All tissues depend on blood supply and the blood supply depends on healthy vessels. The wall of mature blood vessels such as arteries and veins has a layered structure with the innermost layer being a single layer of endothelial cells, known also as the vascular endothelium [1]. The smallest branches of the vascular tree are the capillaries. Their size is in the 5-10 µm range [1]. The wall of a capillary consists of endothelial cells wrapped in basal lamina that hold the cells together. The wall is so thin that nutrients can pass through it by diffusion and enter the surrounding tissue [1]. Waste products can diffuse back into the blood to be carried away and removed from the body. Thus, endothelial cells not only line the entire vascular system, but also control the passage of materials into and out of the bloodstream [1].

The adult vascular endothelium is normally maintained in a differentiated, quiescent state [2, 3]. During embryonic development, and in some adult physiological and pathological events, the endothelial cells become activated, thus enabling them to acquire migratory and proliferative properties. This process is angiogenesis: the formation of new blood vessels from existing vessels [4].

Formation of angiogenic vessels requires orchestrated activation of growth factors, integrins, membrane-bound proteinases and extracellular matrix (ECM) components [2, 3, 5]. These factors stimulate extracellular and intracellular signaling pathways that regulate endothelial cell branching, sprouting, lumen formation and proliferation [5]. Anti-angiogenic factors are also produced during the angiogenic process to balance the activities of pro-angiogenic molecules through tightly controlled cell death and survival functions [3, 6-8]. In pathological conditions, that balance is disrupted, ultimately leading to formation of diseased vessels.

The subtle stage of diseased vessel formation is a primary focus of study in the field of vascular biology. It is involved in many aspects of cardiovascular disease as well as growth of tumors. This review highlights the capabilities of confocal microscopy and micro computed tomography (MicroCT) in imaging vessels, particularly with reference to growth of atherosclerotic plaque.

Imaging vasa vasorum

Angiogenesis is associated with more advanced stages of human atherosclerosis [9]. Angiogenic vessels found in plaque are thought to originate from the vasa vasorum [10]. These vessels (Latin, “vessels of the vessels”) form a network that supplies the outer (tunica adventitia) layer and middle (tunica media) layer of the larger blood vessels [11]. The vasa vasorum can expand to the second order forming many angiogenic microvessels that enter the vessel wall to provide arterial blood supply to the arterial wall [12].

The development and expansion of the second order vasa vasorum correlates with atherosclerotic lesion size in hypercholesterolemic animal models and are thought to be the conduit for nutrient supplies to the plaque [13-15]. We and others have demonstrated that inhibition of neovascularization in the vasa vasorum reduces plaque progression [13, 14, 16]. In each case the inhibitor is a breakdown products of an ECM protein that does not have anti-angiogenic function in its normal configuration [17-20]. Interestingly, others have proposed that inhibition of the expanded vasa vasorum leads to plaque progression [21]. The rationale is that loss of oxygen supply from the vasa vasorum to the vessel wall would generate a hypoxic environment, a process that stimulates factors that lead to plaque development. This was supported by data which demonstrated that an occluded vasa vasorum led to atherosclerosis [22-24]. However, these studies were performed in non-diseased
animal models, which may be quite different from those with the disease.

Animal models of atherosclerosis enable examination of disease progression, while advanced imaging technology provides a means of examining the vasculature and how its changes correlate with initiation and growth of the atherosclerotic lesion. Genetically modified mice that develop plaque when fed a high fat diet are commonly used to study these effects. We present imaging techniques that have been applied to studying atherosclerosis in a genetically modified LDLR-/ApoB48 deficient mouse, which lacks the LDL receptor and has a mutation in the ApoB48 gene such that it is non-functional [25]. The mice were fed an atherogenic diet for 14 weeks and then treated with either saline or an angiogenesis inhibitor, rPAI-123, which is a truncated plasminogen activator inhibitor-1 (PAI-1) isoform [17, 18]. Plaque size, plaque cholesterol and vessel wall measurements showed that the inhibitor has a significant effect on reducing plaque growth and, in fact, suggest that it promotes plaque regression [16]. Confocal microscopy and micro CT techniques were used to compare the vasculature in the rPAI-123 treated mice with the saline treatment group [16].

**Confocal microscopy**

Confocal microscopy is an optical molecular imaging technique that provides reconstruction of 3D images (Z-stacks) of specific molecules that are detected when antibodies conjugated to a fluorophore bind to the target molecule. The most common application is to apply a fluorescently-labeled antibody or molecule to a tissue sample to identify the presence of the specific target molecule [26-28]. The technique can show localization of the target molecule and provide a sense of expression levels.

Quantitative data can be obtained by analysis of confocal Z-stacks with image software such as Volocity V.3.7 (Improvision, Coventry, UK). Figure 1 demonstrates application of this approach [16]. Another approach is to probe tissue for two specific target molecules using two antibodies, each specific for a different target and each conjugated to a fluorophore that excites in a different wavelength.

This approach enables identification of co-localized target molecules, as is demonstrated in Figures 2a-c and 3a-c [16]. In this case, a FITC-conjugated antibody to CD31 was used to identify endothelial cells in the adventitia and plaque of atherogenic mice treated with either rPAI-123 or saline. A second antibody specific for smooth muscle actin was conjugated to Alexa Fluor 568 (Invitrogen). This antibody binds smooth muscle cells in the vessel wall and is an indicator of vessel maturity/stability. If the two fluorophores, conjugated to their respective antibodies, co-localize, then the emitted color spectra are seen as merged (Figures 2a-c, 3a-c). Confocal microscopy plays a very important role in correlating and validating results. For example, the confocal images shown in Figures 2 and 3 were collected as Z-stacks, then thresholded to segment out the signal of the fluorophore-conjugated antibody bound to the specific target. Blood vessels in the plaque, adventitia, and vessel wall can be visualized by alignment of confocal Z-stacks images in a 3-D volumetric image.

The resolution of the volumetric data is increased by tri-linear interpolation to yield a 0.254 µm isotropic voxel. The reconstructed Z-stacks are then manually segmented to represent the detected co-localized probes in consecutive axial slices. Contours of blood vessels going all the way through the interpolated volumes are modeled and stacked in 3-D to provide volumetric surface representation (Figures 2d-2f) and (Figures 3d-3f) [16].

**Micro Computed Tomography (MicroCT)**

Micro Computed Tomography is a structural imaging modality that provides differentiation of contrast-enhanced tissues or structures with high attenuation from non-enhanced soft tissues [29, 30]. Traditional MicroCT imaging applications include screening for anatomical abnormalities [10, 31-35] as well as detection and quantification of changes in live animals [36, 37] or tissue samples removed from sacrificed animals [38].

Most current *in vivo* MicroCT scanners have resolutions ranging from 100 to 30 µm, while *ex vivo* scanners have resolutions from 30 to 1 µm. Due to its high spatial resolution, MicroCT has become an important structural imaging modality for vascular applications and angiogenesis [38]. Its high-resolution 3D representation of vascular structures directly reflects the level of angiogenesis or inhibition/development of neovascularature, providing a quantitative means for assessment of tumor growth over time.

The imaging is a two-step procedure. In the first step, 2D X-ray projections of the imaged object at different orientations of the X-ray source or object are acquired by the X-ray detector. In the second step, the acquired projections are first corrected for various image artifacts and/or
Figure 1. Vasa vasorum detected with descending aorta whole mounts. Atherogenic LDLR-/-ApoB48 deficient mice were treated with either (A) saline or (B) an angiogenesis inhibitor for six weeks with continued high fat diet. Following the treatment period, mice were perfused, descending aortas removed and probed for CD31 using a FITC-labeled anti-CD31. Vasa vasorum were visualized in Z-stack confocal images of descending aorta whole mounts. Figure from Drinane et al. [16].

![Figure 1a. Saline.](image1)

![Figure 1b. Angiogenesis inhibitor.](image2)

Figure 2. Blood vessels of the vasa vasorum in the descending aorta (adventitia, wall and plaque) of saline treated atherogenic mice. Atherogenic mice were treated with either saline or rPAI-123, an angiogenesis inhibitor. Descending aorta cross sections were probed for smooth muscle actin (green) and Lycopersicon esculentum lectin (red) and imaged by confocal microscope. Z-stacks were acquired at a physical resolution of 2.54 μm. To visualize blood vessels in the adventitia, vessel wall and plaque, the Z-stacks were manually segmented to represent co-localized probes in consecutive axial slices. The contours obtained were modeled and stacked in 3D for volumetric surface representation. Figure from Drinane et al. [16].

![Figure 2a. Cross section of descending aorta: adventitia.](image3)

![Figure 2b. Cross section of descending aorta: vessel wall.](image4)

![Figure 2c. Cross section of descending aorta: plaque.](image5)

![Figure 2d. Blood vessels in the adventitia.](image6)

![Figure 2e. Blood vessels in the vessel wall.](image7)

![Figure 2f. Blood vessels in plaque](image8)
Figure 3. Blood vessels of the vasa vasorum in the descending aorta (adventitia, wall and plaque) of rPAI-123 treated atherogenic mice. Images were acquired as described in Figure 2. Figure from Drinane et al. [16].

Figure 3a. Cross section of descending aorta: adventitia.

Figure 3b. Cross section of descending aorta: vessel wall.

Figure 3c. Cross section of descending aorta: plaque.

Figure 3d. Blood vessels in the adventitia.

Figure 3e. Blood vessels in the vessel wall.

Figure 3f. Blood vessels in plaque

Figure 4. MicroCT images of second order vasa vasorum. Atherogenic mice treated with saline or an angiogenesis inhibitor, were infused with microfil. Descending aortas were removed and scanned at 6.5 microns and three-dimensional volumetric images of vasa vasorum were reconstructed from mice receiving various diets and treatment. Figure from Drinane et al. [16].

Figure 4a. Atherogenic diet for 14 weeks (T0).

Figure 4b. 20 weeks of atherogenic diet, saline treatment during weeks 14-20.

Figure 4c. 20 weeks of atherogenic diet, rPAI-123 treatment during weeks 14-20.

Figure 4d-f. Reconstructed images rotated to show plaque.
distortions due to sensor nonlinearity and then used to reconstruct the final volumetric data representing the scanned object in 3D.

Depending on the parameters of the imaging protocol, a scan may take from several minutes to hours. During image acquisition, X-rays emitted by the source are attenuated as they pass through the imaged object. This proportionally reduces the original intensity of the X-rays due to absorption or scatter of photons [29, 30]. Objects with low attenuation properties, like most soft tissue, allow most of the X-rays to pass through the object unabsorbed and arrive at the detector on the other end.

However, if the imaged object has high attenuating properties, for example bone, fewer X-rays will have the kinetic energy to arrive at the detector. In imaging of diseased vessels with MicroCT, we are interested in the vascular structures whose attenuation properties differ very little from the surrounding soft tissue. Consequently, vascular MicroCT requires the use of contrast agents such as Fenestra, Microfil, and Bismuth. The contrast agents either circulate in the blood pool or are used to replace it.

Figure 4a-c shows an example of second order vasa vasorum detected by MicroCT [16]. The contrast agent selected for this procedure consisted of a mixture containing silicone rubber compound Microfil Blue (1 ml), Microfil Clear (3 ml), diluent (8 ml) and curing agent (0.6 ml) (Flow Tech, Inc., Carver, Massachusetts). The mixture is infused through an aortic cannula into the ascending aorta of atherogenic mice treated with saline or the anti-angiogenic protein. Once the microfilm polymerizes, the descending aortas are removed for ex vivo microCT scanning followed by 3-D reconstruction of the scanned images. In these experiments, the scanning protocol was optimized for soft tissue imaging before the mice were scanned at a voltage and current of the X-ray tube of 52 kV and 118 mA respectively. The X-ray exposure time for a single projection was set to 1700 ms and a total of 720 projections per scan were acquired at the maximum resolution of the X-ray detector, 6.5 microns. Following the scan, three-dimensional volumetric images were reconstructed from the acquired two-dimensional projections without averaging, yielding a final voxel size of 6.5 microns and data volumes of approximately 1.5 GBs.

Differences in the density of the vasa vasorum among treatment groups are clearly visualized. The correlation between the vasa vasorum density and plaque size were made possible by rotating the MicroCT images to visualize plaque in the luminal side of the descending aorta (Figures 4 D-F) [16].

**Conclusion**

Confocal microscopy and MicroCT are two of the techniques available for imaging angiogenesis. Confocal microscopy provides targeted imaging of specific biological molecules involved in angiogenesis, while MicroCT provides high-resolution structural imaging. As such, the two techniques are complementary. They can be combined with the use of genetically modified mice to perform studies that investigate mechanisms of disease. Further challenges include the development of novel contrast agents and better imaging hardware that can bridge the gap between sensitivity and resolution that exists in the functional imaging modalities today. Another important challenge is the interpretation and processing of the acquired data.

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**References**


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