6

Quantitative Analysis of Atherosclerotic Lesion Composition in Mice

Marilyn P. Wadsworth, Burton E. Sobel, David J. Schneider, Wendy Tra, Hans van Hirtum, and Douglas J. Taatjes

Summary

Comparative quantitation has become an increasingly desirable tool in determining compositional differences of aortic plaque lesion in transgenically altered mice. To this end, methodology has been developed to identify lipid, cellularity, collagen, and elastin components using traditional bright-field microscopy, fluorescence, and polarized light microscopy, employing both confocal and wide-field imaging systems. Subsequent imaging processing and analysis on the digitally captured images reveals differences in compositional components as influenced by diet, age, and gender. This method can be expanded to employ a rich variety of histochemical and immunohistochemical staining protocols.

Key Words: Atherosclerotic plaques; composition; lesion; quantitation; lipid; cellularity; collagen; fluorescence microscopy; polarized light microscopy; confocal scanning laser microscopy; image analysis.

1. Introduction

With the advent of digital image acquisition, image analysis has advanced from a mostly qualitative to a "semi"-quantitative science *(1)*. A variety of commercially produced software packages are available to perform sophisticated analyses on digital format images. For instance, such analyses might be useful in determining the morphometrical effect of a specific treatment or a transgene on cells and tissues. Indeed, we have been interested in developing computer-assisted image analysis methods for the determination of atherosclerotic lesion composition in transgenic mice *(2–4)*. A paradigm developed in the last 15 yr suggests that two types of atherosclerotic plaque exist: (1) highly cellular, stable plaques that attenuate the vessel lumen but are usually benign clinically and (2) plaques that contain a paucity of cells but are rich in lipid. These plaques (also

From: *Methods in Molecular Biology, vol. 319: Cell Imaging Techniques: Methods and Protocols*
Edited by: D. J. Taatjes and B. T. Mossman © Humana Press Inc., Totowa, NJ

referred to as "vulnerable" plaques) are prone to rupture, especially in the thin shoulder regions, resulting in clinical sequlae such as myocardial infarction and stroke *(5–9)*. To address the molecular and biochemical mechanisms involved in plaque formation, sophisticated imaging methods must be developed to accurately assess lesion composition. The methods we are now developing are highly dynamic, and we continue to extend and optimize them.

The methods described in this chapter have been developed to specifically characterize the composition of atherosclerotic plaques in mouse aortic lesions. Mouse models, particularly the apolipoprotein E-knockout mouse ($ApoE^{-/-}$) are used extensively in cardiovascular research *(10,11)*. We have demonstrated that plaques show compositional changes based on diet, age, and gender in $ApoE^{-/-}$ mice compared with control C57BL6 mice *(3)*. The lesion compositional elements we are interested in studying include lipid, total cellularity, collagen, elastin, smooth muscle cells, and macrophages. These techniques employ traditional histochemical staining and immunostaining visualized with confocal scanning laser microscopy, wide-field transmitted, fluorescent, and polarized light digital image capture, and computer-assisted image processing and analysis. Thus, they might be of interest to those working in other fields requiring quantitative analysis of tissue composition.

2. Materials

2.1. General Equipment and Supplies

2.1.1. Fixation and Processing

1. Scale with glassine weigh paper.
2. Weighing spatula.
3. 200-mL Glass beaker with stir bar.
4. Glass vials with plastic snap top.
5. Fine-tip forceps.
6. Razor blade.
7. Plastic tissue-processing capsules.
8. Stirrer/hot plate.
9. Disposable Tissue-Tek embedding cryomolds 10 mm × 10 mm × 5 mm.
10. Liquid nitrogen (LN_2) in a Dewer flask (Cole Parmer Instrument Company, Vernon Hills, IL) (*see* **Note 1**).
11. 2-Methyl-butane.
12. Plastic cup with attached strings for suspension in LN_2 (to hold the 2-methyl-butane) (*see* **Note 2**).
13. Dissecting microscope.
14. OCT (optimal cutting temperature) embedding medium; Tissue-Tek; Sakura Finetek USA, Torrance, CA.
15. Freezer bag or aluminum foil for storage.
16. Solvent-proof pen.

2.1.2. Cryostat Sectioning

1. Cryostat chuck.
2. OCT embedding medium.
3. Disposable cryostat knives.
4. Small paintbrush.
5. Fisher Superfrost Plus-coated slides or equivalent (Fisher Scientific).
6. Slide box.
7. Large dissecting pin.

2.1.3. Staining

1. Scale with glassine weigh paper.
2. 100-mL glass beaker with stir bar.
3. Coplin glass staining jars.
4. 50 mm × 15 mm Glass Petri dishes.
5. No.1 filter paper.
6. Weighing spatula.
7. Wooden applicator sticks.
8. Forceps.
9. Oven.
10. Graduated cylinder.
11. Size 1-½ glass cover slips.
12. Kimwipes EX-L low-lint cleanroom wipes (Fisher Scientific).
13. Slide tray.
14. Plastic transfer pipets.
15. Aluminum foil.

2.2. Reagents and Solutions

2.2.1. Fixation

1. Phosphate-buffered saline (PBS) 10X stock solution.

   Solution A: 2.76 g sodium phosphate monobasic in 100 mL of distilled water (DH_2O).

   Solution B: 14.1 g sodium phosphate dibasic (anhydrous) in 500 mL of DH_2O.

   Mix: Solution A: 95 mL
   Solution B: 405 mL

   Add 90 g of sodium chloride and increase volume to 1 L. The pH of the solution is then adjusted to 7.2–7.4.
   PBS (1X working solution): 0.01 *M* phosphate buffer, 0.15 *M* NaCl. Dilute one part of the 10X stock solution with nine parts of DH_2O and adjust pH to 7.4, if necessary.
2. 3% Paraformaldehyde (PFA): 3 g PFA, 100 mL PBS, stirred and heated to 60°C (do not boil) until the PFA dissolves completely into solution. Cool to room temperature before using. This fixative should be freshly prepared as required. For

light microscopy studies, we store this fixative for up to 1 wk at 4°C. Always prepare formaldehyde solutions in a fume hood and wear protective gloves.

2.2.2. Tissue Processing

1. 5% Gelatin: 5 g gelatin Type A from porcine skin (Sigma Chemical Co., St. Louis, MO), 100 mL of DH_2O, dissolve with low heat.
2. 10% Gelatin: 10 g Gelatin Type A from porcine skin, 100 mL of DH_2O, dissolve with low heat.

2.2.3. Histologic Stains

1. Oil red O (lipid stain).

   Oil red O stock solution: 2.5 g Oil red O (CI 26125; *see* **Note 3**) (Polysciences, Inc, Warrington, PA), 500 mL of 98% isopropanol, mix well.
   Oil red O working solution: 24 mL Oil red O stock solution, 16 mL of DH_2O. Mix well and let stand for 10 min before filtering. Filtered solution is stable for several hours.
2. Aldehyde Fuchsin (elastic stain).

   Alcoholic Basic Fuchsin, 0.5% stock solution: 2.5 g Basic Fuchsin (CI 42500; *see* **Note 4**) (Sigma Chemical Co., St. Louis, MO), 500 mL of 70% ethyl alcohol. This solution is stable for several months when stored at room temperature.
   Aldehyde Fuchsin working solution: 50 mL 0.5% alcoholic Basic Fuchsin, 2.5 mL acetaldehyde (Aldrich Chemical Co., Milwaukee, WI) (*see* **Note 5**), 1.0 mL hydrochloric acid, concentrated. Mix the three solutions, cover, and allow to sit overnight at room temperature. Filter, cover tightly, and store at 4°C. This solution is stable for 3 wk. Warm to room temperature before using. Use suitable precautions for flammability, ventilation, and body protection.
   Light Green solution.
   Light Green stock solution: 0.2 g Light Green SF yellowish (CI 42095) (Fisher Scientific, Fair Lawn, NJ), 100 mL DH_2O, 0.2 mL glacial acetic acid, mix well.
   Light Green working solution: 10 mL Light Green stock solution, 50 mL of DH_2O.
3. Picrosirius red (collagen stain). Picrosirius red working solution: 0.1% Sirius red F3BA (CI 35780) (Pfaltz and Bauer, Inc., Waterbury, CT) in saturated aqueous picric acid, pH 2.0. Solution is stable at room temperature for 1 yr (*see* **Note 6**).
4. 0.01 *N* HCl.
5. 70% ETOH.

2.2.4. Fluorescent Stains

1. PBS.
2. PBS/1.0% bovine serum albumin (BSA, Fraction V; Sigma Chemical Co.)/0.1% Triton X (10 µL in 10 mL PBS).
3. PBS/1.0% BSA.
4. 10% Normal serum (from secondary antibody host) diluted in PBS/1.0% BSA.
5. Glycerol-based mounting media containing antifade reagent.

6. Primary antibody: α-smooth muscle actin, asm-1 mouse monoclonal antibody (Chemicon International, Temecula, CA), 5 µg/mL final concentration diluted in PBS/1%BSA.
7. Secondary antibody: Alexa 568 goat anti-mouse IgG (Molecular Probes, Eugene, Oregon), 10 µg/mL final concentration diluted in PBS/1%BSA.

3. Methods

3.1. Fixation/Processing Methods

Mouse heart and aorta are dissected out and immersion fixed overnight in 3% PFA/PBS at 4°C as previously described ***(2,3)***. During fixation, the hearts are bisected with a cut parallel to both atria. Residual external fat coating the aortic region is carefully removed with forceps under a dissecting microscope. Following fixation, the hearts are removed from the fixative and placed into plastic processing cassettes and rinsed for 1 h under running water. The container can be covered with gauze and a rubber band to prevent the processing cassettes from washing out. Retain the fixative for use later in the procedure. Capsules are immersed in 5% gelatin for 2 h at 42°C and then transferred into 10% gelatin and infiltrated overnight at 42°C. The following morning, the capsules are refrigerated at 4°C for 3 h to solidify the gelatin. The gelatin-encased hearts are removed from the cassettes and trimmed into a rectangle parallel to the primary atria/heart cut and immersed into 3% PFA/PBS for 2 h to harden the gelatin. The capsules are then transferred into PBS to rinse for 30 min before freezing. The hearts are embedded with OCT medium in labeled embedding molds (cut side up so that the first sections taken will reveal the sinus area) and snap-frozen in 2-methyl butane cooled in LN_2 and stored at –80°C until the time of cryostat sectioning.

3.2. Sectioning

This method is based on the technique of Paigen and co-workers ***(12)***. All sectioning is done in a cryostat using disposable knives. OCT-embedded tissue blocks are carefully mounted so that the first sections taken are from the side showing the bisected heart face. Sections are examined at step intervals until the aortic sinus area is revealed. The aortic sinus area is characterized by the presence of valves and a bulging shape. Orientation adjustments are made as needed until sections show the end of the aortic sinus area, identified by the thinning of the connecting valves and prominence of three valve cusps. Ten-micrometer sections are collected onto Fisher Superfrost Plus-coated slides as follows. Six slides each hold five step sections collected over a well-defined 320-µm area of interest. This area is defined by three prominent valve cusps at the juncture of the aortic sinus region to the end of the valve region, when the valves disappear and the aorta becomes more rounded in appearance. The first slide holds sections 1, 7, 13, 19, and 25. The second slide holds sections 2, 8,14, 20, and 26; the third through sixth

slides continue in the same fashion. Several additional single-section slides are collected at the end of the 300 µm area to act as controls for subsequent staining procedures. Because this technique requires absolute collection of serial sections, compressed or wrinkled sections must be corrected by immediately covering with a drop of water and straightened using a dissecting microscope and teasing needle. The sections on the slides are finally air-dried for 30 min to ensure proper adhesion before being stored in a slide box at –80°C.

3.3. Staining for Lipid and Cellularity (DNA Binding Dyes: DAPI, SYTOX Green, and Propidium Iodide)

Oil red O method for neutral lipids: Pearse method (*see* **Note 7**)

1. Cut 10-µm frozen sections, place on coated slides, and allow to air-dry at room temperature for 30 min before staining (*see* **Note 8**).
2. Stain sections for 10 min in a Coplin jar containing filtered Oil red O working dilution.
3. Gently wash in distilled water.
4. Dry the slide area around the tissue section and incubate with 5 µg/mL DAPI for 15 min in a moist, light-protected chamber (easily created by lining a large Petri dish with moistened filter paper, and inserting two orange sticks on which rest incubating slides). Cover the top lid with aluminum foil to protect the slides from light. DAPI can be replaced with a DNA-binding stain of choice depending on the wavelength desired (*see* **Note 9**).
5. Rinse 2X 5 min in PBS.
6. Mount in glycerol-based mounting medium containing antifade reagent (*see* **Note 10**).

Results: Lipid is stained bright red (*see* **Fig. 1A,B**). Other tissue components are stained faint pink to pale yellow. The DNA component is fluorescent in the desired wavelength (*see* **Fig. 2A**; *see* Color Plate 5, following p. 274) (*see* **Note 11**).

3.4. Elastic Stain

Aldehyde fuchsin elastic stain (method)

1. Air-dry slides for at least 30 min.
2. Rinse the slides in distilled water 1X 5 min.
3. Rinse in 50% ethanol, 5 min.
4. Rinse in 70% ethanol, 5 min.
5. Place sections in Aldehyde Fuchsin solution for 15 min at room temperature.
6. Rinse the slides in 70% ethanol for 10 dips and check differentiation with the microscope. Elastic fibers should be deep blue to purple.
7. Put the slides in distilled water for 5 min to stop the differentiation. At this point, the staining can be continued with the Picrosirius red stain for collagen, counterstained with a Light Green working solution for 1–2 min, or dehydrated and cover-slipped for permanent mounting.

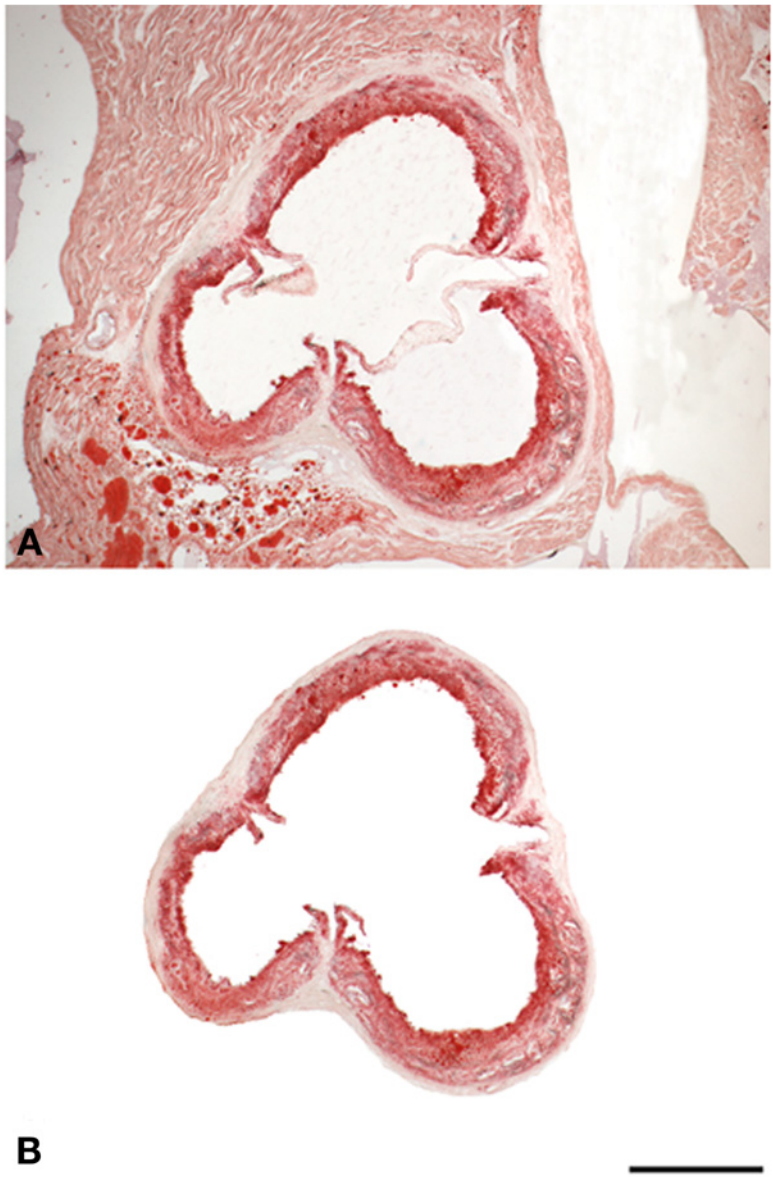

Fig. 1. **(A)** Cryostat section from mouse aorta stained with Oil red O and imaged with a 4X objective lens on a wide-field light microscope. **(B)** Final appearance of cropped image isolating the aorta from surrounding tissues for quantitative compositional analysis. Scale bar = 500 µm.

8. Dehydrate with 10–15 dips in two changes each of 95% alcohol, 100% alcohol, and three changes of xylene and mount with a synthetic mounting medium.

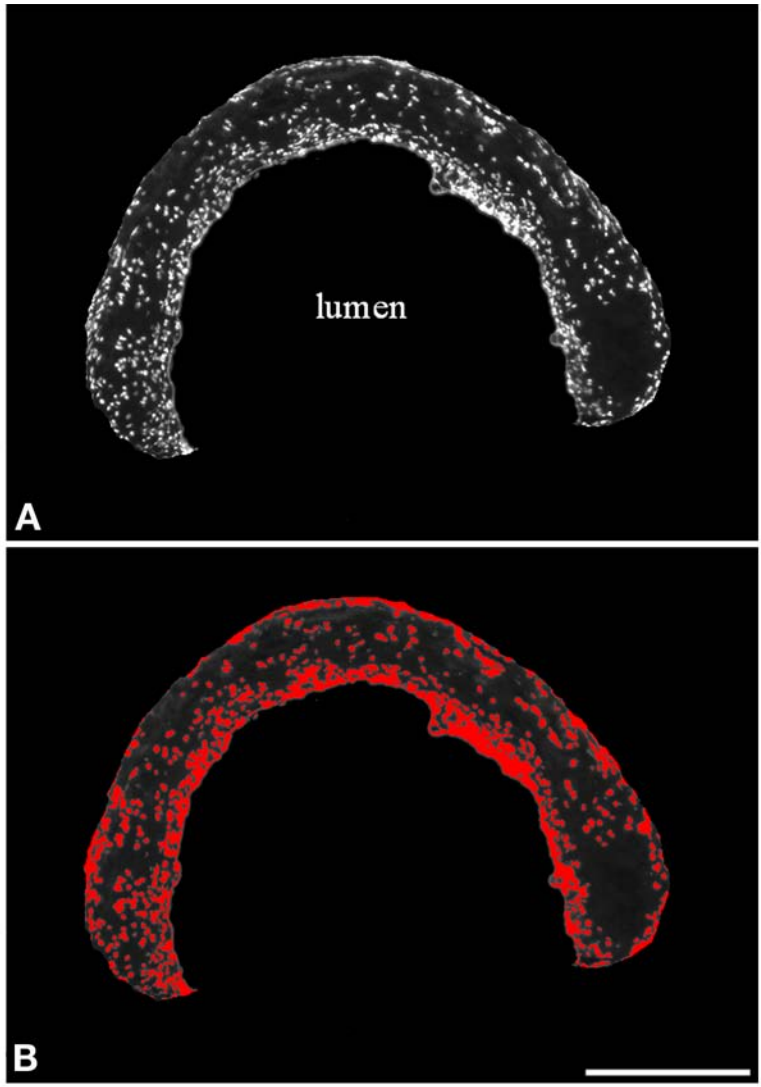

Fig. 2. **(A)** Gray-scale fluorescent capture of DAPI-stained nuclei in one portion of the lesion. The media has been cropped away, revealing the lesion cellularity only. **(B)** Red pseudocolor overlay of the image in **(A)** indicating all pixel intensities included in the threshold. Scale bar = 250 μm. (*See* Color Plate 5, following p. 274.)

Results: Elastic fibers should be purple to deep purple and other tissue elements green if Light Green counterstain is used. If combined with Picrosirius red stain, collagen fibers will be red and can also be viewed with polarized light.

3.5. Collagen

Picrosirius red stain: Dolber and Spach Method (13)

1. Air-dry cryostat sections for at least 30 min prior to staining.
2. Hydrate sections in distilled water for 2 min.
3. Stain for 90 min in filtered 0.1% Sirius red F3BA/saturated picric acid at room temperature.
4. Wash in 0.01 N HCl for 1 min.
5. Rinse in 70% ETOH.
6. Dehydrate with 10–15 dips in two changes each of 95% alcohol, 100% alcohol, and three changes of xylene and mount with a synthetic mounting medium.

Results: In bright field, collagen is stained red and cytoplasm is stained yellow (*see* **Note 12**). For more accurate determination of collagen using this stain, the sections are viewed by polarized light microscopy *(14)*.

3.6. Immunostaining

1. PBS/1.0%BSA/0.1% Triton X-100, 15 min.
2. PBS/1.0% BSA, 10 min.
3. 10% Normal serum directed against the host of the secondary antibody, diluted in PBS/1%BSA, 30 min.
4. Primary antibody, diluted in PBS/1% BSA overnight at 4°C.
5. Rinse with PBS/1% BSA, 2X 10 min at room temperature.
6. Secondary antibody diluted with PBS/1% BSA, 60 min.
7. PBS/1%BSA 2X 5 min.
8. Nuclear stain, 15 min.
9. PBS/1% BSA 2X 5 min.
10. Mount in glycerol-based mounting medium containing antifade reagent (*see* **Note 13**).

3.7. Imaging Procedures and Digital Capture

3.7.1. Confocal Scanning Laser Microscopy

Digital images are captured using a Bio-Rad 1024 confocal laser scanning microscope using Lasersharp 4.2 software (Bio-Rad Laboratories, Hercules, CA). The laser power, iris, gain, and offset for each channel were set using the *Set col* overlay as an intensity guide (*see* **Note 14**). Images are captured using a 1024×1024 box size for increased resolution and in sequential capture mode to avoid channel-to-channel bleed-through. Images are Kalman filtered three times on a final capture for possible noise reduction. The Bio-Rad .pic format is converted to .tif format using a Photoshop plug-in, or by using the export function available in Lasersharp software.

3.7.2. Wide-Field Bright-Field Microscopy

Digital images are captured using an Olympus BX50 upright light microscope (Olympus America, Inc., Lake Success, NY) with an attached Optronics MagnaFire digital camera (Optical Analysis Corp., Nashua, NH) and associated MagnaFire software (version 2.0). The captured images are displayed in 1280 × 1024 pixel RGB format. The objective lenses used are first cleaned and the microscope aligned for Koehler Illumination. The condenser aperture is set at one-half open, and the LBD, ND6, and ND25 filters are engaged. The microscope illumination potentiometer is set at 5 and the beam splitter is pulled out fully to deliver 100% of the signal to the camera. Exposure is set appropriately using a clip detect mode (*see* **Note 15**), an infrared (IR) cut filter is selected to filter out infrared light from the subject, and a white balance is done on a blank area of the slide.

3.7.3. Wide-Field Fluorescence Microscopy

Fluorescence images are captured using the Olympus BX50 microscope as described above. The exposure is set using the clip detect mode, and the white balance preset is set to "fluorescence." The IR cut filter is chosen and images are captured in 1280 × 1024 8-bit gray-scale format (*see* **Fig. 2A**).

3.7.4. Polarized Light Microscopy (see ***Fig. 3***)

Polarized images are captured in 1280 × 1024 8-bit gray-scale format and combined with an additional gray-scale unpolarized image captured by turning the polarizer to an unpolarized position using the clip detect function to avoid oversaturation and inserting a neutral density filter. An RGB image is created from the gray-scale images using the *color/merge* function. The unpolarized image is dropped into the green and blue channels and the polarized image is cropped into the red channel (*see* **Fig. 3A;** Color Plate 6, following p. 274). Dropping the unpolarized image into two channels considerably lightens the final image and assists in more accurately discriminating lesion borders during the subsequent image cropping (*see* **Fig. 3B**). Combining the Picrosirius red stain (imaged with polarized light) with the Aldehyde Fuchsin stain (imaged unpolarized in bright field) provides definition to the elastic lamina. After cropping, the green and blue channels are discarded, leaving an 8-bit gray-scale image of the polarized red channel (*see* **Fig. 3C**).

3.8. Computer-Assisted Image Processing and Analysis

3.8.1. Image Processing

Image processing is easily accomplished with Adobe Photoshop (Adobe Systems Inc., San Jose, CA). Images are opened and the *lasso* and *magic wand* tools are used to selectively highlight and fill unwanted areas with black.

Cropping can be assisted with the addition of a WACOM tablet and Intuos 2 stylus (CDWG, Vernon Hill, IL), which is a more elegant drawing tool that replaces the traditional mouse and mouse pad. Using this tool, the artery (*see* **Fig. 1B**) and, subsequently, the lesion are isolated from the surrounding tissue for analysis (*see* **Note 16**).

3.8.2. Image Analysis

Although we originally performed the image analysis in Adobe Photoshop using a modified version of the protocols published by Lehr and co-workers (***15***,***16***), it is now done using MetaMorph (Universal Imaging Corp., Downingtown, PA) software. Cropped digital images are opened and the appropriate (precalibrated) objective calibration is set by choosing *Measure/Calibrate Distance/Apply*, followed by minimizing this window. This will allow for area measurements to be expressed in calibrated square microns. Pixel values will be displayed at the bottom of the screen.

3.8.2.1. Measurements of the Artery and Lesion Using an RGB Threshold

Color thresholding is applied by choosing the following software options:

1. Color threshold "HSI."
2. Hue range "inclusive."
3. Hue 0–255.
4. Saturation 0–255.
5. Intensity 1–250.
6. State "inclusive."

3.8.2.2. Measurements for Lipid

1. Color threshold "HSI."
2. Hue range "exclusive."
3. Hue 10–240.
4. Saturation 55–255.
5. Intensity 0–255.
6. State "inclusive."

3.8.2.3. Measurements for Cellularity Using Gray-Scale Threshold (**Fig. 2B**)

1. Gray-scale 90–255.
2. State "inclusive."

Once the image is thresholded, the *Integrated Morphometry* feature will measure the thresholded area and log the area values onto a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA). The logged values are then converted into an Excel chart for presentation (*see* **Fig. 4**). Animal comparisons are calculated and expressed as both area and percent values.

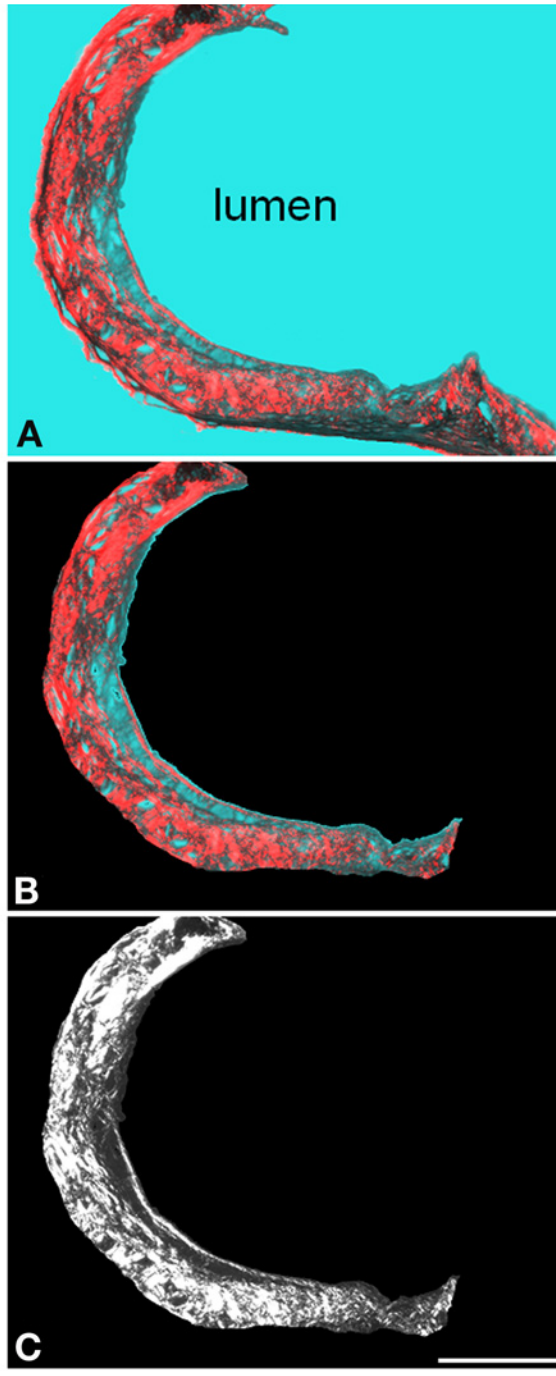

Fig. 3.

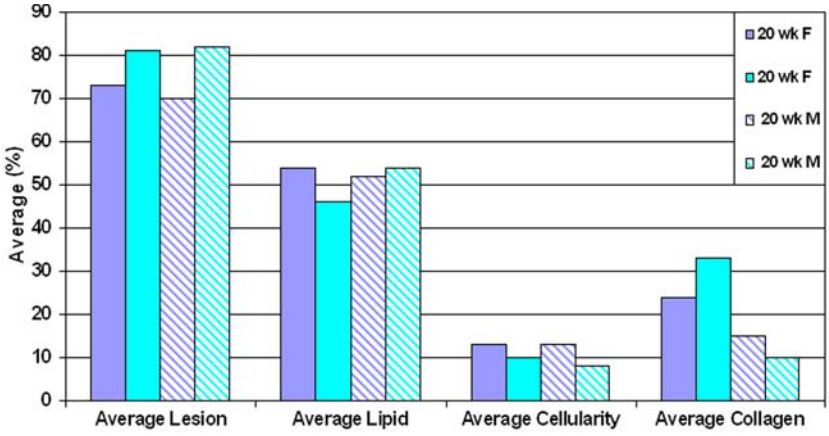

Fig. 4. Microsoft Excel chart displaying the quantitative evaluation of atherosclerotic lesion composition in 20-wk-old male and female Apo $E^{-/-}$ mice fed a high-fat diet for 16 wk. Note the large increase in lesion collagen content in the female mice as compared to their male counterparts.

4. Notes

1. There are a wide variety of insulated flasks available. For safety, any flask used must be able to withstand the low temperature of liquid nitrogen. For our use, a 1-L-capacity flask works well.
2. We made this by punching a hole and attaching a long string on either side of a 150-mL plastic cup. The cup is gently suspended in the LN_2 and the strings are secured to the carrying handle of the container. One hundred milliliters of the 2-methylbutane are added very slowly to the cup. The nitrogen will boil vigorously until the liquid is cooled, so use precautions such as eye protection and pour very slowly.
3. Stains can be purchased from a variety of sources and can be identified by their unique color index (CI) number.
4. Basic Fuchsin is also known as Pararosaniline and Basic Red 9.
5. Acetaldehyde is a highly volatile substance and great care must be taken during its use. It is used in this elastic stain as a substitute for paraldehyde, which must be

Fig. 3. **(A)** Picrosirius red stain from the atherosclerotic lesion captured in the gray scale with polarized light and merged into the red channel with a bright-field unpolarized image that was merged into the green and blue channels. These channels are used for cropping purposes for orientation and will be discarded later. **(B)** Cropped image with the media removed revealing only the isolated lesion. The image was cropped by turning off the red and blue channels and using the green channel's bright-field image to identify the elastic lamina as a landmark for cropping purposes. **(C)** Gray-scale image of the red polarized channel after the green and blue channels have been discarded. Scale bar = 250 µm. (*see* Color Plate 6, following p. 274.)

obtained with a DEA (Drug Enforcement Administration) number. Moreover, paraldehyde must be freshly opened, with the remainder of the container discarded.

6. When weighing and using chemicals refer to the Material Safety Data Sheet (MSDS) for that chemical and use the appropriate precautions, including working in a fume hood and wearing protective gloves and mask.
7. Oil red O is a physical method of staining that relies on the greater affinity for solubility of the dye in lipid than the dye solvent. Alcoholic fixatives should be avoided because they act as lipid solvents. A positive control is usually not needed because most tissue contains some fat.
8. Oil red O staining must be performed on cryostat sectioned frozen tissue. Lipids are solubilized by the organic solvents used in traditional paraffin embedding.
9. The excitation (Ex)/emission (Em) spectra for some of the DNA-binding dyes we have used are listed as follows: DAPI (4′ ,6-diamidino-2-phenylindole dihydrochloride), 359 nm Ex/461 nm Em; SYTOX Green, 504 nm Ex/523 nm Em; propidium iodide (containing RNase), 535 nm Ex/617 nm Em. All of these dyes can be excited by a conventional mercury arc lamp on a wide-field fluorescence microscope. However, for the confocal microscopic excitation of DAPI, an ultraviolet laser is required.
10. Aqueous mounting media must be used for cover-slipping, because resin-based mounting media contain lipid-extracting solvents. When adding a cover slip to the slide, care must be taken to not apply pressure on the cover slip, which might displace the lipid droplets. To optimize resolution, match the cover slip thickness being used with the appropriate corrected objective.
11. Oil red O can be viewed with transmitted light, and it has fluorescent properties when excited by far-red light. This stain is compatible with other fluorescent staining procedures, such as DNA labeling and immunostaining for cellular identification. Thus, with a confocal microscope with three excitation wavelengths, multiple components can be examined. We found fluorescent comparisons are best made with sections stained and imaged in a single batch. Fluorescent staining and imaging, done over time, can introduce slight variability in intensity as a result of fluorophore age, bulb life, imaging conditions, and other variables, which can influence intensity comparisons.
12. Picrosirius red staining can be performed on either fixed paraffin or cryostat sections. Possibly because of the highly acid nature of the Picrosirius red stain, we have been unsuccessful in combining it with immunofluorescent dyes. However, we have successfully used it in combination with hematoxylin stains and the Aldehyde Fuchsin stain for elastic fibers. Picrosirius red can be viewed with transmitted, fluorescent (yellow light excitation), or polarized light (preferred).
13. Primary antibodies of interest can be used in cocktail combinations and combine nicely with a DNA-specific fluorophore such as DAPI, SYTOX Green, propidium iodide, or TOTO. Note that for widefield imaging, the bleedthrough of SYTOX Green nuclear stain can also be detected with a wide-band green-filter cube.
14. The Bio-Rad *Set col* is a look-up table (LUT) that assigns specific colors to pixel intensity ranges on an 8-bit scale. In this case, pixel intensities from 0 to 5 are

depicted as green, 6 to 249 are depicted as gray, and 250 to 255 are depicted as red. This LUT is a useful guide to ensure that a full dynamic range of gray scale is depicted in the image. An optimized image would contain a few green and red pixels, with the majority represented by gray. This method produces the same effect as adjusting a histogram of intensity values on an image.

15. The "clip detect" feature in the MagnaFire software is similar in function to the Bio-Rad *Set col* LUT described in **Note 14**. Essentially, this feature represents saturated pixels by the color red. The exposure time can then be adjusted to allow for only a few red pixels (saturated intensity) in the field.
16. Cropping of the images can also be done in MetaMorph by using the WACOM tablet and stylus and the MetaMorph *drawing tool* option to draw around unwanted areas, followed by the *Display/Graphics/Paint outside* (or inside) region to fill the unwanted areas. The highlighted area must then be reselected and deleted.

References

1. Oberholzer, M., Ostreicher, M., Christen, H., and Bruhlmann, M. (1996) Methods in quantitative image analysis. *Histochem. Cell Biol.* **105**, 333–355.
2. Taatjes, D. J., Wadsworth, M. P., Schneider, D. J., and Sobel, B. E. (2000) Improved quantitative characterization of atherosclerotic plaque composition with immunohistochemistry, confocal fluorescence microscopy, and computer-assisted image analysis. *Histochem. Cell Biol.* **113**, 161–173.
3. Wadsworth, M. P., Sobel, B. E., Schneider, D. J., and Taatjes, D. J. (2002) Delineation of the evolution of compositional changes in atheroma. *Histochem. Cell Biol.* **118**, 59–68.
4. Taatjes, D. J., Schneider, D. J., Bovill, E. G., and Sobel, B. E. (2001) Microscopy-based imaging of the pathogenesis of cardiovascular disease. *Microsc. Anal.* **50**, 21–23.
5. Falk, E. (1985) Unstable angina with fatal outcome: dynamic coronary thrombosis leading to infarction and/or sudden death: autopsy evidence of recurrent mural thrombosis with peripheral embolization culminating in total vascular occlusion. *Circulation* **71**, 699–708.
6. Davies, M. J., Bland, M. J., Hangartner, W. R., Angelini, A., and Thomas, A. C. (1989) Factors influencing the presence of acute coronary thrombi in sudden ischemic death. *Eur. Heart J.* **10**, 203–208.
7. Sobel, B. E. (1999) Increased plasminogen activator inhibitor-1 and vasculopathy: a reconcilable paradox. *Circulation* **99**, 2496–2498.
8. Sobel, B. E. (1999) The potential influence of insulin and plasminogen activator inhibitor type 1 on the formation of vulnerable atherosclerotic plaques associated with type 2 diabetes. *Proc. Assoc. Am. Physicians* **111**, 313–318.
9. Newby, A. C., Libby, P., and van der Wal, A. C. (1999) Plaque instability—the real challenge for atherosclerosis research in the next decade? *Cardiovasc. Res.* **41**, 321–322.
10. Breslow, J. L. (1996) Mouse models of atherosclerosis. *Science* **272**, 685–688.

11. Warden, C. H., Hedrick, C. C., Qiao, J. -H., Castellani, L. W., and Lusis, A. J. (1993) Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science* **261**, 469–472.
12. Paigen, B., Morrow, A., Holmes, P. A., Mitchell, D., and Williams, R. A. (1987) Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* **68**, 231–240.
13. Dolber, P. C. and Spach, M. S. (1993) Conventional and confocal fluorescence microscopy of collagen fibers in the heart. *J. Histochem. Cytochem.* **41**, 465–469.
14. Junqueira, L. C. U., Bignolas, G., and Brentani, R. R. (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem. J.* **11**, 447–455.
15. Lehr, H. -A., Mankoff, D. A., Corwin, D., Santeusanio, G., and Gown, A. M. (1997) Application of Photoshop-based image analysis for quantification of hormone receptor expression in breast cancer. *J. Histochem. Cytochem.* **45**, 1559–1565.
16. Lehr, H. -A., van der Loos, C. M., Teeling, P., and Gown, A. M. (1999) Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-based image analysis. *J. Histochem. Cytochem.* **47**, 119–125.