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A Chicken Embryo Cardiac Outflow Tract Atlas for Registering Changes Due to Abnormal Blood Flow*

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Abstract

Subdivision-based image registration has previously been applied to co-localize digital information extracted from *rigid* structures in biological specimens, such as the brain. Here, we describe and demonstrate the creation and application of a two-dimensional subdivision-based atlas representing a *dynamic* structure: the outflow tract of the developing chicken heart. The atlas is designed to segment three different anatomical layers of the outflow tract, and is demonstrated on the characterization of collagen XIV in both control and induced abnormal flow specimens. Abnormal blood flow in the embryonic developing heart can lead to congenital heart disease. Comparing local cellular and sub-cellular changes that are caused by abnormal flow can assist in understanding the molecular pathways involved in maladaptations of the heart and congenital defects. This study demonstrates the approach and potential for more extensive applications of the subdivision-based atlas for the embryonic chicken heart.

I. INTRODUCTION

Congenital heart disease (CHD), affecting approximately 1% of newborns, is the leading cause of death in children that does not involve infection [1]. Only a small fraction of CHD cases can be attributed to genetic causes, prompting the belief that the majority of CHD cases derive from environmental causes. Abnormal blood flow through the heart is an example of an environmental cause that has been linked to CHD. Blood flow is essential for proper heart development [2], and abnormal blood flow conditions in embryonic animal models of cardiac development lead to heart defects that resemble those in CHD patients [3,

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4]. Because blood flow conditions can be influenced by external stimuli such as nutrition, understanding the mechanism of CHD development from abnormal blood flow is critical to determining appropriate interventions.

Understanding the biological mechanisms that underlie the relationship between hemodynamic stimuli and cardiac adaptation has the potential for improving clinical intervention. The chick embryo is frequently used as an animal model of cardiac development due to ease of access and similarities with human cardiac development. At very early stages of cardiac development, the heart of both humans and chicks is a tubular structure with no valves or chambers. These early stages are very sensitive to hemodynamic conditions. To experimentally alter blood flow in the chick heart, outflow tract banding (OTB) can be employed [3]. In OTB, a suture is put in place and constricted around the heart outflow tract (OFT) (Fig. 1). OTB leads to cardiac remodeling and cardiac defects.

Surgical interventions to alter blood flow conditions have been performed in different animal models, resulting in a spectrum of cardiac defects, e.g. [3, 4]. The initial adaptation steps that lead to the formation of heart defects, however, are not known. Adaptation frequently involves changes in extracellular matrix composition. We are thus interested in identifying changes in collagens in the OFT of chick embryos after OTB. The OFT is an important cardiac segment, because it gives rise to the intraventricular septum and semilunar valves. Further, cardiac cushions, which are protrusions of extracellular matrix towards the lumen, form in the OFT at early stages and act as primitive valves. Changes in the composition of cardiac cushions, in particular, seem to determine the fate of valves and septum. A chick OFT atlas will facilitate visualization and analysis of collagen content changes with various interventions. Here we describe a novel approach for identifying the spatial distribution of extracellular matrix proteins using a subdivision-based atlas of the OFT.

Subdivision is a geometric modeling technique designed for the generation of smooth geometries from coarse meshes [5]. In the case of quadrilateral subdivision, each quadrilateral in the coarse mesh (subdivision level zero) can be subdivided into four quadrilaterals (subdivision level one) using the two-step process of quad-splitting and centroid averaging. The nature of a subdivision mesh lends itself to another function – the organization of spatial information in a multi-scale fashion. In this role, the subdivision mesh serves as an atlas [6]. Past work has demonstrated the use of subdivision meshes in the role of an atlas for the mammalian brain [7–9] and bones [10]. For those examples, the subdivision atlas was used to characterize, compare, and disseminate gene expression patterns, metabolites, and mineral densities. Subdivision mesh atlases have been constructed in both 2D and 3D. In previous work using subdivision atlases, the changes in anatomical shape between specimens were limited. We hypothesized that a subdivision atlas could be used to assist in organizing, tracking, and comparing features in the developing heart OFT, even when the heart shape is changed after experimental interventions. To test this, we have constructed a subdivision atlas of the OFT and registered the atlas to OFT images indicating levels of collagen XIV with and without OTB.

II. Procedure

A. Specimen Preparation

Preparation of embryos followed standard methods in which fertilized White Leghorn chick eggs were incubated blunt end up for ~3 days at 38°C to the Hamburger-Hamilton (HH) stage 18 [11]. Embryos were exposed by removing shell and inner shell membrane at the air sac. For this study, OTB [3] was performed on two embryos (approximately 40% constriction for OTB1, 25% for OTB2) and a sham (control) procedure on one embryo. The egg shells were then re-sealed and embryos incubated for 24 hrs (to HH24 [9]) prior to imaging.

At HH24 whole embryos were collected for immunolocalization of collagen XIV. HH24 embryos were fixed in 4% paraformaldehyde, dehydrated in a graded methanol series, and blocked of peroxidase activity in methanol/dimethyl sulfoxide/hydrogen peroxide. Embryos were rehydrated, washed with phosphate buffered saline Tween-20 milk (PBSTM), and blocked with PBSTM + 4% goat sera before overnight treatment with collagen XIV antibody. Embryos were thoroughly washed in PBST prior to treatment with a fluorescent Rhodamin Red secondary antibody. Specimens were cleared with 1:2 benzyl alcohol: benzyl benzoate prior to imaging.

B. Imaging

Confocal images were collected using a Zeiss/BioRad Radiance 2100 confocal laser scanning system. 512×512 pixel 2D images were acquired every 10 microns over the entire OFT volume. Both multichannel epifluorescence and Green-spectrum autofluorescence were imaged with a 50 mW argon ion laser (488 nm wavelength). Epifluorescence was imaged with a 1.5 mW green HeNe laser (543 nm). Epifluorescence images are shown in Fig. 2.

C. Atlas Construction

We constructed a 2D subdivision-based atlas for the section of the OFT containing cushions by utilizing the method previously described in [7]. Briefly, this approach applies the 2D subdivision mesh atlas construction tool [6] to denote the vertices and edges of a quadrilateral mesh. In addition, each quadrilateral is associated with a specific region, denoted by color. In this work, the constructed atlas was designed to explicitly denote the visually-defined anatomical layers of the OFT including the myocardial wall, the cardiac jelly, and the OFT cushion (Fig. 3).

D. Atlas Fitting

To properly associate protein (collagen XIV) localization from confocal images with the OFT atlas, we used the atlas deformation tool [6] to adjust atlas shape to match the anatomy of the OFT in each of the 3 datasets: sham control, OTB1, and OTB2. This involved manually moving the control points of the atlas. Control points were moved to locations that allowed the explicit region boundaries of the atlas to properly match the corresponding boundaries in the datasets. The fitted atlas is shown for all three datasets in Fig. 4.

E. Measuring Collagen

With the atlas deformed to accurately overlay each image and region boundaries, each quadrilateral in the atlas at subdivision level 4 was associated with the respective underlying information in the image. For this study, the indicator of collagen was the red channel of the RGB image. As a measure of collagen density, we associated the mean intensity of the red channel across the area defined by each of the subdivision level 4 quadrilaterals. To determine the overall expression value of an OFT region, we calculated the area-weighted mean intensity value across all quadrilaterals in the atlas region, except those quadrilaterals showing the band itself which excluded using a mask-based approach [12].

III. Results and Discussion

Using the OFT atlas, we were able to segment the 3 sub-regions of interest from the different specimens. The representative regional collagen XIV densities are plotted in Fig. 5 for each dataset. We found increased levels of collagen XIV in the myocardial wall, the cardiac jelly, and the OFT cushion as a result of the application of the band. These changes may be in response to the altered blood flow.

Importantly, the research described here suggests that a subdivision-based atlas can be leveraged to segment the regions and sub-regions of the embryonic chick heart. Thus, this establishes a method for additional investigations in which the spatial relationships between changes in levels of relevant extracellular matrix proteins (collagens, proteoglycans, *et cetera*), expression of genes, and subregion-specific changes in tissue mechanics will be examined.

Future work will first focus on additional specimens, proteins, and genes in order to establish significant relationships between mechanics and changes in matrix composition (indicative of changes in cell behavior). After identifying critical elements, research will look at changes over time to determine long term consequences. Further, the atlas itself can be extended beyond the OFT to include the developing heart ventricle and atrium. As more datasets are acquired, approaches to automate atlas fitting can potentially be employed [13–17].

One of the advantages of using a subdivision atlas approach is it is an established approach for online dissemination of organized spatial information. Thus, one could directly extend future work into an online spatial database of genes and proteins associated with the development of the heart. Such a database would allow queries of molecular components in user-defined regions [18, 19]. Additionally, the atlas will facilitate clustering of proteins and anatomical space [20]. The assignment of genes and proteins to functional regions can be leveraged toward analysis of molecular pathways [21].

A step beyond a 2D heart atlas is a 3D heart atlas developed with 3D subdivision techniques [22–24]. A 3D atlas has the advantage of incorporating data from any plane of imaging [25]. This allows the 3D atlas to be less dependent on imaging planar topologies that identically connect anatomical regions. A 3D atlas will allow a more precise spatial localization of extracellular matrix changes in response to hemodynamic conditions, and thus is an

important step towards elucidating the mechanisms by which the developing heart adapts to hemodynamic conditions, and how this adaptation can lead to cardiac defects.

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Figure 1. Sub-regions of the outflow tract. The lumen (L) is marked as well as the layers of the outflow tract: myocardial wall (M), cardiac jelly (CJ), and cushion (C). The band applied to alter flow is also indicated.

Figure 2.
Single slice view of acquired image data of control (top) and altered (middle and bottom) collagen XIV datasets.

Figure 3.

Atlas of the outflow tract wall. Sub-regions explicitly defined in the atlas are the myocardial wall (red), the cardiac jelly (orange), and the OFT cushion (yellow). Atlas was constructed using control dataset as a template. Levels of subdivision from 0 to 2 are illustrated.

Figure 4.
Atlas deformed to fit anatomy for control (top) and altered (middle and bottom) collagen XIV datasets.

Figure 5. Collagen XIV by region using atlas-based segmentation. Mean intensity of the red channel in each image calculated using the atlas to define regional boundaries for each of the three datasets.