Proceedings of the Australian Physiological and Pharmacological Society (2004) ***34:*** *129-132* *http://www.apps.org.au/Proceedings/34/129-132*

Microscopic imaging of extended tissue volumes

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Summary

1. Detailed information about 3D structure is key to understanding biological function

2. Confocal laser microscopy has made it possible to reconstruct 3D organization with exquisite resolution at cellular and subcellular levels

3. There have been few attempts to acquire large image volumes using the confocal laser scanning microscope.

4. We have previously used manual techniques to construct extended volumes (several mm in extent, at 1.5 μm voxel size) of myocardial tissue.

5. We are now developing equipment and efficient automated methods for acquiring extended morphometric databases using confocal laser scanning microscopy.

Introduction

The association between form and function is a central principle of the biological sciences and one that has contributed to the growth of the field over many years. The linkage is probably more important today that ever before. It is widely accepted that detailed information about three-dimensional (3D) structure is key to understanding biological function from molecule to organ and with the development of new imaging modalities there has been an explosion in the quality and volume of data that can be acquired at each these scales. For instance, the confocal laser microscope has made it possible to reconstruct three-dimensional organization with exquisite resolution at cellular and subcellular levels. Moreover, using the array of immuno-histochemical techniques now available, it is also possible to probe the link between structure and function directly, for instance by quantifying the co-location of labelled proteins such as gap junctions or receptors with other anatomic structures.

For the most part, confocal imaging has not been used to reconstruct 3D tissue organization in a systematic fashion and there have been few attempts to acquire large image volumes as has been done with MRI or micro-CT. This reflects the physical constraints on the technology. Acquisition rates are limited by the sensitivity of photodetectors and the need to scan points sequentially throughout the tissue volume, while the dimensions that can be imaged are set by the working field of the microscope objective and critically, with respect to Z direction, by absorption and scattering of light in the tissue investigated. That said, there is a clear need for databases that incorporate structural information across the scales addressed by confocal microscopy for normal and pathologically changed organ systems at different developmental stages. Extended morphometric databases of this kind are required to characterize more fully the structural changes associated with various dysfunctional states and to support the computer models that are increasingly being used to integrate experimental information at cell, tissue and organ levels.

Manual technique for acquiring extended confocal microscope images of biological samples

In the remainder of this section, we summarize ongoing research at the University of Auckland which is directed toward the development of efficient methods for acquiring extended morphometric databases using confocal laser microscopy. The work flows from initial studies in which high-resolution volume images were assembled of myocyte arrangement and connective tissue organization across the heart wall[1]. Transmural segments (800 μm × 800 μm × 4.5 mm) were cut from the left ventricle free wall of rat hearts, previously perfused with Bouin's solution for fixation and then with the dye picrosirius red which binds non-sterically to collagen[2]. The specimen was dehydrated with a graded series of alcohols, embedded in epoxy resin and the upper surface of the block was then planed flat using an ultramicrotome. A motorized stage was used to control the horizontal position of the specimen and contiguous z-series image stacks were acquired at different x-y locations. In this way, an extended volume image was generated over the upper surface of the transmural specimen to a depth of around 60μm. The block was then removed from the microscope and mounted in an ultramicrotome where the upper 50μm was removed. The specimen was then returned to the microscope and the cycle of imaging and trimming was completed sequentially until the complete volume was imaged. Painstaking alignment of the upper surface of the tissue block in both the microtome and the confocal microscope was required at each stage in this process to ensure that image registration was, as far as possible, preserved (See Figures 1 and 2). Moreover, further *post-hoc* spatial transformation of image sub-volumes was still required to optimize registration when assembling the complete image volume.

Digital reslicing, segmentation and volume rendering methods can be applied to the resulting volumes to provide quantitative structural data about the 3D organization of myocytes, extracellular collagen matrix and the vascular network. These data have not previously been available and provide a powerful basis for further analysis of function. For example, it is a relatively trivial matter to quantify the transmural variation of perimysial collagen once it has been

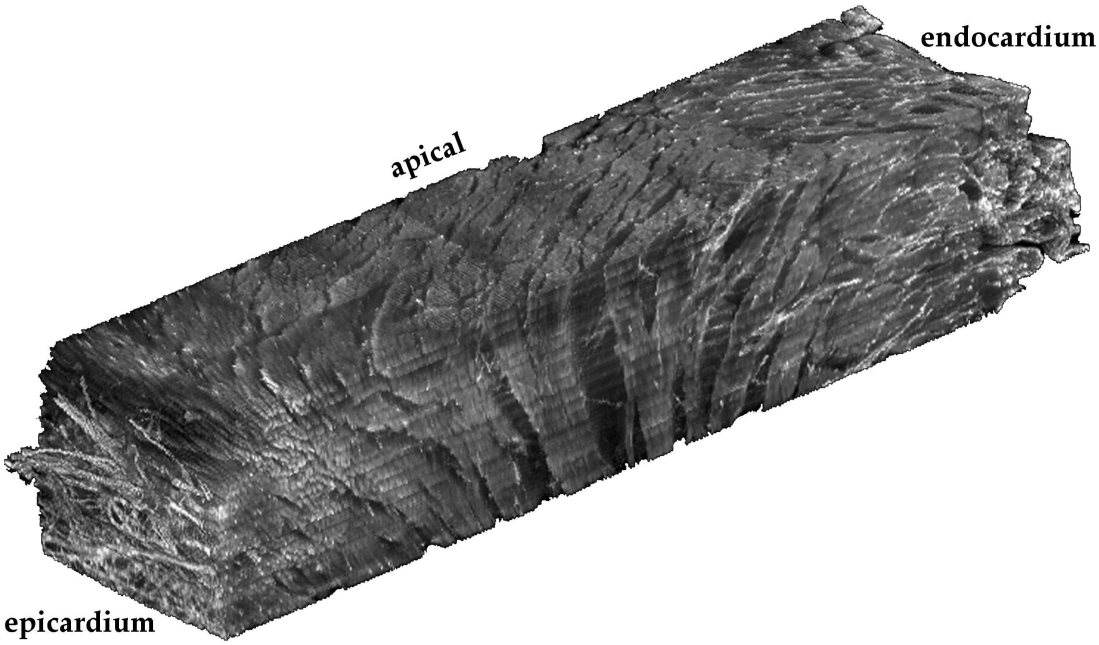

Figure 1. Oblique view of extended volume image from left ventricle of rat heart obtained using confocal microscopy. *Note the laminar organization and collagen (white) interconnecting layers of myocytes. The epicardial collagen weave is clearly seen along with cleavage planes between myocardial layers. (From 1, with permission from the Royal Microscopical Society).*

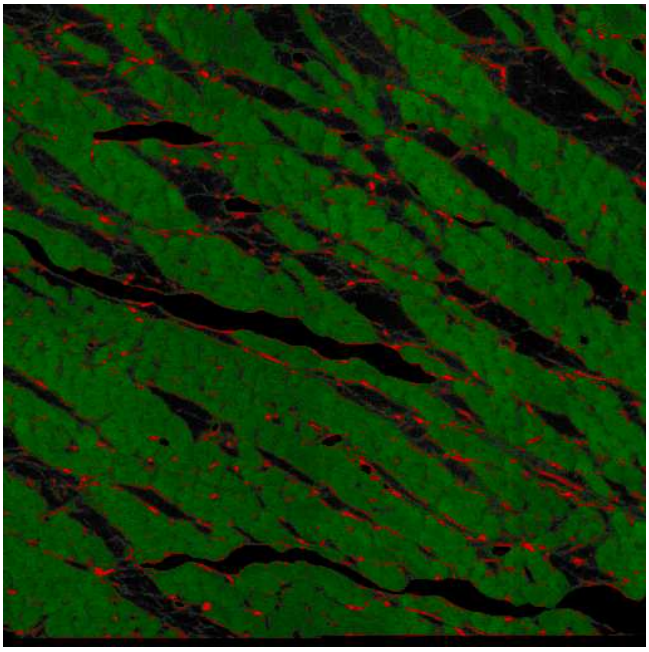

Figure 2. Image slice from left ventriclar midwall of rat heart (800 × 800 μm) illustrating laminar organization of myocytes. *Plane of optical section is perpendicular to myocyte axis. Red dots are perimysial collagen cords running parallel to myocyte axis.*

segmented out of an extended volume image as shown in Figure 3b. The heart wall remodelling associated with many types of cardiac disease involves changes in the extent and distribution of collagen and detailed information about the time-course of these changes is necessary to better understand the disease processes involved. It follows that extended volume imaging provides a pathway for systematic acquisition of such data. It can also be used for the development of computer models, which make it possible to examine the effects of myocardial structure on the function of the heart. For instance, we have extracted the 3D arrangement of cleavage planes and myocyte orientation from an extended volume image of rat left ventricular myocardium (3.8mm × 0.8mm × 0.8mm at 1.5μm pixel size, 0.72×10^9 voxels).

This application illustrates well the utility of being able to gather detailed microscopic information over extended volumes. The myocardial layers and cleavage planes are defined by connective tissue and myocytes interconnections that are visible at levels of a few micrometers and it is details of these structures that are needed to define the local electrical and mechanical properties of the laminar myocardium. However, the cleavage planes can extend for two to three millimetres. In order to fully describe the structure and associated material properties, for instance when developing a computer model of myocardium it is necessary to have information across a wide scale range, the system we have developed provides the tools to acquire this information. The extended volume image of rat myocardium has been incorporated into a structurally detailed, finite element model of ventricular myocardium that has been used to study the influence of discontinuous myocyte organization on the propagation of electrical activation in the heart[3].

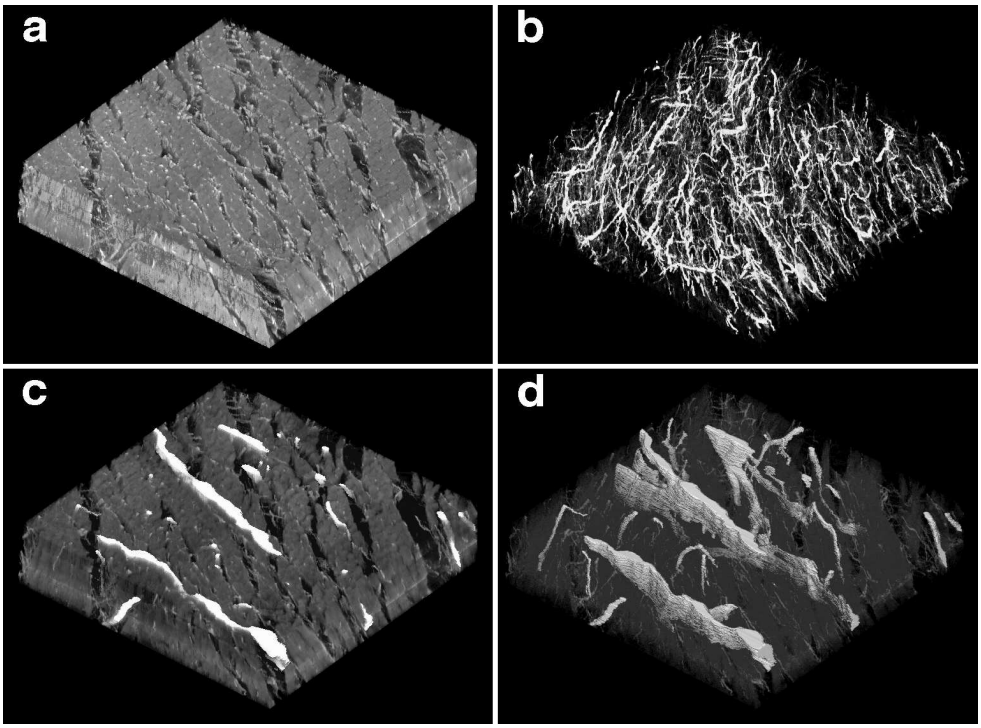

Figure 3. Reconstructed subvolumes (800 × 800 × 100 μm). *In the upper panel collagen is segmented and rendered (a) with and (b) without background due to myocytes. In the lower panel venous sinuses are segmented and rendered, (c) with, and, (d), without background. (From 1, with permission from the Royal Microscopical Society).*

Development of automated techniques for acquiring extended volume images

Unfortunately, acquisition of an image volume such as that presented in Figure 1 requires weeks of painstaking work and this precludes the use of the manual approach outlined above for systematic morphometric analysis. For this reason, we are now developing an automated system that provides for computer controlled confocal imaging and milling of embedded tissue samples over extended volumes. The system consists of (i) a confocal microscope (Leica TCS 4D) with a Kr/Ar laser (Omnichrome) (ii) a variable speed Ultramill (Leica) which cuts to 1μm over a 75mm path using diamond or tungsten carbide tips, and (iii) a three-axis translation stage (Aerotech) with xyz movement of 1000, 200 and 75mm, respectively at 100nm step size. The stage controls the positioning of specimens for imaging and milling (See Figure 4). Microscope and mill are supported above the translation stage using rigid mounting systems designed to facilitate alignment of imaging and cutting planes. The system is mounted on an anti-vibration table (Newport). Z-stack volume images are acquired for overlapping x-y areas that cover the region of interest. The imaged volume is then milled off and the process is repeated. A major advantage of this method is that alignment of the sample elements is maintained throughout the imaging and milling operations, thereby preserving spatial registration and making reconstruction of the complete volume image easier and faster.

The system is controlled using a dedicated computer (Dell P4, 1.8GHz, 1GB RAM, Windows 2000) using custom software written using the LabVIEW™ programming language. A single user interface has been developed that enables image acquisition and milling to be controlled interactively or automatically and allows the operator to process, reconstruct and visualize the image volumes. The flexible user interface provides the ability to image chosen sub-volumes at high resolution, but placing them within the context of a large volume imaged at lower resolution.

Preliminary studies carried out with cardiac tissue specimens demonstrate that the system has the capacity to acquire 62.5 million voxels per hour, each averaged over 8 scans. This means that a fully-registered $1mm^3$ image volume can be acquired at 1μm resolution (10^9 voxels) with 8× averaging, in 16 hours. This is a more than ten fold improvement with respect to our initial manual approach and further more modest improvement is seen to be possible with optimization of scanning and signal acquisition protocols.

The imaging rig is currently being used in a series of different projects including a longitudinal study of cardiac remodelling in spontaneously hypertensive rats throughout the course of their progress to heart failure and an analysis of the 3D organization of renal tubules and blood vessels in nephron segments. Particular emphasis is being given to extending the range of embedding and staining techniques that can be used with this automated volume imaging system.

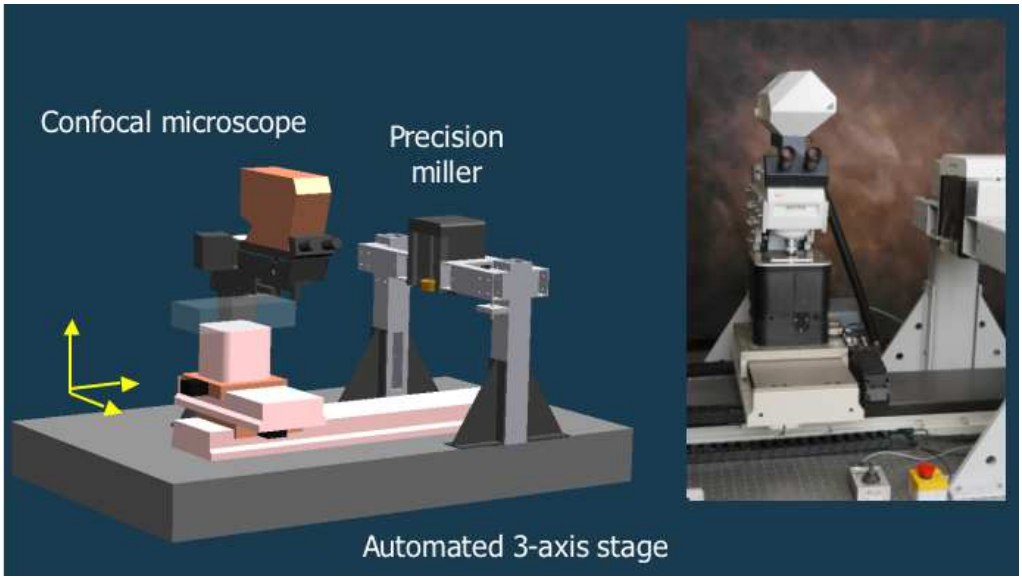

Figure 4. The automated confocal imaging rig. *The schematic indicates the arrangement of the confocal microscope, the ultramill and the three-axis translation stage. The inset photograph shows all three components and the anti-vibration table on which they are mounted.*

Acknowledgments:

Development of this imaging system was made possible by a grant from the Wellcome Trust.

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Received 27 July 2004, in revised form 17 August 2004.
Accepted ?? August 2004.

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