# Application of Confocal Microscopy for 3D Assessment of Carotid Plaque Structure: Implications for Carotid Blood Flow and Stroke Research

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## Abstract

*Background:* Little information is available on how forces resulting from fluid flow interact with structural stability of carotid atherosclerotic plaque and how such interactions may impact on stroke prevention; investigation of the 3D structure of plaque could help in such studies. The aim of this study was to investigate whether confocal microscopy can be used to obtain 3D visualization of the structure of atherosclerotic carotid plaques.

*Methods:* Carotid plaque specimens were collected from routine end-arterectomy surgical operations. Both bright-field microscopy and Laser Scanning Confocal Microscopy (LSCM) were used to generate 3D image data-sets and visualizations of surgically removed carotid plaques.

*Results:* Evidence of carotid plaque vulnerability was demonstrated by reduced fibrous cap thickness and large lipid-necrotic core with evidence of cracking.

*Conclusion:* The generation of 3D images of carotid plaques could help in: (i) investigating key features that affect plaque structural stability; (ii) comparing 3D microstructure of the plaque with clinical imaging assessment and blood flow investigations; and (iii) developing markers to identify patients requiring clinical intervention.

*Keywords:* Carotid plaque; 3D images; LSCM; Blood flow; Plaque vulnerability

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## Introduction

Stroke is the third leading cause of death and a leading cause of long-term disability; it is estimated that around 6,500,000 stroke survivors are alive today (2,600,000 males and 3,900,000 females) in the United States alone.[1] Each year, about 795,000 people suffer a stroke and over 137,119 people died from stroke in 2006 in the United States.[2]

In the United Kingdom, strokes account for more than 8% of deaths in men and 12% of deaths in women,[3] with the total cost to the National Health Service estimated to be over £7 billion per year.[4] Around 70-80% of strokes are ischemic in character, caused by the obstruction of the major arteries in the cerebral circulation.[5] Although stroke is generally understood as a medical phenomenon, it is also a physical event influenced by fluid dynamics.[6] However, little is known about the etiology of stroke and how mechanical forces interact with patho-biologic aspects. In particular, the relevance of geometry, strength and internal architecture of carotid plaque on their long-term stability in the fluid flow field are poorly understood; this reinforces the need to better understand the physical processes involved in stroke.

Ultrasound imaging of atheromatous plaques in patients with carotid disease has revealed a spectrum of lesions ranging from plaques with predominantly echo-lucent properties to those which are densely echogenic. The clinical application of carotid plaque characterization lies in identifying patients who require clinical intervention. Although fibrous plaques are essentially stable lesions, whereas lipid-laden plaques are prone to intimal tearing (the commonest event initiating embolization and stroke),[7] it has not yet proved possible to show any definite link between a specific plaque type and cerebrovascular events. Such an association is probably multi-factorial and mediated by a combination of the degree of stenosis, plaque morphology, strength and internal (3D) structure, fluid flow and biological factors. What is clear is that critical interaction between these factors can result either in embolization (or hemodynamic compromise) or beneficial stabilization.[8] Plaques, particularly if soft, can rupture causing stroke; since most are usually

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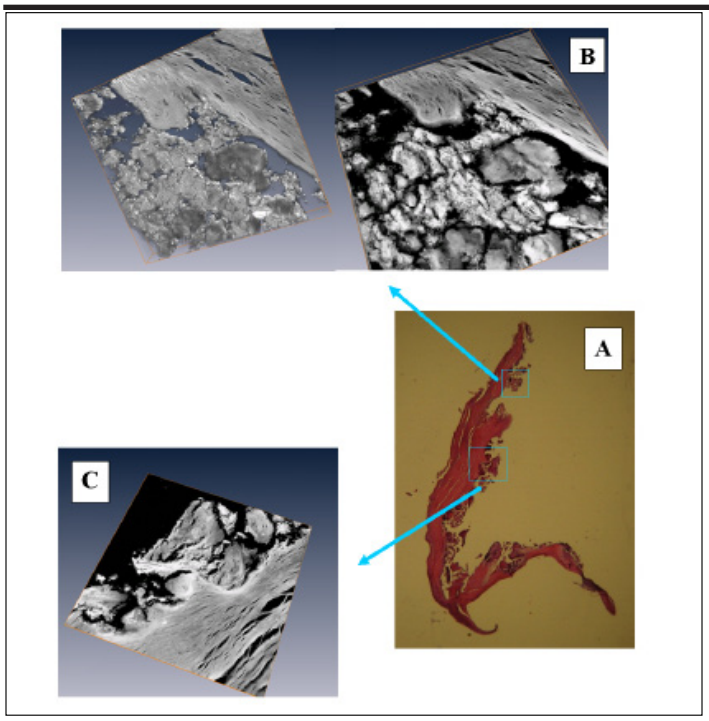


**Figure 1:** Image (A) represents a low magnification image of cross section of carotid plaque specimen stained with H&E stain. Image (B) represents x20 objective orthoslice and intensity projection visualization of part of carotid plaque specimen, using LSCM. The lower image (C) is from an intensity projection visualization using a x10 objective.

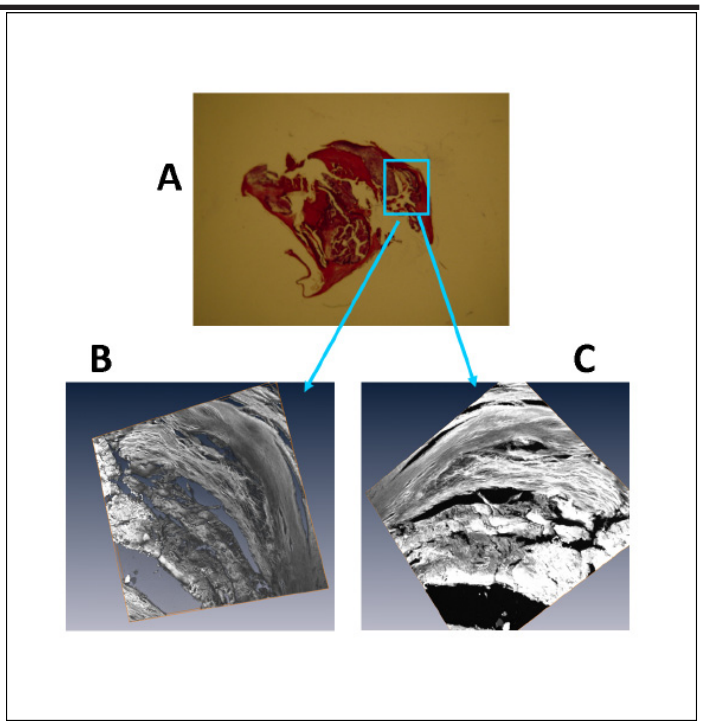


**Figure 2:** Image (A) represents a low magnification image of cross section of a second carotid plaque specimen stained with H&E stain. Images (B & C) represent x20 objective orthoslice and intensity projection visualization of part of carotid plaque two, using LSCM.

asymptomatic, it is difficult to know which plaques should be treated and which can be safely left alone.

Laser Scanning Confocal Microscopy (LSCM) uses laser light to generate a series of 2D images of parallel planes in a sample. With software it is thus possible to generate 3D visualization of such scans in order to help to understand the structure of the sample. If this technique could be applied to examine carotid plaque specimens, it could provide a means of characterizing plaque for comparison with ultrasound imaging.

The aim of this study was to investigate whether confocal microscopy can be used to obtain 3D visualization of the microstructure of atherosclerotic carotid plaques obtained from surgical end-arterectomy specimens.

## Materials and Methods

### Carotid plaques

Carotid plaque specimens were collected from routine end-arterectomy surgical operations. All participants gave informed consent and the study protocol was approved by the National Health Service local research Ethics committee. Carotid plaques were removed en bloc during surgery to preserve the entire plaque structure.

### Histological examination

Carotid plaque specimens were first washed with saline (immediately after removal) in order to remove any excess blood then fixed in 10% buffered formalin. After decalcification, each plaque was sectioned transversely into 3 parts and paraffin embedded. For each paraffin-embedded part, a number of sections with 5, 20 and 30 micrometre thickness were cut. Each slide was sequentially numbered in order to reconstruct the entire plaque length later on.

Haematoxylin and Eosin (H&E) staining was performed to stain the slides used for morphological studies by light microscopy. Slides containing 5 micrometre sections ere used for this purpose. Images of these slides were captured using a Stemi 2000 Stereo-microscope (Carl Zeiss, Germany) and Canon 7.1 Mega-pixel digital camera using a zoom of 0.8. Six carotid plaque slides stained with H&E were examined by light microscopy in order to characterize the following histological features: necrosis, calcification, fibrous cap, erosion or ulceration, hemorrhage, fibrous tissue and lipid content.

Slides containing 20 and 30 micrometre thick sections were stained with saturated aqueous fluorescein for a few minutes and then dehydrated, cleared and mounted in DPX. Six specimens were then examined and analyzed using Laser Scanning Confocal Microscopy (Leica SP1, Leica Microsystems GmbH, Germany); a 488 nm laser was used for excitation of the fluorescein; images

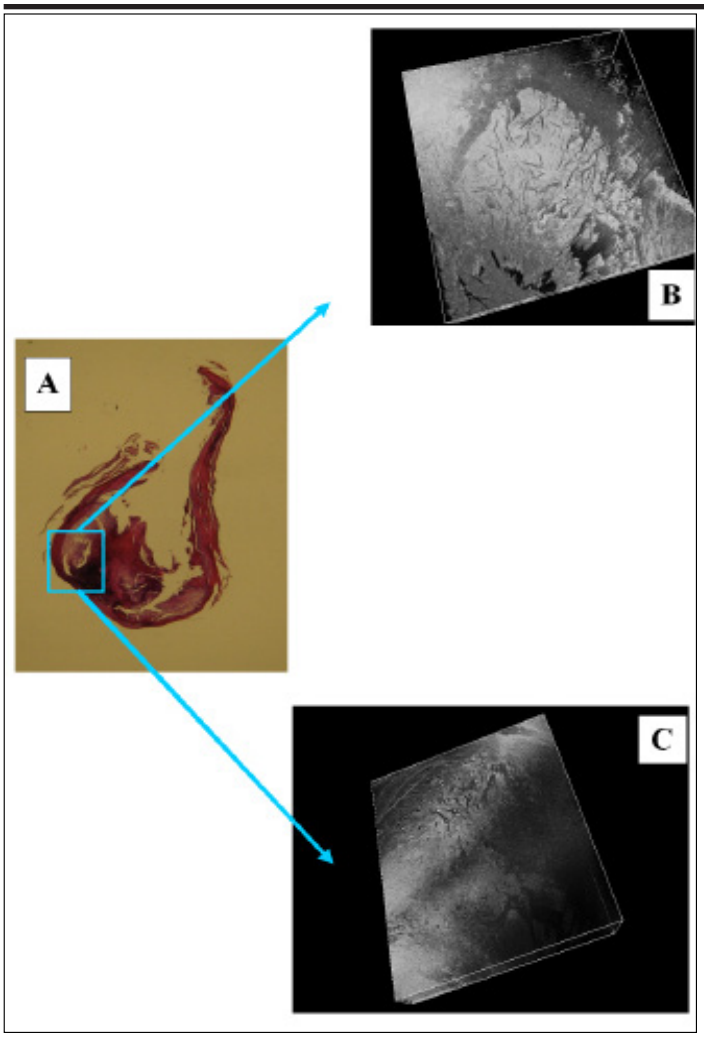


**Figure 3:** Image of H&E stained plaque (A), showing amorphous pink material with slit-like "cholesterol clefts" of lipid material (B, C). There is overlying haemorrhage on the plaque, with a thrombus formed on top of the plaque (A). 3D visualization using IRIS Explorer.

were captured with an image size of 1024 x 1024. Two objectives lenses a x10 (NA 0.3) and a x20 (NA 0.5) were used for the confocal microscopy.

### 3D visualization

Z-stacks from the confocal microscopy (x10 and x20 objective lenses) were visualized using Amira v4.0 (Mercury Computer System Inc, USA) and IRIS Explorer (NAG Ltd., UK). Two methods of visualization were used, stacking z-slices whilst making black transparent (with IRIS Explorer v5.0 and Amira v4.0) and secondly using intensity projection (maximum and average) Figures 1, 2 and 3 using Amira v4.0.

## Results

The images shown in Figures 1, 2 and 3 comprise examples of visualization of plaque sections using both light microscopy and LSCM.

Figures (1A, 2A, 3A) represent low magnification images of cross sections of three carotid plaque specimens stained with H&E stain. Figures (1B & 1C, 2B & 2C, 3B & represent the 3D visualizations of specific regions within these plaques stained with fluorescein stain and visualized by LSCM (as demonstrated in each diagram).

Fibrous tissue was present in all of the plaque samples in varying degrees. The three samples were predominately composed of lipid material, with necrotic core composed of amorphous debris and cholesterol clefts. Regions of actual fibrous cap disruption and some ulceration were observed in all of the samples. The extent of breaks in the fibrous cap varied with each plaque.

The specimen shown in Figure 1 was predominately composed of lipid material, with necrotic core (Figures 1B & 1C) comprised of amorphous debris and lipids.

Fraying of the fibrous cap was notable in one of the plaques (Figure 2A). In the same plaque there was fibrous cap erosion with exposure of the underlying necrotic core to the lumen (Figure 2A). Evidence of carotid plaque vulnerability (to rupture) as demonstrated by a reduced fibrous cap thickness, a large lipid-necrotic core and an increased inflammatory cells infiltrate was shown in Figure 2A. The 3D images showed lipid necrotic core with evidence of a crack in the middle of the core (Figures 2B & 2C).

Figure 3 showed ruptured carotid plaque, with amorphous pink material (Figure 3A) and a 3D slit-like "cholesterol clefts" of lipid material (Figures 3B & 3C). There is overlying hemorrhage on the plaque, with a thrombus formed on top of the plaque (Figure 3A).

## Discussion

In a nation where the aging population is growing and stroke is the third-leading cause of death, the study of carotid plaque morphology and blood-flow dynamics is of primary concern for stroke physicians, neurologists and vascular surgeons. Clinicians are in desperate need of quantitative treatment guidance and fundamental understanding of vascular abnormalities. A significant number of patients with carotid artery disease will require end-arterectomy as the disease is likely to progress.[9] Therefore, a more detailed understanding of the structure of carotid plaque and how this could be affected by blood flow dynamics is necessary.

This present study has shown that carotid plaques can be imaged using confocal microscopy and hence enabling examination of the internal 3-dimensional microstructure and geometry of the plaques. We were thus able to obtain 3D visualizations from different carotid plaque specimens. Most specimens were predominately composed of lipid material, comprising necrotic core of amorphous debris and cholesterol clefts, with varying degrees of fibrous tissue present in all plaques. Regions of actual fibrous cap disruption and some ulceration were also seen. Fraying of the fibrous cap was notable with fibrous cap erosion

and exposure of underlying necrotic core to lumen. Evidence of carotid plaque vulnerability as demonstrated by reduced fibrous cap thickness and large lipid-necrotic core with evidence of cracking was also seen in the 3D visualization.

Research suggests that shear stress generated by the nature of blood flow (whether laminar or turbulent) within the carotid artery might be responsible for stimulating a number of enzymes which have been implicated in the acute disruption of the carotid plaque leading to the onset of clinical ischemic events and stroke.[10,11] In addition, purely mechanical effects due to shear impeded by fluid flow can also result in instability and rupture.[12,13] Studying blood flow in areas of plaque induced vessel constriction with flow models may help to investigate prediction of carotid plaques vulnerability.[14,15] Work by Li and co-workers[13] has highlighted the importance of determining fibrous cap thickness, which results in an increase in plaque stress in arteries.

Our studies show that confocal microscopy can be successfully used to examine the 3D structure of carotid artery plaque specimens; such 3D microscopic imaging can be compared with ultrasound imaging (captured by routine diagnostic procedures such as Duplex ultrasound or if appropriate intra-vascular ultrasound "IVUS" techniques) in order to attempt to improve plaque characterization.

The extensive use of IVUS during the last 20 years, particularly in North America stems from its ability to reach and visualize deep structures of the artery, including the cross-sectional area of the atherosclerotic plaque. However, in addition to its invasive nature, its use has shown some difficulties and limitations in classifying plaque composition. Although recent advances in IVUS technology, such as the use of radio-frequency signal analysis, integrated backscatter and elastography have improved the capability of IVUS for plaque characterization, some of these limitations still pose significant challenges which limited the accuracy and clarity of the images produced by IVUS. The limited resolution of IVUS precludes accurate evaluation of relevant micro-structural features of the plaque. One area which might be explored is establishing a correlation between ultrasound images generated by IVUS and 3D images captured by confocal microscopy (as described in this study). Therefore, it could be argued that 3D image visualization can help to clarify structure which may be difficult from 2D images alone; this may help to overcome the lack of plaque characterization.

On the other hand, Optical Coherence Tomography (OCT) is a relatively new imaging technique that offers potential in the identification of, as well as the distinction between, stable and unstable atherosclerotic plaques. However, there seems to be too many false-negative diagnoses (for both lipid-rich plaques and fibro-calcific plaques) frequently being reported with OCT. These were mainly attributed to the limited penetration depth of OCT.

It is possible that the information gathered through the use of IVUS and OCT may ultimately reveal the true mechanism of plaque formation. As this knowledge is currently being applied in the field of 2D imaging histology using light microscopy, it might be interesting to compare 3D confocal microscope images of carotid plaque with prior diagnostic investigations carried out using conventional Duplex scanning or intra-vascular ultrasound or even OCT imaging. This may help to investigate possible characterization of such images including looking into the influence of resolution. Such an approach will lead to improvement in detection of vulnerable plaques from image characterization and may thus help to reduce stroke morbidity and mortality.

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