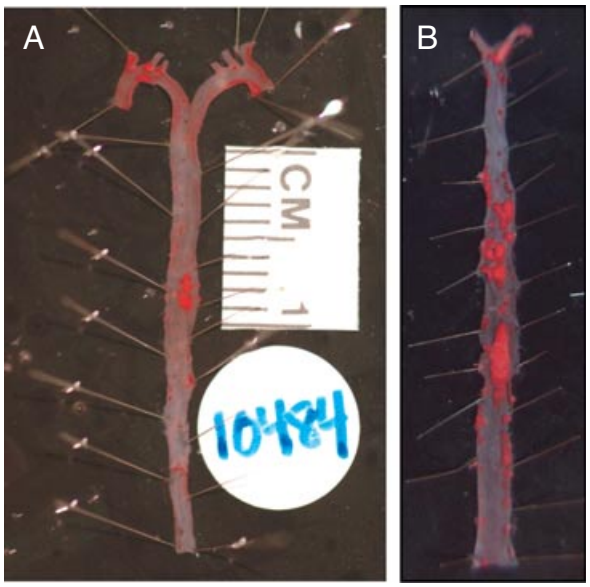


**Figure 15.24.4** En face Oil Red O (**A**) and Sudan IV (**B**) staining for lipids within the wall of atherosclerotic aortas from *ldlr*$^{-/-}$ mice. The Sudan IV staining picture is courtesy of Dr. Galina K. Sukhova from Brigham and Women's Hospital, Department of Medicine. For the color version of this figure go to *http://www.currentprotocols.com/protocol/im1524*.

33. Store dishes indefinitely at 4°C, keeping aortas covered in PBS.

34. Add a marker bar showing three 15-mm segments pinned next to the aorta.

35. Digitally photograph the stained aorta.

***ALTERNATE PROTOCOL 2***

## SUDAN IV STAINING OF WHOLE AORTAS FOR EN FACE ANALYSIS

Sudan IV ($C_{24}H_{20}N_4O$) is a lysochrome (fat-soluble dye) diazo dye used for the staining of lipids, triglycerides, and lipoproteins on frozen sections or en face preparation of the aorta. Sudan IV and Oil Red O have both been used for many years to quantify the lesion. Although Sudan IV is favored by some laboratories, Oil Red O provides a more intense red color for easier detection of lesional lipids.

### *Materials*

Sudan IV (Sigma)
70% (v/v) ethanol (Fisher)
Acetone (Fisher)
Distilled water
Light microscope

1. Dissect, fix, and pin out aortas on silicon (see Basic Protocol 3, step 28)
2. Rinse briefly in 70% (v/v) ethanol.
3. Immerse in 0.5% (w/v) Sudan IV in 35% ethanol/50% acetone for 15 min at room temperature with continuous shaking.
4. Destain in 80% ethanol until the nonlesional aorta is clear.
5. Wash with distilled water.
6. Examine the aortas using a light microscope.

   *Lipids are stained in red color with a more orange shade than Oil Red O (see Fig. 15.24.4B).*

***BASIC PROTOCOL 4***

## QUANTIFICATION OF ATHEROSCLEROTIC LESIONS

This protocol offers three alternate procedures for atherosclerotic lesion quantification. The quantification of lipid-positive areas on frozen sections of aortic sinus or aortic arch and en face preparations of aortas is commonly used as an estimate of atherosclerotic lesion size. Lipids are stained with Oil Red O or Sudan IV (see Basic Protocol 3 and Alternate Protocol 2) and the stained areas are quantified by digital image analysis.

### *Materials*

ORO-stained sections of aortic root and aortic valve (see Basic Protocol 3)
Sudan IV-stained sections (see Alternate Protocol 2)
IMAGEPRO PLUS software (Media Cybernetics)

### *Analyze aortic root/aortic valve sinus lesions*

1. Collect slides with alternate cryosections from the aortic root in which three aortic valve cusps are clearly seen (Fig. 15.24.2).

   *Other slides with adjacent sections are used for immunohistochemistry.*

2. Capture digital images of alternate sections and quantify ORO-stained area, total intimal area, and total cross-sectional area, using IMAGEPRO PLUS software.
3. Express the result as percent of the total cross-sectional vessel wall area.
4. Calculate the mean of six sections per mouse (Gotsman et al., 2007).

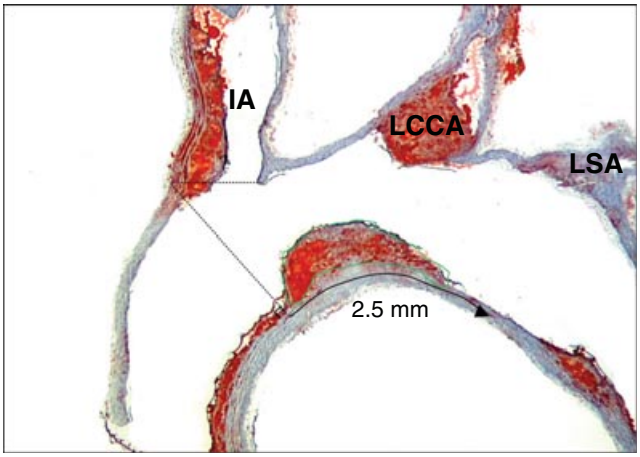


**Figure 15.24.5** Measurement of lipid-stained area in lesser curvature of *ldlr*$^{-/-}$ mice. A frozen section of aortic arch was stained with Oil Red O (ORO) and digitally photographed. A 2.5-mm segment of the lesser curvature of the aortic arch image is defined proximally by a line dropped from the right side of the innominate artery origin (dashed line) that is perpendicular to the medium of the lesser curvature. The ORO-positive area within the 2.5-mm stretch of intima is calculated by digital image analysis (inside green line). For the color version of this figure go to *http://www.currentprotocols.com/protocol/im1524*.

***Analyze lesional area or wall thickness of lesser curvature of aortic arch (optional)***

5. Define a 2- to 3-mm segment of the lesser curvature, proximally by the aortic root and distally by a perpendicular axis from the distal side of the innominate artery origin. (Fig. 15.24.5).

6. Calculate the wall thickness, or the lipid-positive area, in this 2- to 3-mm stretch, for each mouse by computerized image analysis (Mach et al., 1998; Buono et al., 2003).

***Analyze en face aortas stained with ORO or Sudan IV (optional)***

7. Pin a 15-mm scale marker next to the aorta (Cybulsky et al., 1999). Obtain images of the aorta and determine the extent of atherosclerosis using IMAGEPRO PLUS software.

## COLLAGEN STAINING OF MOUSE AORTAS

***SUPPORT PROTOCOL 2***

Collagen is the main component of lesional matrix. Human lesions with a high risk of rupture have high lipid content, numerous inflammatory cells, and a thin fibrous cap with reduced collagen and vascular smooth muscle cells (Libby, 2004). Collagen deposition is often stained and quantified in order to phenotype lesions in different experimental groups of mice. Picrosirius Red staining of type I and III is a widely used technique of collagen histochemistry (Puchtler et al., 1973; Junqueira et al., 1979).

### *Materials*

Mice with atherosclerotic disease (Basic Protocol 1)
Tap water
Distilled water
Sirius Red F3BA (Polysciences)
Picric Acid (VWR)
HCl (Sigma)
Xylene (Fisher)
Appropriate aqueous mounting medium

Slide rack
Light microscope

Additional reagents and equipment for removing aortas (Basic Protocol 2) and fixing frozen sections in formalin (Basic Protocol 3)

***Remove aortas and prepare frozen sections***

1. Remove aortas (see Basic Protocol 2) from mice with atherosclerotic disease (Basic Protocol 1) and prepare fixed frozen sections in 10% neutral buffered formalin for 10 min (see Basic Protocol 3).

   *Alternatively, paraffin sections may be used, in which case, first deparaffinize in xylene, hydrolyze in alcohols, and then rinse in distilled water.*

2. Rinse in 200 ml tap water for 10 min at room temperature.
3. Rinse in 200 ml distilled water for 2 min at room temperature.
4. Incubate in 200 ml 0.05% solution of Sirius Red F3BA in saturated aqueous solution of picric acid (this solution should be freshly prepared) at room temperature.

   *The incubation time for mouse aortic arch is 4 to 4.5 hr.*

5. Rinse two times, each time in 200 ml 0.01 N HCl. Dip the rack with slides into the first portion of HCl to get rid of drops of Sirius Red dye and then in 0.01 N HCl.
6. Rinse in 200 ml distilled water.
7. To dehydrate first incubate in 200 ml 70% ethanol for only 30 to 45 sec, and then in 200 ml xylene at 100°C for 5 min.
8. Coverslip with any aqueous mounting medium.
9. Acquire images with a light microscope with polarizing filters.

   *Picro-sirius red is used to enhance the birefringence of collagen fibers, which is largely due to co-aligned molecules of type I collagen. When examined through crossed polars, the larger collagen fibers are bright orange or red (Fig. 15.24.6). It is necessary to rotate the slide in order to see all the fibers, because in any single orientation the birefringence of some fibers will be extinguished. This inconvenience can be solved by equipping the microscope for use with circular rather than planar polarizing filters (Whittaker et al., 1994). The disadvantage is a loss of a completely black background.*

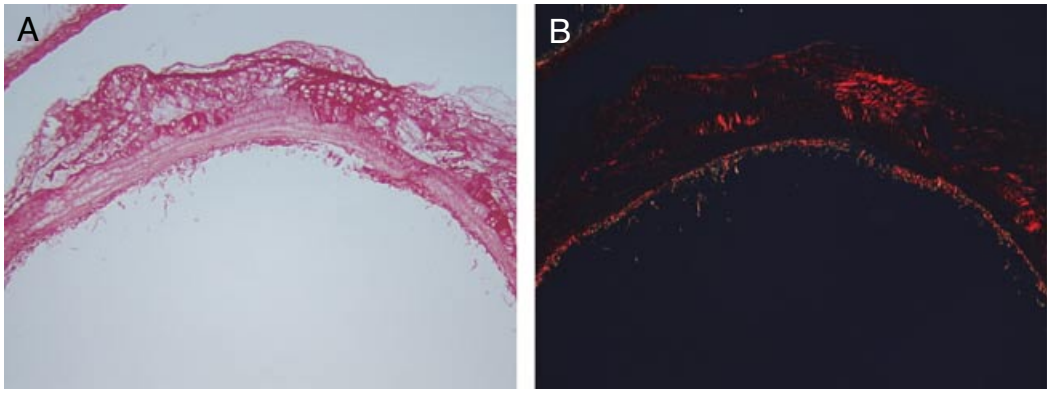


**Figure 15.24.6** Picrosirius Red staining for collagen in cryosections of aortic sinus from hypercholesterolemic *ldlr*$^{-/-}$. Picrosirius Red staining under regular light microscope (**A**) or using polarized light (**B**). These pictures are courtesy of Dr. Galina K. Sukhova from Brigham and Women's Hospital, Department of Medicine. For the color version of this figure go to *http://www.currentprotocols.com/protocol/im1524*.

## IMMUNE CELLS DETECTION IN AORTIC TISSUE



The presence of immune/inflammatory cells in atherosclerotic lesions can be detected and quantified in microscopic sections stained by immunohistochemical sections.

### *Materials*

Sections of aorta (see Basic Protocol 2)
Acetone
Calcium- and magnesium-free phosphate-buffered saline (CMF-PBS, Invitrogen, cat. no. 10010-023)
Normal serum (serum of the species that the secondary biotinylated antibody is raised in; use heat-inactivated serum)
Wash fluid (see recipe)
Avidin/Biotin Blocking kit (Vector) containing:
  Avidin solution
  Biotin solution
Primary antibody (e.g., rat anti-mouse CDY, clone RM4-5; Pharmingen)
Secondary antibody (e.g., biotinylated goat anti-rat Ig; Invitrogen)
Hydrogen peroxide
ABC complexes (Vector)
0.1 M acetate buffer, pH 5.2 (see recipe)
AEC solution (see recipe)
Gill's Hematoxilin No. 2 (Fisher)
Aqueous mounting medium

Coverslips
Light microscope

### *Fix and block the sections*

1. Fix sections of aorta (see Basic Protocol 2) in 200 ml acetone for 6 min at room temperature.
2. Air dry the slides for 30 sec. Do not let the slides dry after this step.
3. Wash the slides twice, each time in 200 ml CMF-PBS.

   *PBS used throughout this procedure is calcium and magnesium free.*
4. Block with 10% normal serum/CMF-PBS for 10 min at room temperature.
5. Wash the slides in 200 ml CMF-PBS and in 200 ml 1:1 wash fluid/PBS.
6. Block with 0.5 ml avidin solution and then block with 0.5 ml biotin solution (for each slide) for 10 min each in a moist chamber at room temperature.
7. Wash in 200 ml CMF-PBS and 200 ml 1:1 wash fluid/PBS.

### *Perform immunostaining*

8. Incubate with primary antibody for 1 hr in CMF-PBS and then with secondary antibody biotinylated at the appropriate dilution in CMF-PBS for 40 min, in a moist chamber at room temperature.
9. Wash in 200 ml CMF-PBS.
10. Inactivate endogenous peroxidases by incubating slides in 1% hydrogen peroxide/CMF-PBS for 10 min at room temperature.
11. Wash in 200 ml CMF-PBS. Do not include any type of sera or BSA in these last washes to avoid background problems.

***Detect cells***

12. Incubate with avidin-biotin complexes (ABC) for 40 min at room temperature in a moist chamber. Prepare the ABC not less than $\frac{1}{2}$ hr before using. Follow the manufacturer's instructions.
13. Wash with 200 ml CMF-PBS.
14. Wash in 200 ml 0.1 M acetate buffer, pH 5.2, for 4 min at room temperature.
15. Incubate in 200 ml 3-Amino-9-ethylcarbazole (AEC) working solution for 10 min at room temperature.

    CAUTION: *AEC solution is carcinogenic.*

16. Wash with 200 ml 0.1 M acetate buffer, pH 5.2, for 3 min at room temperature.
17. Wash in 200 ml distilled water.
18. Stain with 200 ml Gill's Hematoxylin no.2 (nuclei staining) diluted in distilled water (2:1) for 1 min at room temperature.
19. Wash with warm tap water for 4 min for several changes until the water clears.

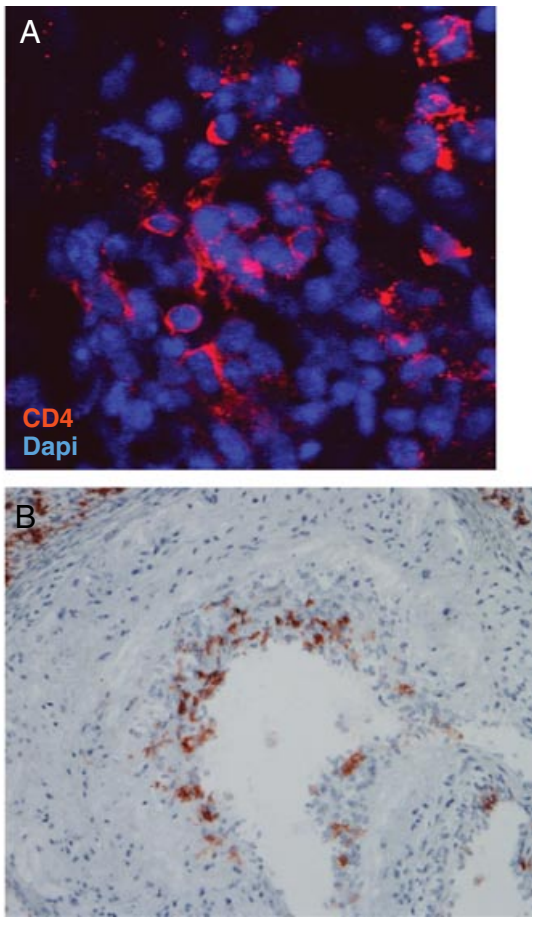


**Figure 15.24.7** CD4+ T lymphocytes stained within the arterial wall of hypercholesterolemic *ldlr*$^{-/-}$ mice. Staining of lymphocytes in en face aortic arch by immunofluorescence (**A**) and in cryosections of the lesion of aortic sinus by immunohistochemistry (**B**). Nuclei are stained blue in both images. For the color version of this figure go to *http://www.currentprotocols.com/protocol/im1524*.

20. Coverslip with aqueous mounting medium.

21. Acquire images with a light microscope and quantify with six sections from a single mouse and different mice.

    *Specifically stained cell markers appear red/brown and nuclei in blue colors, (Fig. 15.24.7B).*

## IMMUNOFLUORESCENCE MICROSCOPY

***SUPPORT PROTOCOL 4***

In this protocol, aorta samples are first prepared and stained using Oil Red O and filipin (Basic Protocol 3) and then stained using a primary antibody of interest, followed by a fluorescently labeled secondary antibody.

### *Materials*

4% paraformaldehyde
En face sections of the aortic arch (see Basic Protocol 2)
Phosphate-buffered saline (PBS; Invitrogen, cat. no. 10010-023)
Bovine serum albumin (BSA; American Bioanalytical)
Primary antibodies (e.g., rat anti-mouse CD4; Biolegend)
Secondary antibodies (e.g., Alexa 555-labeled goat anti-rat Ig; Invitrogen)
Immunofluorescence microscope

1. Fix en face sections of the aortic arch (Iiyama et al., 1999) with 1 ml (per slide) 4% paraformaldehyde overnight at 4°C.
2. Permeabilize the samples (as described in Basic Protocol 3, step 16).
3. Block the samples with 1 ml PBS+1% BSA or with 1 ml 5% serum in PBS, corresponding to the source of the species of the secondary antibody (heat-inactivated serum), for 1 hr at 22°C in a moist chamber.
4. Incubate with the primary antibodies diluted 1:50 in PBS overnight at 4°C in a moist chamber.
5. Wash with 1 ml PBS.
6. Incubate with secondary antibodies against the source species of the primary antibody, in PBS for 45 min at 22°C in a moist chamber.
7. Analyze the samples with an immunofluorescence microscope (see Basic Protocol 3, step 20; Fig. 15.24.7A).

## FLOW CYTOMETRIC ANALYSIS OF AORTIC DIGESTS

***SUPPORT PROTOCOL 5***

The digestion of the aortas with a cocktail of enzymes allows the dissociation of the tissue and the release of the immune/inflammatory cells present in the aortic wall (Galkina et al., 2006). This method does not distinguish between cells in intimal lesions from cells in the adventitia.

### *Materials*

Mouse aortas (see Basic Protocol 2)
Digestion buffer (see recipe)
Phosphate-buffered saline (PBS; Invitrogen, cat. no. 10010-023)
Fluorescently labeled antibodies (e.g., APC-labeled rat anti-mouse CD3; Pharmingen)

Surgical scissors
70-μm strainer (BD Bioscience)
3-ml syringes
Centrifuge

1. Cut aortas (Basic Protocol 2) into small pieces with surgical scissors.
2. Digest the aortas (pool two to three aortas) with 2 ml digestion buffer (for each aorta) for 1 hr at 37°C.
3. Mash the aorta through a 70-μm strainer using a 3-ml syringe.
4. Add 5 ml 1× PBS to wash the cells and spin down in a centrifuge for 5 min at 300 × *g*, 4°C.
5. Incubate the final cell suspension with fluorescently labeled antibodies and analyze by flow cytometry using standard methods.

*It is important to mention that some cellular surface markers (e.g., CD4) are not detectable after the aortic digestion. In order to solve this problem some investigators use cytoplasmic staining protocols for these markers.*

## REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2A****; for suppliers, see* ***APPENDIX 5****.*

### *Acetate buffer, pH 5.2, 0.1 M*

1 M acetate buffer, pH 5 (Bioworld, cat. no. 40120004)
Dilute 1:10 in distilled water
Adjust pH to 5.2 using glacial acetic acid (Fisher, cat. no. BP2401)
Prepare fresh

### *AEC solution*

*Prepare stock solution*
Combine 0.4 g 3′-amino-9′-ethyl-carbazole (Sigma) in 100 ml *N*′,*N*′-Dimethyl Formamide (Fisher)
Mix well and keep in a dark bottle up to 6 months at 4°C.
*Prepare working solution*
Combine 300 ml 0.1 M acetate buffer, pH 5.2 (see recipe), with 15 ml of AEC stock solution (see recipe above)
Filter through No. 1 Whatman Filter Paper (Whatman)
Prepare fresh
Just before use, add 150 μl of 30% hydrogen peroxide (Fisher)

### *Digestion buffer*

Add the following to Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen, cat. no. A1285801) containing 20 mmol/liter HEPES (Invitrogen):
125 U/ml collagenase type XI (Sigma)
600 U/ml hyaluronidase type I-s (Sigma)
60 U/ml DNase1 (Sigma)
450 U/ml collagenase type I (Sigma)
Prepare fresh

### *ORO solution*

Saturate 0.3 g ORO powder in 10 ml of 100% isopropanol in a 250-ml beaker
Cover with aluminum foil and let stand for 3 days at room temperature

*continued*

Agitate and place in a 50-ml tube and centrifuge for 15 min at 900 × *g*, room temperature

Take the liquid supernatant and store up to 6 months at room temperature as stock solution

Dilute the stock solution 6:4 with distilled $H_2O$

Agitate and filter through a 0.45-mm filter.

Store up to 6 months at room temperature as working solution for staining

### *Wash fluid*

*Component A*

Dissolve the following in 3 liters of distilled water:

32 g NaCl (Sigma)

0.8 g KCl (Sigma)

4.6 g $Na_2HPO_4$ (Sigma)

0.8 g $KH_2PO_4$ (Sigma)

0.4 g sodium azide (Sigma)

*Component B*

Dissolve 8 g gelatin (Fisher) in 1 liter of distilled water by heat

Mix A and B together

Adjust pH to 7.2 to 7.4 using 0.1 N HCl or NaOH

Store up to 6 months at 4°C

## COMMENTARY

### Background Information

Atherosclerosis is a disease of the cardiovascular system in which cholesterol is deposited in the walls of arteries, leading to chronic obstruction and reduced blood flow to organs, as well as increased risk of blood clots causing heart attacks and strokes. The major risk factors for atherosclerosis include high levels of LDL-cholesterol, hypertension, smoking, diabetes, and a family history of atherosclerotic disease. Moreover, many research studies have demonstrated that inflammation driven by immune responses in the arterial wall also have profound influences on atherosclerosis. The accumulation of lipids and immune cells, mainly T lymphocytes and macrophages, contributes to the inflammatory processes and promotes plaque formation as well as changes in plaque stability.

Two genetically modified mouse lines, *ldlr*$^{-/-}$ and *apoE*$^{-/-}$, have been most commonly used to study atherosclerosis. The advantage of using either *ldlr*$^{-/-}$ or *apoE*$^{-/-}$ mice is the extensive amount of published data available on which to base experimental protocols. Both mutant alleles have been bred onto C57BL/6 and BALB/c backgrounds, although the C57BL/6 background is more favorable for disease development and is more widely used. The basis for choosing between the *apoE*$^{-/-}$ versus *ldlr*$^{-/-}$ mice is not straightforward. The *ldlr*$^{-/-}$ model allows for more experimental control of the atherogenic process and associated immune responses, because atherogenesis does not begin to any appreciable degree in young *ldlr*$^{-/-}$ mice until they are started on a cholesterol-containing diet, and lesion progression can be slowed or stopped by change in diet. In *apoE*$^{-/-}$ mice, spontaneous severe hypercholesterolemia is present at birth, even on chow diet. The lipoprotein profiles of normal mice differ significantly from humans, with a higher proportion of total cholesterol in the high-density lipoprotein (HDL) fraction than in both the low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) fractions. The reverse is the case for humans. Both ApoE and Ldlr deficiency result in marked changes in the mouse lipoprotein profile, but in different ways. The profile in *ldlr*$^{-/-}$ mice more closely resembles that in hypercholesterolemic humans, with the majority of the cholesterol found in the LDL fraction. In *apoE*$^{-/-}$ mice, the majority of the cholesterol is in the VLDL-sized particles. Furthermore, the ApoE lipoprotein is reported to have intrinsic immunomodulatory activities; therefore, its deficiency may introduce confounding variables in immune responses unrelated to what occurs in human disease. Despite these various features, which suggest the *ldlr*$^{-/-}$ model may be a better choice, some investigators find that lesions in the *ldlr*$^{-/-}$ remain predominantly lipid rich longer with

less progression to lesions with thick fibrous caps and necrotic cores typical of advanced human lesions, while *apoE*$^{-/-}$ mice are more likely to develop such lesions. Nonetheless, there are many published examples showing that histopathology of lesions in both lines recapitulate the major features of human lesions, and robust influence of innate and adaptive immune responses on atherosclerosis have been demonstrated in both models.

The choice of diet (Table 15.24.3), the amount of cholesterol in the diet, and the duration of feeding depend on the experimental design. The more cholesterol, the less time it takes for quantifiable lesions to develop. However, the strikingly high levels of blood cholesterol in atherogenic-diet fed *apoE*$^{-/-}$ mice may obscure physiologically relevant modulatory effects of the immune system. Cholate, which inhibits biliary secretion of cholesterol, was used in the early studies on fatty steak formation in C57BL/6 mice, and is a component of the widely used Paigen diet (Paigen et al., 1985). The disadvantage of using cholate is that it causes liver injury and inflammation, and is not necessary for lesion development in *ldlr*$^{-/-}$ or *apoE*$^{-/-}$ mice (Lichtman et al., 1999; An et al., 2009), thus most investigators in the field now avoid its use.

Here we describe the most common strategies to prepare aortic tissues for quantifying lesion size, lipid content, cells, and matrix. Most previous studies have performed lipid or immune cell staining by histochemistry, with the disadvantage of not being able to simultaneously stain for multiple immune markers, and therefore the need for many sections per mouse to perform comprehensive immunophenotyping of lesions. However, new approaches such as immunofluorescence staining and confocal microscopy give the ability to visualize multiple fluorescent dyes on single tissue sections (Taatjes et al., 2000). The most common stains used for lipids in en face preparations of aorta are Oil Red O and Sudan IV staining. Sudan IV provides much deeper red color, and the stains are therefore much easier to see.

## Critical Parameters and Troubleshooting

The major critical parameter is choosing the right mouse model (Table 15.24.1). The basis of choosing between the *apoE*$^{-/-}$ or *ldlr*$^{-/-}$ mice depends on the control of the atherogenic process that is needed. As mentioned before, atherogenesis begins in *ldlr*$^{-/-}$ mice when the mice are started on a cholesterol-rich diet and in *apoE*$^{-/-}$ mice hypercholesterolemia is present at birth. It is important to start feeding the animals with cholesterol or chow diet when they are young (6 to 8 weeks old) and be sure that all mice are the same age before the start of the experiment.

Given the chronicity and complexity of the atherosclerotic process, it is not surprising that animal-to-animal variability in the amount of lesion development over a defined time can be high. Power analyses conducted in different laboratories based on the historical variability usually indicate that a minimum of ten to fifteen mice per group is required for two group comparisons, and more for multiple group comparisons.

During the aorta isolation for en face staining, it is extremely important to thoroughly clean the aorta, removing all the periadvential adipose tissue. If this is not done, the Oil Red O or Sudan IV staining of the adipose tissue will obscure atherosclerosis lesion staining.

In general, the methods described in this summary are straightforward and rarely fail. However, it is important to follow step by step, and not to modify any incubation time, to get clean staining and avoid false positives. When comparing groups of mice with different diets, genetic manipulations, or treatments, all mice need to be sacrificed the same day and the staining of samples from all groups should be performed at the same time in order to avoid introduction of systematic errors that affect one group and not another. Alternatively, if it is not feasible to process all mice in one day, equal numbers of mice from each experimental group should be done on each day of processing.

## Anticipated Results

Hypercholesterolemic mice survive well over a year. The mice rarely die of myocardial infarction or stroke, which are the main causes of death in humans with atherosclerosis, unless they are extremely old.

The staining of cholesterol esters and triglycerides (Oil Red O and Sudan IV staining), together with the staining of smooth muscle cell $\alpha$ actin and collagen by histochemistry, in the aorta or aortic root, will allow one to determine the area and major cellular and matrix constituents of the lesions. The analysis usually shows an increase in the lesion size compared to mice fed a chow diet and this increase correlates with an increase of total cholesterol levels in serum.

Different cell populations of the immune system appear within the lesion in *ldlr*$^{-/-}$ or

*apoE*$^{-/-}$ mice fed a cholesterol diet. Analysis of the cellular composition in the aorta, by immunohistochemistry and immunofluorescence using one or more antibodies, reveal macrophages, dendritic cells (DCs), and T lymphocytes within early lesions after 4 weeks of cholesterol diet. $CD4^+$ T cell are more abundant than $CD8^+$ T cells, although some genetic manipulations result in increased numbers of both subsets. Some studies report the presence of NKT cells, mast cells, and rarely neutrophils.

## Time Considerations

The time required to set up experiments depends upon the number of mice (minimum ten to fifteen mice per group) and the duration of feeding with chow or saturated fat/cholesterol diets. The time required also depends on the genetically altered mouse model chosen. A*poE*$^{-/-}$ mice fed a cholesterol diet result in very high levels of blood cholesterol and more rapid development of quantifiable lesions. If the investigator decides to use *ldlr*$^{-/-}$ mice, these mice need to be fed a cholesterol diet for at least 4 weeks in order to see a small lesion and up to 18 to 20 weeks to detect larger lesions within the aorta. If *apoE*$^{-/-}$ mice are used, the same period of time as *ldlr*$^{-/-}$ mice or longer is recommended when mice are fed a chow diet, and a shorter time when they are fed a cholesterol diet.

The analysis of the lesion size and cellular composition can require 3 to 4 days to complete, depending upon the number of mice and tissue samples or sections to be analyzed. One day is necessary to collect the samples from all groups of mice. With experience, the harvesting of one aorta requires at least 20 min. Typically, twelve slides with three cryosections per slide can be obtained in ~20 to 30 min. The staining of six to twelve frozen sections, by Oil Red O or Sudan IV staining, immunohistochemistry, and immunofluorescence, takes 1 day. Photography and image analysis of twelve slides requires another full day. Analysis of en face Oil Red O and Sudan V staining of the aorta also requires 2 to 3 days of staining and 1 day for analysis. En face immunofluorescence staining of the aortas will require 2 to 3 days, depending on the number of primary or labeled antibodies used for the staining. If the antibodies are from the same species, the staining needs to be done in separate 45-min or overnight incubations.

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