A Framework for Quantification of Regional Cardiac Fibrosis from Serial Sections using 3D Whole Slide Imaging

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Abstract— **Pathological cardiac fibrosis is important in predisposing the heart to arrhythmia and mechanical failure. The regional distribution of fibrosis is often described qualitatively and quantitatively in histological studies of animal hearts after staining collagen with specific colored stains. Currently this description is often piecemeal, as it lacks rigorous spatial registration, matching and methodological standardization between animals and between study groups. We propose a strategy for the quantification of regional fibrosis using the American Heart Association (AHA) cardiac segmentation model. We quantify fibrosis after whole heart 3D histological reconstruction in one normal rat heart and in one rat heart in right heart failure induced by monocrotaline. We then assess the minimum spaced histological sampling which allows for accurate assessment of regional fibrosis. We show that using every section of a set of 5 µm serial sections quantifies regional right ventricular fibrosis, with highly significant ($p < 0.001$) differences between heart failure and control hearts. We show that the absolute error of collagen quantification is low when sections are taken spaced by up to 100 µm (error 5.7±5.8%). Likewise, absolute error associated with sectioning starting position is low for sections spaced up to 100 µm (error 13.3±17.2%). Above 100 µm section spacing quantification error is large (tending to 50%) and error associated with sectioning starting position is large (tending to 60%).**

I. Introduction

Knowledge of the extent, nature and distribution of myocardial fibrosis is important in the understanding of mechanical heart failure and of cardiac conduction disturbances. It is particularly important in the assessment of heart failure treatments and of cardiac antifibrotic agents [1]. In these studies section based histology sampling is often the method of choice for quantifying myocardial fibrosis due to: (i) readily available histological stains with excellent signal to noise ratio (SNR); (ii) high acceptance of the method; and, (iii) the detailed structural information obtained. MRI contrast based methods do exist for global cardiac fibrosis quantification but SNR is low compared to histology. Three-dimensional histological methods exist but are time consuming and require highly specialized equipment [2]. A problem with the current application of 2-dimensional histology imaging to quantify fibrosis is that there are no defined methodologies or standards for cardiac sampling. Often sub-regions of the heart are excised and prepared for histology, and after histological processing sections are often selected form these tissue blocks in a piecemeal fashion. It is not uncommon to see different myocardial regions quantified between control and intervention animal groups, as evidenced by very different cardiac fiber orientation between images. The process is manual, and there are multiple stages where bias can affect results. The recent advent of high-throughput whole slide imaging scanners ("virtual slide scanners") enables whole heart serial histological imaging. We demonstrate a fully automated method for whole right and left ventricle (RV, LV) myocardial fibrosis quantification which uses novel 3D histological reconstruction, serial slide registration to an MRI model, followed by standardized segmentation into AHA myocardial regions [3] and automated image analysis. Furthermore, we explore if volumetric subsampling (sectioning the whole heart, but only collecting and imaging sections at regular depth intervals) can be used to reduce the time and cost of histology whilst preserving the accuracy of the quantitative results. We explore these approaches in the rat monocrotaline induced right heart failure model and in a normal control rat [4].

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II. Methods

A. Heart Preparation and Perfusion Fixation

Full details are provided in [4], briefly, a male normal Wistar rat (weighing 345 g) and a male Wistar rat in right heart failure (weighing 336 g) were euthanized in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986, the hearts removed and retrograde perfused with: (i) HEPES-Tyrodes to clear blood; (ii) BDM to prevent contraction; then (iii) MRI contrast agent (0.1% Gd-DTPA) and fixative (4% formaldehyde for 20 minutes). The hearts were imaged 2 hours after fixation immersed in fixative solution, and were processed for sectioning 1 week after MRI.

B. MRI

The High Resolution MRI (HR-MRI) was carried out using a FLASH (Fast Low Angle SHot) MRI sequence in a Bruker (Ettlingen, Germany) 9.4T spectroscope with 20 averages and echo time (TE) = 7.9 ms repetition time (TR) = 50 ms, with 20 averages taking 18h to acquire at a resolution of 50 x 50 x 50 µm. Full details are provided in [5].

C. Serial Histology

After HR-MRI the hearts were processed for histology using standard histological preparation. Briefly, the hearts were (i) dehydrated, equilibrated with histoclear, mounted in paraffin wax, sectioned into serial 5µm thick sections, and sections lifted onto Suprafrost slides (Leica Microsystems, UK). Slides were stained using Picrosirius Red stain for collagen as described in [5]. Slides were imaged using an Aperio AT scanner (Aperio, Vista, CA) with a 20× Objective to produce whole slide images with a final resolution of 0.46 microns per pixel. To minimize stain variation, all sections were taken by the same technician on the same microtome and stained in batches. All slides were scanned on the same scanner to minimize inter-instrument variation in color.

D. Three-dimensional Histological Reconstruction

A consistent problem in previous work has been that whole heart deformation from histological preparation and slice-wise independent deformations due to histological sectioning result in poor volumetric reconstruction of histology. We propose a novel histology to MRI registration approach based on our previously presented approach for registering differently stained histology images [6]. This involves generating synthetic tissue channel images using image feature (texture, color, intensity, etc.) co-occurrence of roughly aligned image pairs. This approach consists of 4 stages:

i) **Manual localization of central section to MRI:** We developed an interactive GUI within our in-house Volume Viewer to localize the oblique MRI slice corresponding to the central histology section. This was based on manually defining a long axis (two clicks) and manually selecting the particular long axis view using a slider to control the angle about the long axis.

ii) **Automated 3D localization of a small number of 3D sections:** One histopathology image 50 sections (~ 250 µm) above, and one histopathology image 50 sections below the central section are roughly aligned in 2D to the central section using the rigid registration method described in [7] and initialized in 3D space parallel to the central slice with an assumed out of plane separation based on the approximate section separation (~5 µm) multiplied by the number of sections separating the section from the central section. A variation on the stack transform [8] is used within a stochastic optimization framework to optimize the individual in-plane rotations and translations of the 3 slices, the separation between slices, and the global 3D rotation and translation w.r.t. the MRI volume. The metric used is the mutual information between quantized local feature clusters (see [6] for a description of how these clusters are formed).

iii) **Automatic registration of all other sections:** Given the registered sections from part ii), the initial planes to use to register all other sections are defined as parallel to the initial 3 sections, with offset from the central slice determined by linear regression from slice number to z-position ($P_z = K_1 \times \text{slice\_no} + K_2$) estimated in a least squares minimizing way from the initial 3 sections. The rotation, translation, and subsequently non-rigid transform of sections within this 2D plane are estimated using the method described in [6], in which mutual information of clustered features is used to generate multiple derived "tissue channel" images from both the histopathology and MRI. These are rigidly registered individually on a 2D 256×256 block-by-block basis and registrations over all channels and blocks combined into a single 2D non-rigid (rotation + translation + B-spline) transform using a robust estimator. At this stage approximately 7×7 blocks are used to calculate a B-spline transform with 3×3 internal knots. The out of plane offset of the section is subsequently optimized by the method described above (ii) using the 3 (fixed) reference slices - only optimizing the single out of plane offset. Finally the out of plane slice number to distance regression function is re-estimated from all sections, and the out of plane offset from center updated.

iv) **Global coherence optimization (smoothing):** The previous stages rely only on the MRI data to position and warp the histopathology images. At the final stage both the corresponding oblique MRI slice, the two histopathology sections above, and the two histopathology sections below are used in the 2D non-rigid registration process. The individual registrations (5×Number of blocks) are the same as previously described, and all registrations over all 2D image pairs are combined to form a single 2D non-rigid transform. This process is repeated iteratively at increasing image magnification and numbers of knots of the B-Spline.

E. Cardiac Ventricular Segmentation

The cardiac ventricular segmentation was carried out by manually fitting a RV and LV segmentation model to the HR-MRI. The segmentation model was modified from the standard AHA LV segmentation [3], but adapted for the RV as well as the LV, with the septal segments allocated to the LV only. The resulting segmentation has 8 RV and 17 LV segments and is shown in Fig. 1. The localized regions of the whole heart serial histology section images were isolated for each segment, and used for automated collagen quantification.

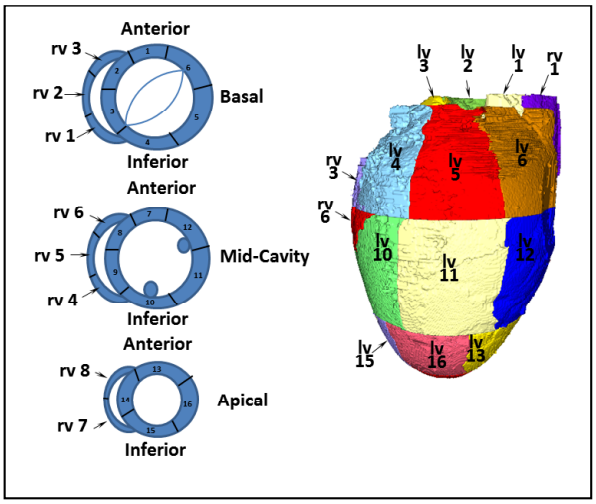

Figure 1. *AHA Based Cardiac Segmentation*

F. Three-dimensional Quantification of Regional Fibrosis

The collagen was segmented from the surrounding tissue using a simple rule based thresholding approach on the red, green and blue (R G B) channels of the histology images.

Simple collagen thresholding rules (as used in [9]]) were determined and optimized based on manual segmentation of 10 randomly selected histology images. Pixel counts were then taken, and the percentage of collagen to myocardial cells determined.

G. Minimum Sampling to Quantify Regional Fibrosis

As the registration method first aligns histology slices into the MRI volume, it is possible to have an accurate histology registration with only a small subset of all the consecutive serial 5 µm images. In order to explore if it is possible to accurately determine regional fibrosis using spaced sections the analysis was repeated for all right ventricular segments with inclusion of only one section per 10, per 20, per 50, per 100 or per 200 sections (corresponding to 50, 100, 250, 500 and 1000 µm between sections). In one right ventricular segment (RV1) the dependency of collagen quantification on the sectioning starting position was determined by exploring variation in error depending on the selected spaced-section image subset.

III. Results

A. Three-dimensional Histological Reconstruction

High-quality volumetric reconstruction was achieved using the serial section registration method (Fig.2) as evidenced by close approximation of myocardial blood vessels and cleavage planes/myolaminar between the MRI and histology images. Registration error is generally less than one MRI voxel dimension (50 µm). This does result in some image jitter between adjacent histology section planes, but it is not a cause of significant misclassification of tissue segments and is therefore not relevant to this study, as collagen quantification is in the section plane images only.

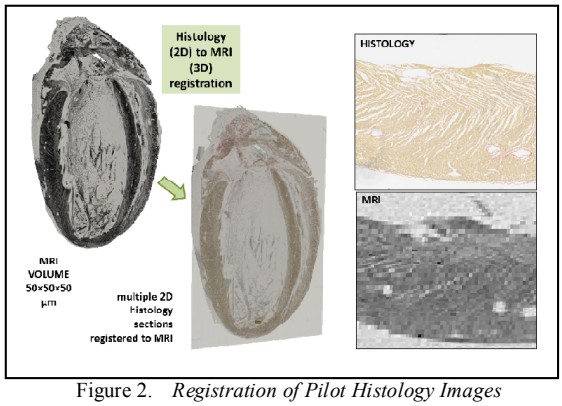

Figure 2. *Registration of Pilot Histology Images*

B. Three-dimensional Quantification of Regional Fibrosis

The segmental collagen fractions are shown for the left ventricle and right ventricle for the control and failing heart in Fig. 3. The collagen fraction is expressed as the percentage of collagen pixels of myocardial pixels. In the right ventricle there is universally greater collagen fraction in the failing heart (average across all RV segments: CONTROL: 4.5±1.6, FAILING x10.9±2.7, $p < 0.0001$). In the left ventricle the global pattern is the same (average across all LV segments: CONTROL: 7.57±3.1, FAILING 10.6±6.9, $p < 0.0001$), however, there are individual segments where there is greater collagen in the normal heart than the failing heart.

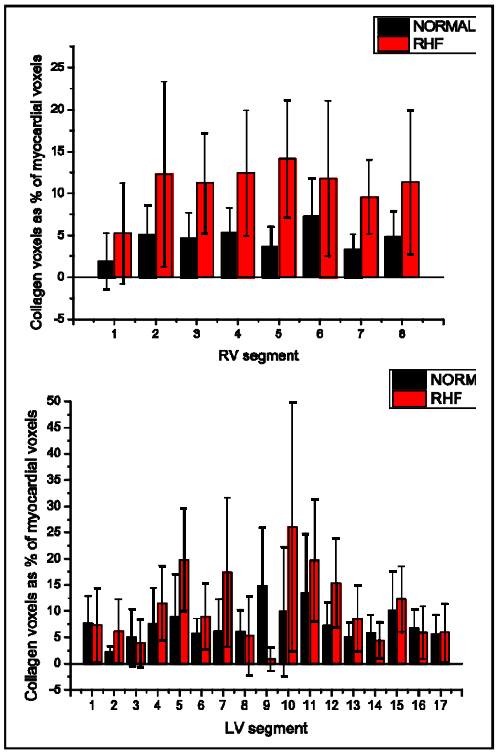

Figure 3. *Percentage of collagen content in RV and LV segments*

C. Minimum Sampling to Quantify Regional Fibrosis

The absolute difference between determined mean collagen fraction from all 8 RV segments for spaced sections was expressed as a percentage of the collagen fraction including all sections. This is referred to as absolute error (%) and is shown in Fig. 4. Error is low for section spacing of 50 and 100 µm (at 100 µm section spacing average absolute error (%) across all RV segments: CONTROL: 5.7±5.8, FAILING 3.5±3.2). Error increases linearly, tending to 40% at 1000 µm spacing. If spaced/stepped sections are used then it is possible that there is dependence upon the starting position of sectioning. This was explored in segment RV1 in the normal heart (Fig. 5). Changing the starting position of the spaced sections had minimal impact for sections spaced at 50 and 100 µm. For 100 µm spacing mean absolute error was 13.3±17.2%. Above 100 µm spacing the error is large (greater than 30%).

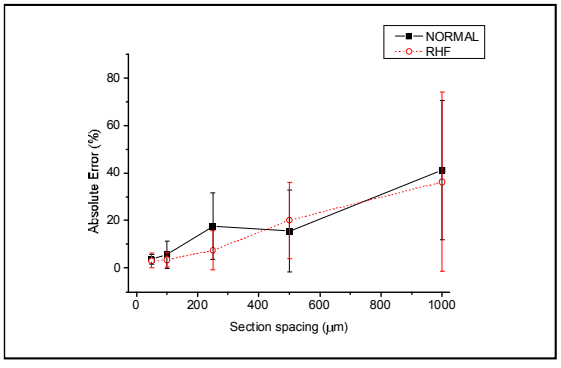

Figure 4. *AHA based cardiac segmentation*

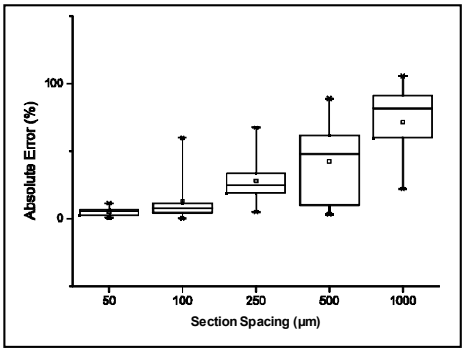

Figure 5. *Dependance of error on starting position in segment RV1*

IV. Discussion

We have demonstrated that serial section histology with contrast enhanced MRI aided slice registration provides a robust method quantifying whole heart and regional fibrosis. The pattern of fibrosis revealed is in agreement with previous studies on this heart failure model using conventional histology methods with manual point counting determination of fibrosis [10]. There are certain applications where it may be desirable to collect and image every sequential 5 µm section, and this will provide the most precise quantification of total and regional fibrosis. However, for many applications the use of more widely spaced sections (step sectioning) will be more appropriate. We provide the first quantitative analysis of the statistical consequences of the use of differently spaced sections and we show that error is <6% using 100 µm spaced sections, which is likely to be acceptable for many applications. Sectioning and imaging rat hearts with 100 µm spaced long-axis sections is highly feasible, as the cardiac short axis diameter is ~10000 µm therefore requiring ~100 sections. The simple rule based threshold method used here to segment collagen from myocardial cells is primitive from an image analysis perspective. However, such approaches are widely applied in the cardiology literature, exampled by: (i) point counting based on manual user assessment of the color at grid points on native histology images (see [9] and associated citations including [10]); (ii) digital thresholding on RGB values [9] (as here); and, (iii) entirely qualitative assessment of a small number of selected representative myocardial regions with no quantification [11]. The collagen segmentation framework could be enhanced through stain normalization by color deconvolution [12] and by a more sophisticated collagen segmentation algorithm using spatial information [13]. This would improve the quality of segmentation and additionally reduce the impact of two current limitations of our approach: (i) section damage (e.g. folding) can be misinterpreted as fibrosis; and, (ii) different histological types of fibrosis cannot currently be distinguished, such as perivascular fibrosis and interstitial fibrosis.

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