Visualisation of intramural coronary vasculature by an imaging cryomicrotome suggests compartmentalisation of myocardial perfusion areas

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Abstract—*A technique is presented for the 3D visualisation of the coronary arterial tree using an imaging cryomicrotome. After the coronary circulation of the excised heart was filled with a fluorescent plastic, the heart was frozen and mounted in the cryomicrotome. The heart was then sliced serially, with a slice thickness of 40 μm, and digital images were taken from each cutting plane of the remaining bulk material using appropriate excitation and emission filters. Using maximum intensity projections over a series of images in the cutting plane and perpendicular plane, the structural organisation of intramural vessels was visualised in the present study. The branching end in the smallest visible vessels, which define tissue areas that are well delineated from each other by 1–2 mm wide bands populated only by vessels less than 40 μm in diameter. The technique presented here allows further quantification in the future of the 3D structure of the coronary arterial tree by image analysis techniques.*

Keywords—*Vascular visualisation, Coronary circulation, Coronary tree, Cryomicrotome*

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1 Introduction

KNOWLEDGE OF the organisation of the intramural coronary vessels is important for the understanding of flow and oxygen distribution within the myocardium and the location and extent of areas at risk in the case of a coronary stenosis.

Several techniques have already been applied to visualise intramural coronary vessels. Silicone elastomers have been injected into the main coronary arteries, after which tissue was made transparent by dehydration with heavy alcohols (BASSINGTHWAIGHTE *et al.*, 1974). Those studies provided insight into the organisation of the capillary network within the myocardium and its connection to the arterial tree. No full description of the branching patterns of the coronary arterial tree could be obtained by these studies.

Corrosion cast methods resulted in statistical descriptions of the morphology of the coronary tree. Such information has been used in some studies to describe the coronary structure mathematically, to predict perfusion distribution and localised effects of control properties (CORNELISSEN *et al.*, 2000; 2002; KASSAB *et al.*, 1993; VANBAVEL and SPAAN, 1992). However, 3D information from these morphometric studies is lacking. Micro CT or other X-ray techniques permit accurate measurement of the arterial tree but are limited to small hearts (MOHLENKAMP *et al.*, 2002; TOYOTA *et al.* 2002).

The purpose of this technical report is to present a method for the collection of detailed information on the structure of the coronary arterial tree. This method is based on the imaging cryomicrotome (BERNARD *et al.*, 2000) used to slice hearts serially while imaging the cutting plane of the bulk with a set of excitation and emission filters. The coronary arterial system was filled prior to freezing with a fluorescently labelled plastic. The resulting images are of such high quality that reconstruction of the intramural coronary tree is possible. Initial results provide an estimate of the width of tissue separating different perfusion areas. With the development of more sophisticated image analysis software, this method could result in a full 3D reconstruction of the coronary arterial tree of all vessels larger than 40 μm.

2 Methods

Goat hearts were obtained after non-cardiovascular experiments in our institution. The left main stem of the excised heart was dissected free, cannulated and perfused with a crystalloid solution containing 10^{-5} mol dm^{-3} adenosine to dilate the vascular bed maximally. The coronaries distal to the

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cannula were then filled at a pressure of 80 mm Hg with Batson no. 17 plastic replica material consisting of a monomer base solution (methyl methacrylate), a catalyst and a promoter. For each millilitre of base solution, 5.8 μl promoter and 166 μl catalyst were used. The filling solution was made fluorescent by 39 μg Potomac yellow* per millilitre base solution.

The replica material was allowed to harden for 24 h, and the heart cavities were then filled with Cryoblock† mixed with Indian ink (1%). The heart was then submerged in a cylindrical container filled with 5% carboxymethylcellulose sodium solvent and frozen at −20°C. After at least 24 h, the heart was placed in an imaging cryomicrotome‡ that had been improved by our workshop for mechanical and electronic stability.

The frozen hearts were serially sectioned from base to apex, parallel to the base, into 40 μm slices. After each cut, images were taken from the cutting plane of the remaining bulk by a digital camera** equipped with a Nikon lens (70–180 mm) with a resolution of 2000 × 2000 pixels, each pixel representing 40 × 40 μm. The fluorescence was measured by application of an excitation filter†† in the light path towards the cutting plane and an emission filter‡‡ in the light path from cutting plane to camera. Individual tissue slices were normally wasted, except in incidental cases when a slice was collected to confirm the filling of smaller arterioles by replica material using a microscope.

Images were processed using custom-made software written in Delphi# using the Imaging Toolkit of MCM§. We generated 2D maximum intensity projections (MIPs) of a stack of consecutive images in the following way. First, image 2 was subtracted from image 1, and then image 2 was added to the subtraction result. As grey levels were clipped at values outside the range between 0 and 255, this subtraction–addition resulted in an image composed of pixels with a grey level equal to the maximum intensity of the two original images at coordinates (x, y). The resulting MIP was then used as the new image 1, and the next image of the stack was used as image 2. The finite result was an image of the maximum pixel intensities at x, y positions in the total stack, analysed without involvement of any grey-level threshold.

From the dataset of stacked images of the cutting planes (x, y) parallel to the base of the heart, new images were constructed in the axial (z) direction. These images were then used to generate (x, z) MIPs orthogonal to the cutting plane. Dimensions were determined manually by virtual calipers on the computer screen.

Three-dimensional rendering of stacks of images was carried out using proprietary image registration and visualisation software of the Netherlands Cancer Institute (VAN HERK *et al.*, 2000).

3 Results

A typical microscope image of a slice of 200 μm collected from the cryomicrotome to check vessel filling is depicted in Fig. 1. This image demonstrates that arterial vessels with a diameter smaller than 10 μm are easily filled with our replica material, but no plastic was found in the capillaries or veins.

A region of interest, (ROI), of a single image is depicted in the left panel of Fig. 2. MIPs of a stack of such images are shown in the other panels in Fig. 2. The middle panel is a MIP over ten corresponding ROIs, and the right panel is a MIP over 30, with the ROI of the left panel in the centre of the stack. With an increasing number of slices, more depth is visualised by the MIPs at the risk, however, of losing detailed structural information.

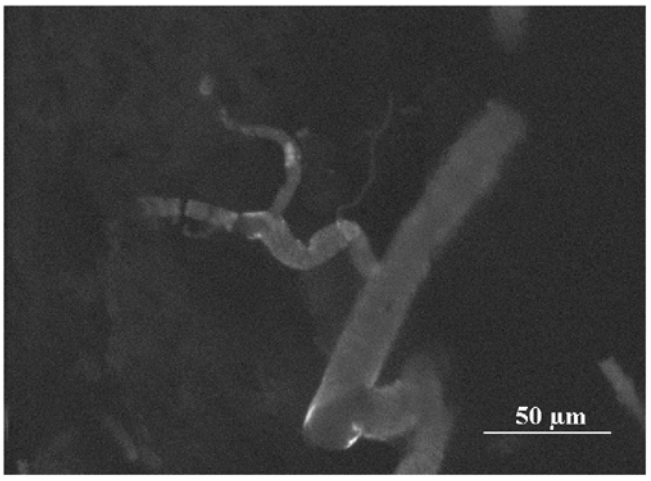

Fig. 1 *Typical microscopic image of a 200 μm thick slice collected from cryomicrotome demonstrating that vessels with diameter smaller than resolution of cryomicrotome camera were filled with replica material*

Fig. 3*a* shows a MIP from a stack of 40 slices (1.6 mm) in the mid-section of a different heart. Note the presence of individual vascular units traversing the myocardium that are separated by bands with no visible filling. These bands were found to be as wide as 1–2 mm, defining a border zone of tissue around vascular structures containing vessels with diameter larger than 40 μm. These rather wide border zones contain vessels smaller than the resolution of the technique, as was confirmed by microscopy of individual collected slices (see Fig. 1).

Fig. 3*b* provides an orthogonal MIP view in the z-direction, through a stack of 423 corresponding original images. This image represents a MIP of 20 reconstructed images (0.8 mm) in the axial direction. The white background bar in the top panel indicates where the orthogonal image crosses the MIP of the cutting plane (0.8 mm wide). Similarly, the white bar in the bottom panel indicates the intersection of the cutting-plane MIP with the orthogonal MIP.

The wide border zones found in the cutting plane MIPs also exist in the orthogonal view.

Examples of 3D reconstructions of the coronary tree that can be obtained with this cryomicrotome system are provided in Fig. 4. This example shows a 512 × 512 × 512 pixel subset of a 3D data set demonstrating the detailed spatial resolution of the vascular structure. Please check our website, http://www.biomedicalphysics.org, for additional material on 3D reconstructions.

4 Discussion

This paper demonstrates that a vascular filling technique combined with serial sectioning by an imaging cryomicrotome is able to provide high-resolution image data allowing the 3D reconstruction of the coronary arterial tree in hearts weighing over 200 g. This tree spans the full range from the main epicardial vessels in the order of 3 mm to arterioles in the order of 40 μm, a range of almost 2.5 orders of magnitude in diameter. Our first results demonstrate that the intramural vascular bed is organised such that vascular structures are clearly delineated by relatively wide border zones that are perfused by only very small (<40 μm) vessels.

*Radiant Color nv, Houthalen, Belgium
†Cryoblock, Klinipath, Duiven, The Netherlands
‡Barlow Scientific, Olympia, WA, USA
**Megaplus model 4.2i, Kodak
††UVND 0.2 D440/20x, Chroma Technology Corp, Rockingham, USA
‡‡UVND 0.2 D505/30 m, Chroma Technology Corp, Rockingham, USA
#Borland, vs. 7.0
§Birkerød, Denmark, http://www.mcm-design.dk

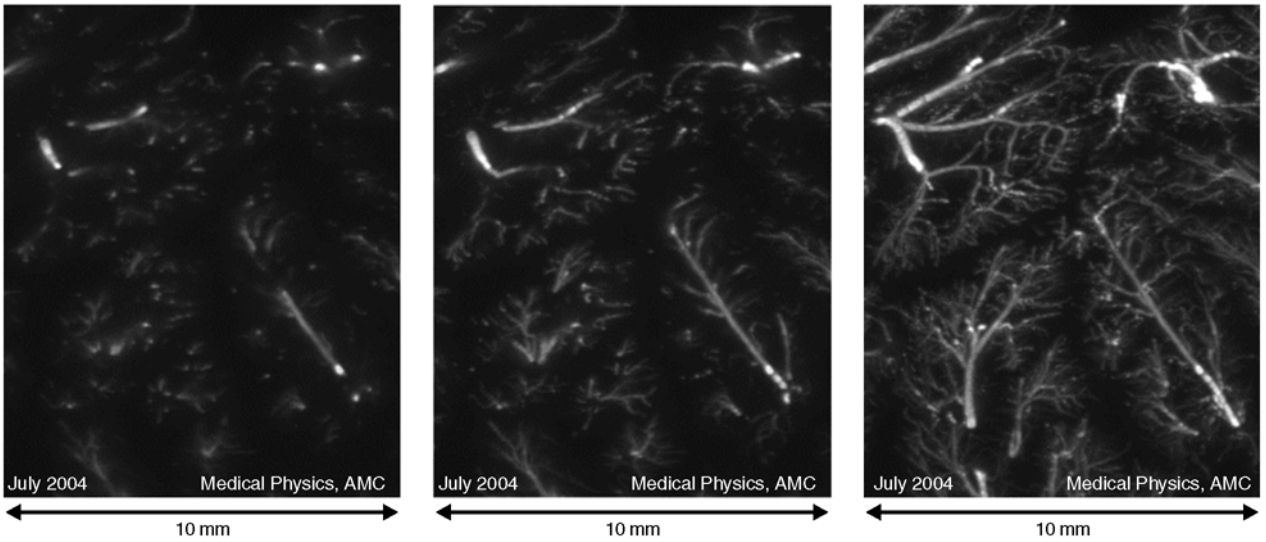

Fig. 2 *Development of MIP image with increasing number of slices used: stacks of 1, 10 and 30 images are depicted from left to right. Note that vascular structure can be better recognised in MIP than in image of single slice, but at expense of detail information when high-intensity structure of one layer coincides with low-intensity structures at same position in other layers. Panels depict region of left ventricular free wall. Note that these images are from different heart to that shown in Fig. 3*

4.1 *Critique of the method*

The first requirement for detection by the imaging cryomicrotome is that vessels are filled by the replica material. We collected incidental slices of tissue for microscope observation and found that, in general, vessels smaller than 15 μm were filled by the replica material.

In terms of resolution, it is necessary carefully to distinguish between the optical resolution defined by the lens system and

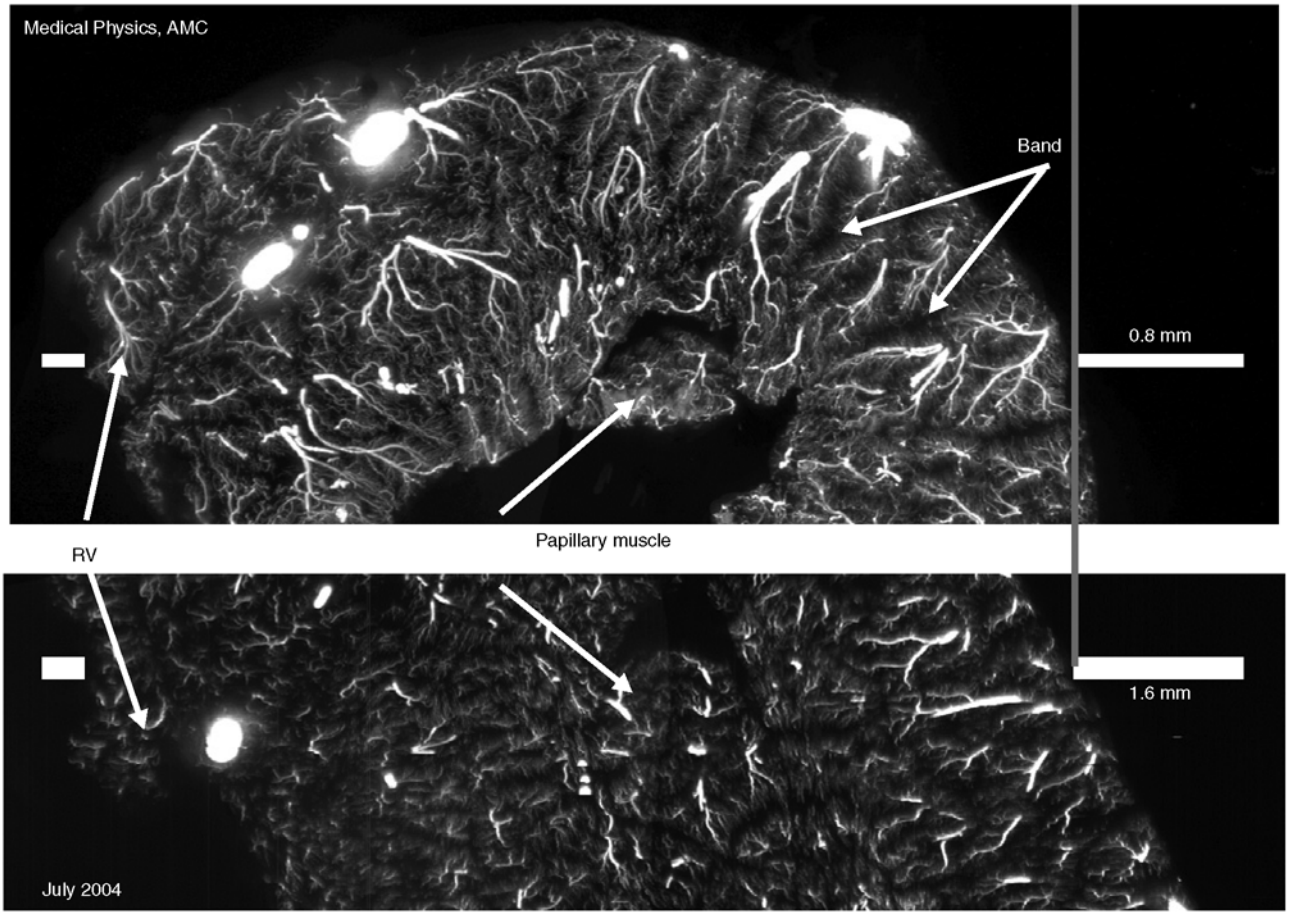

Fig. 3 *(a) MIP from stack of 40 cross-sectional images (1.6 mm of tissue) of heart taken close to top of papillary muscle. Note separation of papillary muscle from endocardium by ventricular space, which should not be confused with dark bands delineating individual vascular units that in some cases extend from epi- to endocardium.*
(b) MIP of tissue slab of 0.8 mm (20 pixels) perpendicular to plane shown in 3a. Position and thickness of corresponding slabs are indicated by white bars on right. Note that, at left ventricular free wall, orientation of larger vessels is radial, whereas end vessels are dominant in middle part of heart. RV = right ventricle

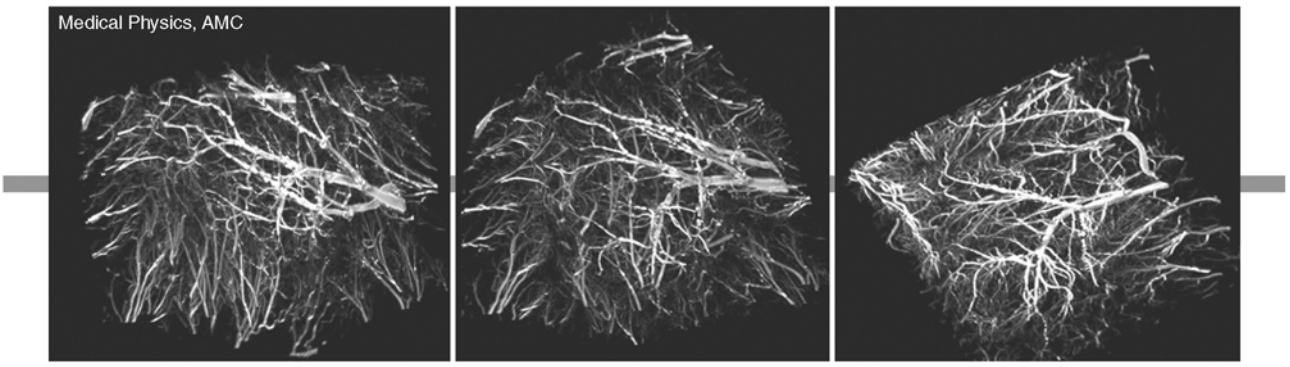

Fig. 4 *3D reconstruction obtained from stacked image set after MIP processing. From left to right, 2 × 2 × 2 cm (500 × 500 × 500 pixels) cube is shown after virtual clockwise rotations around central horizontal axis indicated by grey bar*

the pixel size of the camera. The latter was 40 μm in the present study. However, vessels with a diameter smaller than this size may be visible, as their fluorescence is still detected by the camera, albeit with low intensity and spread out dimensions. In incidental comparisons of the cryomicrotome image with microscopy images of collected slices, vessels of 30–40 μm seen with microscopy could be located in the cryomicrotome images. We could observe smaller diameter vessels with the imaging cryomicrotome at higher camera gains, but, in this case, clipping to the maximum possible image intensity occurred in regions of the larger vessels. Hence, in the present study, the resolution was 40 μm, with smaller vessels filled but not detected owing to the combination of the low fluorescence level with respect to the background of the smaller vessels and the resolution limits of the imaging system.

4.2 *Compartmentalisation of imtramural vascularity*

The bands of tissue in which only very small arteries exist between vascular compartments suggest watersheds between them. Such watersheds have been demonstrated earlier, but then between perfusion areas of major epicardial arteries. In such border regions, so-called capillary end loops were found rather than continuous capillary networks connecting perfusion areas (OKUN *et al.*, 1979). This suggestion is consistent with the observations of micro necrosis with distinct borders in human hearts and microsphere inducible infarctions in animals (ENG *et al.*, 1984; OKUN *et al.*, 1979).

Earlier studies measuring NADH autofluorescence at the epicardium of rat hearts demonstrated that specific 'band-like zones' of ischaemia are induced under conditions of restricted perfusion (INCE *et al.*, 1993). These functional observations could well be related to the present anatomical observations. These considerations lead to the hypothesis that, in normal conditions, the heart contains well-defined perfusion areas without direct vascular communication, and the border regions between them are the first to become ischaemic in conditions of restricted perfusion. When intramural collaterals are formed under ischaemic conditions, these tissue bands have to be bridged by newly formed vessel segments. Further studies combining functional and anatomic measurements are needed to test this hypothesis.

4.3 *Future developments*

Our ultimate aim is the full 3D reconstruction and detailed quantification of the geometry of the coronary arterial tree. This requires a more precise calibration of vascular diameter measurements. A special issue in this case is the anisotropy in the point-spread function. This function describes mathematically how an infinitesimally small point in space radiating light equally into all directions is observed in the surrounding space with a given imaging system. In the case of the cryomicrotome, the amplitude of this function is smaller within the cutting plane (x, y) than in the sampling direction (z). This difference can be appreciated from a comparison of the images shown in Fig. 3. The same vessel segment in these images appears to have a larger diameter in the z-direction than in the x, y-plane.

More sophisticated image analysis is needed to quantify the full three-dimensional structure of the coronary arterial tree. Such analysis requires skeletonisation and branch point identification, tree-branch diameter measurement in any orientation, and labelling of individual branch segments. Such detailed processing has been done for the lungs (PALAGYI *et al.*, 2003) and for neural branching structures (STREEKSTRA and VAN PELT, 2002). The quality of the images obtained with the cryomicrotome certainly allows the application of these techniques to the quantitative reconstruction of the coronary arterial tree.

5 Conclusions

This initial study provides promising evidence that the imaging cryomicrotome could be a valuable instrument for generating 3D datasets of the entire coronary tree of large hearts with unprecedented resolution. The maximum intensity projections suggest the organisation of the tree in vascular units surrounded by relatively thick areas devoid of vessels larger than 40 μm. The imaging cryomicrotome could be a very useful instrument in studies directed at physiological or pharmacologically induced angiogenesis in acute animal models.

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JOS SPAAN (1945) is head of the Dept of Medical Physics at the Academic Medical Center of the University of Amsterdam. He also leads a research group which aims to understand the mechanics, control and distribution of myocardial perfusion in health and disease. He considers himself lucky to have such a proficient and enthusiastic group. Please visit our website http://www.biomedicalphysics.org/for a more detailed look at our work.