Whole heart detailed and quantitative anatomy, myofibre structure and vasculature from X-ray phase-contrast synchrotron radiation-based micro-CT

Anna Gonzalez-Tendero PhD¹, Chong Zhang PhD²,³, Vedrana Balicevic⁴, Rubén Cárdenes PhD¹,², Sven Loncaric PhD⁴, Constantine Butakoff PhD², Bruno Paun², Anne Bonnin PhD⁵, Eduard Gratacós PhD¹, Fatima Crispi PhD¹, Bart Bijnens PhD²,⁶

¹ Fetal i+D Fetal Medicine Research Center, BCNatal – Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Deu), IDIBAPS, University of Barcelona, and Centre for Biomedical Research on Rare Diseases (CIBER-ER), Barcelona, Spain; ²PhySense, DTIC, Universitat Pompeu Fabra, Barcelona, Spain; ³CellNetworks, Heidelberg, Germany ⁴Faculty of Electrical Engineering and Computing, University of Zagreb, Zagreb, Croatia; ⁵European Synchrotron Radiation Facility, Grenoble, France (Now at Paul Scherrer Institut, Villigen, Switzerland); ⁶ICREA, Barcelona, Spain

Address for correspondence:

Prof. Bart Bijnens
Universitat Pompeu Fabra - DTIC
Carrer de Tànger, 122-140
ES–08018 Barcelona (Spain)
Tel. +34 61 501 6853
e-mail: bart.bijnens@upf.edu
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e-mail: bart.bijnens@upf.edu

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ABSTRACT

Background: While individual cardiac myocytes only have a limited ability to shorten, the heart efficiently pumps a large volume-fraction thanks to a cell organization in a complex 3D fibre structure. Subclinical subtle cardiac structural remodelling is often present before symptoms arise. Understanding and early detection of these subtle changes is crucial for diagnosis and prevention. Additionally, personalized computational modelling requires knowledge on the multi-scale structure of the whole heart and vessels.

Methods and results: We developed a rapid acquisition together with visualization and quantification methods of the integrated microstructure of whole hearts using synchrotron based X-ray (phase-contrast) tomography. These images are formed not only by X-ray absorption by the tissue, but also by wave propagation phenomena, enhancing structural information, thus allowing to raise tissue contrast to an unprecedented level. We used a (ex-vivo) normal rat heart and fetal rabbit hearts suffering intrauterine growth restriction as a model of subclinical cardiac remodelling to illustrate the strengths and potential of the technique.

Conclusions: We have developed a novel, high resolution, image acquisition and quantification approach to study a whole heart at myofibre resolution, providing integrated 3D structural information at microscopic level without any need of tissue slicing and processing. This opens up new possibilities for a systems approach towards analysing cardiac structure and function, providing rapid acquisition of quantitative microstructure of the heart in a near native state.

Key words: myocardial remodelling; myofibre structure; coronary vasculature; synchrotron phase-contrast CT;
INTRODUCTION

For structural/functional quantification, cardiac chambers are often simplified to ellipsoid-like volumes. In reality, macroscopic shape is variable and the endocardium shows complex trabeculations. Additionally, intra-cavitary structures, such as valve apparatuses, with papillary muscles and tendinous chorda as well as false tendons are present.

At microscopic level, myocardium is made of millions of myocytes aggregated as a 3D mesh within supporting fibrous matrix. Individual myocytes are arranged in fibre-like structures, oriented in a very specific way. The whole of fibres creates the cardiac geometry with specific curvature/thickness. This complex organization is what allows the heart to efficiently pump a large volume-fraction with individual myocytes only shortening 10-15%. This structure is interlinked with a coronary tree starting from epicardial arteries, branching into a complex network while penetrating the wall.

Myocytes are aggregated and aligned in a predominant direction, depending on their position within the wall. At the epicardium as well as endocardium, myocytes are tangential to the epicardium and oblique to the long-axis of the heart, with a predominant longitudinal (base-apex) direction, while in mid-myocardium predominantly circumferential. The change in angle with respect to the equator is gradual and this helical angle in epicardial layers is opposite to sub-endocardial layers, tending to approach +90° at endocardium and -90° at epicardium.

Ejection results from complex 3D movement that involves longitudinal contraction, circumferential contraction as well as rotation/twisting. Local deformation is determined by the 3D fibre-organization: while longitudinal contraction is determined by the longitudinal component of fibre shortening (predominantly from endo- and epi-cardium), circumferential contraction is mainly determined by fibres located in mid-myocardium. Rotation and twisting depend on fibre obliqueness.

While (genetic) cardiomyopathies or fibrosis will significantly change local microstructure, more subclinical conditions may lead only to subtle changes. An example is intrauterine growth restriction (IUGR) due to placental insufficiency, affecting 7-10% of pregnancies and major cause of perinatal mortality and long-term morbidity. IUGR-induced low birth-weight is
strongly associated with increased risk of cardiovascular diseases and mortality in adulthood.\textsuperscript{8} Fetal hypoxia and volume/pressure overload induce remodeling which is characterized by decreased longitudinal compensated by increased radial function, together with impaired relaxation.\textsuperscript{9,10} Moreover, cardiac shape has been shown to be more globular in IUGR\textsuperscript{11} as well as preterms.\textsuperscript{12}

The 3D architecture is established early in prenatal life.\textsuperscript{13} The fetal immature heart is very plastic and thus a substrate for microstructural remodelling due to changing intraterterine conditions. Normal fibre architecture in fetal myocardium has been studied using polarized light microscopy and laser-scanning confocal microscopy.\textsuperscript{14,15,16} This showed that fibre orientation is sensitive to changes in mechanical load and hypoxia.\textsuperscript{16,17} However, little is known about changes of architecture during development and under altered haemodynamics. Other IUGR consequences are adaptive changes to coronaries, which may even predispose for adult disease.\textsuperscript{18,19,20} Given the high plasticity of the coronaries in the immature heart, it is highly predisposed to remodel in order to meet oxygen demands.\textsuperscript{18} In particular, the coronaries responds to conditions of chronic hypoxemia by a substantial increase in cross-sectional area\textsuperscript{18} or increasing vessel growth and vascularization.\textsuperscript{21} Therefore, to understand disease progression, integrating information about individual cells and their spatial organization is essential. While a lot is known about physiology as well as regional microstructure, translating this towards clinical medicine, is still challenging. A translational approach based on systems-medicine, integrating multiple scales, was proposed, including attempts at computational modelling. For these initiatives, detailed knowledge on whole heart 3D morphology, integrating all substructures (from organization of myocytes to whole heart anatomy) at a resolution including all relevant details, is essential. Several technical approaches have been used, both through direct visualization (microscopy or micro-MRI/CT) or through inference by assessing local tissue properties (diffusion tensor MRI (DTMRI) or echocardiography). However, detecting orientation of individual myocytes, especially in larger volumes or the whole heart, is still a major challenge.\textsuperscript{22,23} Histology/microscopy provides sufficient detail, but hardly allows imaging whole hearts, and
slicing/processing is required, which is a source of artifacts.\textsuperscript{14,15} High resolution/field MRI (including DTMRI)\textsuperscript{24-26} still has a spatial resolution which is suboptimal (~50 µm) and scan times are extremely long. Classical, absorption-based, Micro-CT, after iodine staining, shows promise,\textsuperscript{28,30} but organ preparation is challenging and extracting integrated microstructure, including myofibres and vessels is hardly feasible.

In this study we propose an integrated, high resolution, image acquisition and quantification approach to study whole hearts at myofibre resolution, providing structural information at microscopic level without need of slice-processing and illustrate how this can be used to assess subclinical remodelling. Imaging is based on X-ray phase-contrast synchrotron radiation-based micro-CT.\textsuperscript{31} Phase-contrast imaging has emerged as a novel X-ray-based approach providing enhanced contrast in some biological tissues and additionally suggesting to improve the diagnostic work-up.\textsuperscript{32}
MATERIAL AND METHODS

New Zealand white rabbits and Wistar rats were provided by a certified breeder. Animal handling/procedures were performed in accordance to regulations and with approval of the local Ethics Committee.

A rat (25 days) was anesthetized with isofluorane 3% and oxygen 2 ml/min. Heparin 500U was administered as well as saturated KCl to arrest the heart. After thoracotomy, a phosphate-buffer saline solution was used to rinse and 10% formalin to fix the heart, which was excised and immersed in formalin.

A validated IUGR rabbit model was reproduced. Briefly, at 25 days gestation both uterine horns were exteriorized and one selected as IUGR, in which selective ligature of 40-50% of utero-placental vessels of each gestational sac was performed. The abdomen was closed and animals were kept in regular conditions and fed a diet of standard chow and water ad libitum. At 30 days gestation, a caesarean was performed. After anaesthesia with intramuscular ketamine and xylazine, the fetal chest was opened and hearts were prepared as above. Before imaging, hearts were dehydrated with ethanol and, to avoid motion artefacts, immobilized in 1% agarose.

Data acquisition

Propagation-based phase-contrast tomography dataset were acquired at the European Synchrotron Radiation Facility (ESRF-beamline ID19), using 19KeV X-rays, and 1100mm propagation distance. Field of view was 5.68x15.96mm, with isotropic pixels of 7.43µm. The sample was at room temperature and placed on a holder. After positioning it at the stage’s centre of rotation, it was rotated over 360º acquiring 2499 projections (exposure time 0.3s). Total time of each acquisition was 14min. Four to five sequential acquisitions (overlap 363 slices - 2,697mm) always from base to apex, were necessary to cover the whole heart along its long axis. Additionally, 41 reference flat-field images (without sample), and 21 dark-images (with shutter closed) were taken for background removal. Therefore, total acquisition time was approximately 1-1.25 hours/sample (4-5 chunks).
Each projection series was reconstructed using filtered back-projection\textsuperscript{34} as well as Paganin’s method.\textsuperscript{35} Reconstructed volumes were converted to 16-bit tiff images and merged into a single dataset. In all cases, the whole heart was in the resulting 3D dataset. An example of an unprocessed dataset will be made available for download.

Images were analysed with Fiji (reslicing/rendering and vessel quantification),\textsuperscript{36} ICY (rendering)\textsuperscript{37} and ilastik (vessel segmentation)\textsuperscript{38} and in-house developed Matlab (The MathWorks, Massachusetts, USA) software for fibre analysis.

**Coronary segmentation**

Coronaries cover only a small part of the large volumetric datasets (~0.5%), and are best distinguished by a boundary that may be incomplete. ilastik\textsuperscript{38} offers a seeded segmentation module, Carving, which provides semi-automated segmentation based on sparse user scribbles.\textsuperscript{39} For each object of interest, i.e. vascular tree, it requires “inside” and “outside” seeds as input to propagate to explain the entire volume, using a biased watershed algorithm. Several iterations maybe needed to achieve segmentation. This workflow also provides the uncertainty estimation of the current segmentation, thus guiding the user to locations where additional input may be helpful.

The vessel local lumen diameter is approximated as the diameter of the largest fitting sphere (Fiji Local Thickness plugin\textsuperscript{40}).
Fibre-orientation quantification

For calculating the myocardial fibre-orientation, the gradient structure-tensor method was used. While in DTMRI, spatial anisotropy of diffusion is assessed,\textsuperscript{24} the structure-tensor calculates the structural anisotropy from the image appearance, thus assessing local dominant directions (i.e. fibres).\textsuperscript{26,27}

For each voxel, oriented gradient magnitudes in x, y and z-directions were obtained using central difference. The local structure-tensor within a 3D neighbourhood is then defined as:

$$\text{ST} = \begin{bmatrix}
\sum g_x^2 & \sum g_x g_y & \sum g_x g_z \\
\sum g_y g_x & \sum g_y^2 & \sum g_y g_z \\
\sum g_z g_x & \sum g_z g_y & \sum g_z^2
\end{bmatrix}$$

where $g_x$ denotes the gradient in the x-axis etc., and $\Sigma$ denotes integration of selected oriented gradients in the neighbourhood defined as a cube surrounding the voxel. Eigen-decomposition of ST transforms the given gradient space into a space defined with three orthogonal vectors encoding the appearance of a tubular structure, i.e. fibre. The smallest eigenvalue corresponds to the vector pointing in the fibre direction (since it is associated to minimal image intensity variation). To calculate fibre angle maps, we transform these vectors from Cartesian to a cylindrical coordinate system, where the axis corresponds to the LV long axis. For the smallest eigenvalue vector, the inclination angle was then calculated as an angle between the transverse plane and the vector projection to the local tangent plane.

RESULTS

All reconstructed datasets covered the whole heart and provided 3D detail that has not been generated for a whole heart at this resolution before. One rat, one normal fetal rabbit and one IUGR fetal rabbit heart were processed.

Figure 1 shows the images obtained from the rat. Figure 1 (A-C) shows the originally reconstructed short axis slices at aortic valve (1A) and mid-ventricular level (1B), as well as a longitudinal reslice through aortic valve and apex (1C). Cardiac substructures can be clearly differentiated (atria, ventricles, great vessels and valves). Intramural vessels as well as local
fibre directions can be recognized (1B). When visualizing data using volume-rendering (Figure 1D-G), the detailed architecture of tricuspid valve apparatus (1D) and aortic valve (1E) can be clearly visualized. Additionally, details of local wall complexity (such as atrial pectinate muscles - 1F/G) and part of the vasculature are visible.

Figure 2 shows a detailed view of the aortic wall (2A) as well as from the RV free wall (2B) and LV lateral wall (2C) from the same rat heart. In the aortic wall, spiral-like structures can be distinguished, corresponding to elastin providing support and elasticity. In the ventricular walls, besides clearly visible vessels, predominant directions of fibres can be observed. Longitudinally oriented cell-aggregates show as dot-like structures in short-axis, while circumferential fibres are line-like. From this, it can be seen that in the LV, in epicardial, as well as endocardial sides, fibres are predominantly longitudinal while in mid-wall, they are more circumferential. However, in the RV, fibres at endocardial sides are mainly longitudinal while mostly circumferential at epicardial sides. The structures seen in this type of images have been shown to correlate well with histology. Video 1 shows the (edge-enhanced) whole rat heart dataset from short-axis slices. The intramural course of coronary vessels is easily traceable and the oblique course of fibres gives the impression of flow within the myocardium with different direction at both edges of the walls. The spiral like arrangement towards the apex is clearly shown.

Figure 3 shows surface rendered images of cuts through the fetal rabbit hearts. The IUGR heart (right) is clearly smaller than the normal (left). Additionally, coronaries are clearly dilated and much more prominent in IUGR. In the RV cut (bottom), both chordal as well as false tendons can be depicted. Video 2 shows cuts through the whole heart, showing all endo- and epicardial structures as well as the vasculature.

Figure 4 (and video 3) shows the segmented arterial trees for both fetal rabbit hearts (top) as well as a colour-visualization of estimated local lumen diameter (bottom) where coronary dilatation in IUGR can be clearly observed. In Figure 4 (right), the normalized histogram of lumen diameter shows that coronary size in IUGR is shifted to the right (i.e. larger diameter) of
the histogram, and the relative amount of vessels with smaller diameters (left side) in normal hearts is higher.

Figure 5 (top) shows local helix angles within a slice (mid ventricular) of control (left) and IUGR (right) hearts, together with a visualization of the resulting 3D fibre structure in IUGR (middle left: fibre angles within one plane; middle right: 3D fibre tracking throughout the wall). The gradual change from predominantly longitudinal at epicardial side, towards more circumferential in mid-myocardium and again longitudinal at epicardial can be observed. A rather abrupt change in mid-septum is present as was suggested earlier.\textsuperscript{41} The bottom plots show the histogram of the distribution of the angles within the LV and RV wall (IUGR). From this distribution, as well as from the slice showing the regional angles, it can be observed that in the LV, fibres are predominantly longitudinal with both positive and negative angles, corresponding to epi- and endocardial regions. However, in the RV, endocardium is clearly predominantly longitudinal, but epicardium is not, resulting in an imbalance of positive and negative large angles in the distribution.

**DISCUSSION**

We described a comprehensive, integrated approach for non-destructive acquisition, visualization and quantification of (sub-)structure of whole hearts at almost cell-level resolution.

This approach allows rapid whole heart imaging to quantify morphological remodelling of all substructures within different cardiac components. Both myocardial tissue and vessels can be extracted and interrelated from the same dataset, with the ability to extract local orientation of myocytes (=fibres) and relate it to location within wall and surrounding vasculature. Additionally, global chamber geometry, including detailed visualization of trabeculations and (false) tendons is provided.

These datasets provide a unique source of information where tissue properties can be quantified/compared within the 3D structure for purposes of describing changes induced by
genetic/acquired disease, even if remodelling is subtle and not detectable by current imaging modalities.

The data richness, together with resolution and image contrast, allows use of state-of-the-art analysis tools to quantify and visualize different substructure of the whole 3D dataset. To extract coronaries, we employed an interactive segmentation using sparse user-specified seeds. The ability to discriminate local predominant direction of myocyte-aggregates allows for quantification of transmural fibre distribution by extracting 3D components of the local structure-tensor using gradient calculations together with fibre-tracking approaches similar to those used in DTMRI. This allows optimal re-use of the vast spectrum of analysis tools, while offering the possibility to combine approaches from high-resolution data with techniques developed for lower resolution (clinical) modalities.

Additionally, both superior resolution as well as the fact that all substructures can be recognized and segmented while originating from single dataset, enable to obtain structural data required for performing computational modelling of the heart as a functional organ. Current approaches often get image-based overall geometry, but have to incorporate fibre structure from low-resolution (ex-vivo) DTMRI or statistical models developed from microscopy\textsuperscript{42-44}. This offers a wealth of new possibilities to improve models, especially towards more individual simulations and comparing control and pathological specimens.

The proposed imaging is based on X-ray phase-contrast tomography, which is available in contemporary synchrotron facilities. It is currently the only non-destructive method that provides the resolution needed to resolve details of the size of individual myocytes (<10 µm). Even if some microCTs can provide the resolution, X-ray absorption imaging does not provide contrast needed to discriminate microstructural details within cardiac tissue. Vessels and fibres can only be detected when a contrast agent is used. However, this is highly artefact prone since a homogeneous contrast distribution along myofibres and within vasculature is very challenging.

With phase-contrast X-ray imaging, not only local X-ray absorption is quantified, but it additionally captures wave-propagation phenomena that enhances subtle differences in
tissues and improves tissue/vessel contrast and thus helps capturing details on local predominant myofibre direction. Therefore, phase-contrast imaging is far superior to (contrast-enhanced) X-ray absorption when studying cardiac microstructure. While a lot of research is going on to allow for phase-contrast imaging based on traditional CTs, currently, high-resolution phase-contrast X-ray imaging is only available in synchrotrons. It is an expanding field in synchrotrons and has shown great promise for non-destructive visualization/quantification of samples from different origins and application fields (paleontology, biology, material-science). Extending the use of these large-infrastructure research facilities, funded by (inter-)national research organization, towards cardiac applications, opens up new possibilities for studying cardiovascular development, pathophysiological remodelling and therapeutic targets.

To illustrate the potential of the approach, besides a healthy rat, we also scanned a control and IUGR fetal rabbit heart. The resolution and absence of complicated preparation and imaging, allowed studying the micro-architecture of the fetal heart, which is challenging otherwise. Additionally, we have shown that integrated assessment of organ morphology, vasculature and fibre-structure provides a way to quantify subtle (sub-clinical) remodelling induced in utero, beyond gross changes induced by genetic alterations. We observed that, while the heart is smaller, coronaries are clearly dilated, most probably as adaptation to haemodynamic challenges induced by hypoxia/hyponutricia. Additionally, the gradual change of fibre-direction can be quantified.

In this report, we describe preliminary results, mainly focusing on direct visualization and quantification of remodelling induced by IUGR which has been demonstrated to induce a remodelling at the organ level, as well as at the cellular and subcellular level. However, the precise mechanisms leading to increased risk of cardiovascular disease in adulthood are still not well understood.
LIMITATIONS

While phase-contrast imaging is highly promising for quantification of the integrated multi-scale cardiac structure and its components, currently it is limited to synchrotron-facilities and therefore not easily accessible. On-going research in CT-technology might lead to more widespread access.

In our study, we examined in-vitro hearts. However, while challenging, the technology should be applicable in-vivo.

Current synchrotrons have limited fields-of-view, making it time-consuming to do hearts larger than rodents.

In this study, we didn’t do a direct comparison of the same tissue with either histology or DTMRI. However, it was already shown that synchrotron microCT does visualise the cardiac fibres and the results from the tracking of our data correspond to as well findings from histology as from DTMRI, but at much higher resolution compared to the latter.

CONCLUSION

In conclusion, we have developed a novel, high resolution, non-destructive approach towards visualizing and quantifying the microstructure of whole hearts at myofibre resolution, providing structural information at microscopic level without need of slice processing. This opens up new possibilities for a systems-medicine approach towards remodelling, providing fast acquisition of hearts in a near native state without processing artefacts. Studying fetal and rodent hearts is particularly challenging due to their size and the proposed approach could help to understand normal organ development and remodelling in disease from earliest stages of life.

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REFERENCES


FIGURES

1. X-ray phase-contrast synchrotron radiation-based micro-CT imaging of a rat heart. A, B and C, Cardiac anatomy can be observed with great detail: aortic and tricuspid valves (A), fibre orientation (B), false tendon or right ventricle moderator band (C). When doing volume rendering of the images, details of tricuspid (D) or aortic valve (E) can be observed; as well as the atrial pectinate muscles (F and G).

2. Detail of aortic and ventricular walls of a rat heart. In the aortic wall layers can be distinguished (A); whereas in the ventricular walls, orientation of fibres can be determined and classified as circumferential (circ) or longitudinal (long) in both right (B) and left (C) ventricles.

3. Volume rendered images depicting detailed cardiac anatomy of an IUGR and a control heart. IUGR fetal heart (top-right) is smaller and with thinner walls compared to the control fetal heart (top-left). Additionally, it can be appreciated that the coronary vessels are clearly dilated and much more prominent in the IUGR heart.

4. Segmentation of the coronary tree. The coronary tree can be visualized in detail when segmented (top left). Differences between control and IUGR rabbit fetal hearts are even more evident when the segmentation is visualized as a quantification of lumen diameter (bottom left). Lighter colours mean larger diameters; therefore dilatation of coronary arteries in IUGR is clearly visible. At the right, the normalized histogram distribution comparing approximated lumen diameter of IUGR versus normal (normalized to total amount of vessels) suggests that in IUGR, there is a shift to the right side, i.e. larger diameter, of the histogram, and the relative amount of vessel with smaller diameters (left side) in normal heart vessels is higher.

5. Fibre orientation. Fibre angles change across the walls within a slice in fetal rabbit control (top-left) and IUGR (top-right) hearts. Fibre angles change from endo- to epi-cardium from about +60° to -60°. In the middle, a visualization of the 3D fibre structure of the IUGR rabbit
heart is shown (left: fibre angles within one slice; right: 3D fibre tracking within the wall). At the bottom, the histogram of fibre angles for left as well as right ventricle are shown.
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Elastin fibres in the aortic wall

Fibre orientation change

Intramural vessel

False tendon

Figure 1
Figure 2

Aortic wall

RV wall

Circ.

Long.

LV wall

Long.

Circ.

Long.
Figure 4

Normal IUGR

Control IUGR

Vessel diameter (μm)

Prevalence (%)
Figure 5

Control

IUGR

LV angle histogram

RV angle histogram