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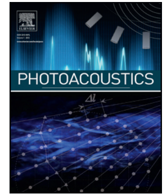

Research article

Multimodal optoacoustic and multiphoton microscopy of human carotid atheroma

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ABSTRACT

Carotid artery atherosclerosis is a main cause of stroke. Understanding atherosclerosis biology is critical in the development of targeted prevention and treatment strategies. Consequently, there is demand for advanced tools investigating atheroma pathology. We consider hybrid optoacoustic and multiphoton microscopy for the integrated and complementary interrogation of plaque tissue constituents and their mutual interactions. Herein, we visualize human carotid plaque using a hybrid multimodal imaging system that combines optical resolution optoacoustic (photoacoustic) microscopy, second and third harmonic generation microscopy, and two-photon excitation fluorescence microscopy. Our data suggest more comprehensive insights in the pathophysiology of atheroma formation and destabilization, by enabling congruent visualization of structural and biological features critical for the atherosclerotic process and its acute complications, such as red blood cells and collagen.
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1. Introduction

Atherosclerosis, a multifactorial disease of the arterial wall, is a major precursor of ischemic heart disease and stroke; two of the leading morality causes worldwide [1]. Ischemic stroke is attributed to thrombosis and cerebral ischemia and often associated with chronic atheromatous plaque accumulating within the sub-endothelial layer (intima) of carotid arteries. Effective plaque prevention or treatment requires understanding of the mechanisms underlying atheroma formation, rupture, and consequent precipitation to acute ischemic events.

Atheroma tissue obtained from patients is a highly valuable source of information regarding the disease. It typically presents an inhomogeneous mass of high structural and biological complexity, usually consisting of lipids, inflammatory and smooth muscle cells (SMC), connective tissue, and calcium deposits [2–4]. Multiple factors (e.g. inflammatory, biomechanical, genetic, environmental) seem to be involved in all stages of atheroma formation and progression [3,4].

The discrete histopathological features of atherosclerotic plaques, reflecting different pathogenetic and pathophysiological mechanisms, allow for classification according to their severity: early, intermediate, and advanced [5]. Early lesions usually start as simple clusters of lipid-loaded macrophages before reforming to so-called fatty streaks and giving rise to discrete lipid pools among the SMC layers along with discrete intimal thickening [6]. Intermediate lesions are the first to be described as atheromas, since they have a well-defined core of lipids usually protruding into the vascular lumen. In terms of clinical incidence, early lesions are asymptomatic while intermediate ones may already cause

Abbreviations: BF, Brightfield; CAE, Carotid thrombendarterectomy; CMR, Continuous multirecord; DAQ, Data acquisition; FOV, Field of view; GM, Galvanometric mirrors; HE, Hemalaun-Eosin; IPH, Intraplaque hemorrhage; LDL, Low density lipoprotein; MAP, Maximum amplitude projection; MPM, Multiphoton microscopy; MPOM, Multiphoton and optoacoustic microscopy; NLO, Non-linear optical; OAM, Optoacoustic microscopy; PMT, Photo multiplier tube; PSR, Picro-Sirius Red; RBC, Red blood cell; ROI, Region of interest; SHG, Second harmonic generation; SMC, Smooth muscle cell; THG, Third harmonic generation; TPEF, Two-photon excitation fluorescence.

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ischemic disturbances [7,8]. However, the presence of symptoms is usually connected to advanced (late) and complicated plaques even if the phenomenon of plaque rupture seems to be uncorrelated to parameters such as plaque size and degree of luminal narrowing [7,9]. Advanced lesions are characterized by a thinning of the fibrous cap and a large lipid core containing an increased amount of collagen, inflammatory cells, as well as affected SMCs. These plaques may evolve into complicated ones with superficial fissure formation, intraplaque hemorrhage (IPH), and acute luminal thrombus occurrence due to direct exposure of lipid core material to blood contents [4,5,8].

From a topographical point of view, each plaque may be roughly divided into sections corresponding to specific structural regions: the cap, the shoulders, and the core (Fig. 2a). According to multiple histological studies, thrombosis is usually the result of cap ulceration and rupture near to one of the shoulders [10–12]. A vulnerable plaque is generally characterized by a large necrotic core (lipids, fibrin, blood inclusions, and macrophages), a thin degraded cap (decreased collagen and SMCs), as well as prominent inflammatory and neovascularization features [13]. Plaque rupture is frequently considered to be a mechanical event. On the one hand, collagen, a basic constituent of the extracellular matrix, seems to be associated with the biomechanical integrity of the cap [14]. Its degradation and disorganization have been correlated to decreased stability of the fibrous cap [14,15], while the retrieval of collagen production (e.g. statin therapy) seems to stabilize the plaque [16]. On the other hand, red blood cells (RBC), either within the plaque's neovessels or in the form of IPH, play a crucial role in atheroma progression, destabilization, and rupture [13].

With respect to the above mentioned histopathological evolution of atheromatous lesions linked to topographical conditions and compositions at specific regions within the plaque, new powerful and accurate imaging tools are required to precisely observe and analyse components influencing the mechanical stability. Within this framework, the accurate identification and characterization of the histological components of an atheromatous sample could increase our knowledge about atherosclerosis and define features related to ischemic events, which would potentially serve as future therapeutic targets.

Studies of atheromas largely rely on histopathology [17–21], which can interrogate several cellular and molecular features of the disease. Conversely, histopathology is invasive and laborious, requires tissue staining, and typically visualizes thin slices and small areas of the specimen. Non-invasive volumetric or quantitative measurements of excised atheromas are studied by tissue-sectioning microscopy, such as confocal or multiphoton microscopy [22–25]. Nevertheless, tissue-sectioning microscopy also requires labels for cellular and sub-cellular moieties. While genetically modified animals using fluorescent reporter labels may be generated for studying atheroma events in mice, imaging of excised human specimens is problematic for use with tissue-sectioning microscopy since it requires diffusion and clearance of a label through the tissue post-mortem. Likewise, the use of fluorescence agents against specific atheroma targets have been used in animal studies [26–29], but are not readily available for human use.

The use of non-linear optical (NLO) microscopy has been suggested for label-free visualization of atheromas, based on optical harmonic detection or tissue autofluoresence. It has been shown that second harmonic generation (SHG) microscopy resolves collagen structures, two-photon excitation fluorescence (TPEF) microscopy probes nicotinamide adenine dinucleotide

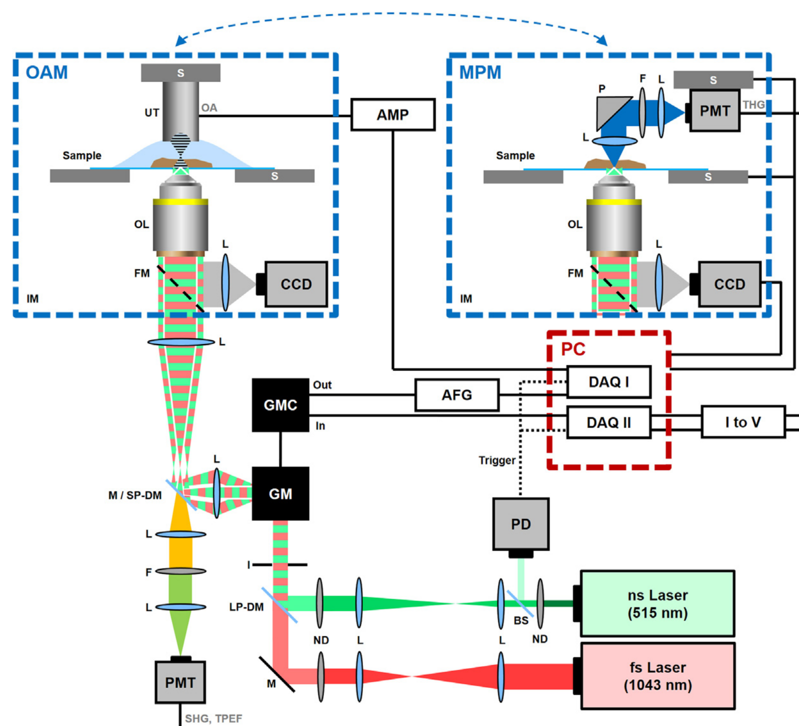

Fig. 1. Schematic depiction of the MPOM system consisting of two interchangeable microscopy systems, namely OAM and MPM. Abbreviations: AFG, arbitrary function generator; AMP, amplifier; BS, beamsplitter; DAQ, data acquisition card; F, optical filter; FM, flippable mirror; GM, galvanometric mirrors; GMC, GM control; I, iris diaphragm; L, lens; LP-DM, longpass dichroic mirror; M, mirror; ND, neutral density filter; OA, optoacoustic signal; OL, microscope objective lens; P, prism; PD, photodiode; PMT, photomultiplier tube; S, xyz stage; SHG, second harmonic generation signal; SP-DM, shortpass dichroic mirror; UT, ultrasound transducer; THG, third harmonic generation signal; TPEF, two-photon excitation fluorescence signal.

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