# Light Sheet Fluorescence Microscopy for Quantitative 3D Imaging of Vascular Remodeling

Neil MacRitchie[1], Pasquale Maffia[1,2,3]

[1]Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8TA, United Kingdom;

[2]Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8TA, United Kingdom;

[3]Department of Pharmacy, University of Naples Federico II, 80131 Naples, Italy.

**Correspondence to:** Dr Pasquale Maffia, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Sir Graeme Davies Building, 120 University Place, Glasgow G12 8TA, UK. Tel. +44 (0)141 330 7142; Email: Pasquale.Maffia@glasgow.ac.uk and Dr Neil MacRitchie, Institute of Infection, Immunity and Inflammation, University of Glasgow, UK. Tel. +44 (0)141 330 5015; Email: Neil.MacRitchie@glasgow.ac.uk

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Vascular pathologies create marked changes in the structure of the vessel wall, with pronounced alterations in the extracellular matrix and vascular cell organization together with infiltration of inflammatory cells. The result is a complex coordinated series of events that, depending on the nature of the condition, resulting in vascular repair or chronic injury and inflammation. In rodent models of vascular disease, *ex vivo* analysis of these changes is frequently performed using destructive or where possible, non-destructive methods. The most widely used method of assessing vascular morphology and spatial localization of cells and disease markers involves traditional histological methods, whereby tissue sections (typically 4-10 μm) are cut and stained either for morphological analysis/scoring or immunohistochemistry/immunofluorescence (IHC/IF) for identifying particular cells and markers of interest. While such tools are particularly useful for identifying the presence or absence of pathology, several factors converge to produce a final data set that is often only semi-quantitative and prone to selection bias[1]. These include the somewhat arbitrary number of tissue sections used for analysis, thus making like for like comparisons between animals problematic. This is an important concern in interpreting datasets since vascular pathologies consist of micro-environments with a non-homogenous distribution of cells and morphology. Since vascular remodeling in response to injury occurs in three dimensions, the above limitations can arise from the inherent 2-dimensional (2D) approach of sectioning and imaging arterial cross-sections across a single axis (e.g. transverse or longitudinal). These limitations may be mitigated by 3-dimensional (3D) imaging of the vessel in a non-destructive manner, thus allowing volumetric analysis, in addition to the more precise analysis of the geometry and topology throughout the site of vascular injury.

One such approach is light sheet fluorescent microscopy (LSFM), that allows 3D structural analysis of intact tissues following illumination with a sheet of laser light across multiple planes. The principles behind this technology are relatively simple[2], whereby the tissue of interest (typically several mms in diameter) is perfusion-fixed, mounted and stained with fluorescently labeled antibodies before undergoing tissue clearing (to induce transparency)

and exposure to LSFM, followed by digital image analysis (Figure 1). Previously, LSFM has been performed to investigate the architecture and function of both zebrafish[3] and mouse hearts[4-6], and only very recently to visualize atherosclerotic plaques and neointima formation in mice[7]. Since optical sections can be acquired across multiple planes, both 2D and importantly 3D quantitative measurements of vessels can be extracted such as medial and intimal hyperplasia volumes.

In this issue of *Cardiovascular Research*, Buglak et al.[8] provide further validation of the technique by quantitatively comparing LSFM with traditional 2D histological staining in two rodent models of vascular injury: the rat balloon angioplasty and the mouse carotid artery ligation. The authors utilize the inherent autofluorescence of the media layer alongside fluorescent antibodies specific for endothelial cells to define the architecture of the vessel. When comparing measurements of stenosis (intima: media ratio) between LSFM and hematoxylin & eosin stained histology slices, a greater degree of precision (lower coefficient of variation (CV%)) was observed for LSFM while mean values were similar. It was also noted that CV% values for stenosis in the rat model decreased as the number of optical sections used for analysis increased. Importantly, since the measurements are performed automatically along the entire section of the vessel, the results are less prone to selection bias than physical tissue sectioning. Additionally, the 3D long spatial view of the injured vessel segment allowed the identification of regions of intimal hyperplasia and quantitative comparison with neighboring regions where hyperplasia was absent. The ability to create both 2D slice images and 3D volumetric rendered images also allowed a more detailed and accurate view of vessel morphology. This is evidenced by the finding of neoangiogenesis in the adventitia of injured mouse carotid arteries, a feature also recently noted by Becher et al., 2020[7]. Furthermore, by utilizing similar laser excitation wavelengths to classical confocal and multiphoton microscopy, the authors employed multi-channel LSFM to identify the presence of CD68+ macrophages within the same adventitial space. One could, therefore, imagine LSFM being used to identify the location of leukocyte aggregates in vascular pathology such as dendritic cell/T-cell interactions which have been shown to play an

important role in experimental atherosclerosis[9-11]. Moreover, as demonstrated by Buglak et al.[8], 3D reconstruction can be very informative in delineating disease-specific changes in the endothelium. This has been expanded recently to assess endothelial erosion on atherosclerotic murine plaques and even small diameter human vessels[7].

The advantages of LSFM in comparison to classical histology/microscopy can be summarized as follows: 1) greater precision; 2) imaging in three dimensions and hence greater extraction of information; 3) increased imaging depth; 4) faster data capture and less photobleaching/phototoxicity compared with laser scanning fluorescence microscopy; 5) reduction in user bias; 6) easier to standardize between experiments/laboratories than arbitrary physical sectioning. On the contrary, confocal and multi-photon microscopy display higher spatial resolution[12-14] allowing more detailed examination of events at the cellular level but this limitation of LSFM may be mitigated to some degree by the ability to prepare physical sections and perform IHC/IF following LSFM[8]. As noted by Buglak and colleagues[8], the process from vessel isolation to analysis is also longer (although less user intensive) than physical sectioning. It is also worth considering that LSFM cannot be applied *in vivo* and hence longitudinal imaging of evolving vascular pathologies cannot be performed. However, LSFM could act as a supplemental tool for post-analysis following *in vivo* imaging by other high-resolution modalities such as magnetic resonance imaging (MRI) or ultrasound.

Increased experimental rigor and techniques that allow reproducibility are highly desired given the lack of reproducibility of many animal models[15]. By extracting larger and more accurate datasets over comparable methods, LSFM may reduce the number of animals required per study and therefore be aligned with the principles of 3Rs (Replacement, Reduction and Refinement), as well as improve the translation of new therapeutics for vascular disease.

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**Conflict of Interest**

None declared.

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