

Iodine staining outperforms phosphotungstic acid in high-resolution micro-CT scanning of post-natal mice cardiac structures

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Abstract

Purpose: Micro-computed tomography (micro-CT) scan provides high-resolution three-dimensional images of mineralized tissues in small animal models. Contrast enhancement is essential to visualize non-mineralized tissues with micro-CT scan. We attempted to compare the two most common contrast agents to stain and image mouse cardiac structures.

Approach: *Ex-vivo* micro-CT scan images of the mouse hearts were obtained following staining by potassium iodide or phosphotungstic acid (PTA). PTA-stained samples were imaged after various durations following staining (14 days, 25 days, 187 days, and 780 days), whereas iodine-stained samples were imaged after 72 hours. We compared median staining intensity between PTA and iodine at 0.1-mm intervals from the edge using the Mann Whitney test with correction for multiple comparisons.

Results: Sixty post-natal mice hearts were stained with either PTA or iodine and imaged using micro-CT scan. Iodine proved to be faster and more uniform in complete enhancement of cardiac tissue in as short as 72 h, whereas PTA required a significantly longer time period to penetrate mouse cardiac structure (>150 days). Median staining intensity with iodine was strongly higher than that with PTA from 0.1- to 1.5-mm distance from the epicardial edge (2-tailed *P* value <0.01 or lower throughout).

Conclusions: Iodine-stained soft tissue imaging by micro-CT scan provides a non-destructive, efficient, and accurate visualization tool for anatomical analysis of animal heart models of human cardiovascular conditions. Iodine is more efficient compared to PTA to achieve complete murine myocardial staining in a significantly shorter time period.

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1 Introduction

Soft tissue visualization at the micrometer scale is paramount for investigation of structure, function, and development of organs.¹⁻³ Moreover, meticulous histopathological assessment of potentially affected tissue is crucial for the investigation and treatments of diseases.^{4,5} Histological sectioning of stained tissues yields high-resolution information in the sectioned plane in a two-dimensional (2D) view. Three-dimensional (3D) reconstruction and visualization of serially sectioned tissues is possible.^{6,7} However, laboratory procedures of both data

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procurement and analysis are labor-intensive, and the process of serially sectioning tissue can distort samples producing artifacts, such as creating holes, folding, and missing parts.

Another promising new histological technique is episcopic fluorescence image capture, which could generate a 3D dataset from sectioned material.^{8,9} However, this method and light microscopy histological visualization both are invasive and destructive.

On the other hand, non-destructive imaging techniques, such as high-frequency ultrasound,¹⁰ micro-magnetic resonance imaging (micro-MRI),^{11,12} and micro-computed tomography (micro-CT),¹³ are available for detailed and high-resolution visualization of small animals' soft tissues. High-frequency ultrasound has emerged as a non-invasive imaging modality of living mouse hearts and can provide high-resolution 2D/3D image. However, it is time-consuming and it is difficult to assess some cardiac and aortic arch anomalies using this imaging modality.¹⁴ Micro-MRI provides high-resolution 2D and 3D data with isotropic spatial resolution without the need for staining with a contrast agent.¹ However, micro-MRI setups are generally very expensive, have long image acquisition time, and are not readily available.

X-ray absorption micro-CT has recently attracted great attention in *in-vivo* and *ex-vivo* visualization of small animal models and provides high-resolution 3D data, at a micrometer scale.^{3,15} As opposed to micro-MRI, micro-CT scan techniques are versatile, available, and easy to perform at a large scale with a detection sensitivity similar to that of micro-MRI. Micro-CT scanning was initially applied to mineralized tissues such as teeth and bone,^{16,17} where high intrinsic x-ray attenuation provided high-resolution imaging that was lacking when the same technique was applied to soft tissues. High-resolution imaging of soft tissues is possible with contrast agents such as iodine, osmium, or tungsten.^{18,19}

Micro-CT scan has the capability for rapid 3D image reconstruction and provides the means to perform virtual autopsies with spatial resolution at near cellular levels ($<1\ \mu\text{m}$).^{20,21} The acquired images can be presented in multiple imaging planes and rapidly reconstructed thereby allowing a detailed assessment of cardiac anomalies without sample destruction. This technique is non-invasive and can be conducted without compromising the ability to further pursue downstream histological analysis.^{20,22}

In this study, we compared two common micro-CT scan staining agents for enhancement of soft tissues and their application for investigation of post-natal murine cardiac structures as part of a project on identifying genes that contribute to the left ventricular hypertrophy in the collaborative cross (CC) mice.^{23,24} The ability to study the CC strains using fixed tissue allows access to more strains at a greatly reduced cost compared to shipping live animals.

2 Methods

2.1 Compliance with Ethical Practice

All tissues and animals used in this study were handled in compliance with The Australian Code for the Responsible Conduct of Research, 2007, and Australian National University Animal Experimentation Ethics Committee.

2.2 Sample Preparation

We studied 60 post-natal mouse hearts in this experiment. They were harvested from 18- to 77-week post-natal mice. Hearts and connected vessels were fixed in a 4% formalin solution until imaging preparation.

Formalin-fixed hearts were then dehydrated in ethanol in order to remove formalin from heart tissue. To prevent a further and abrupt shrinkage of the fixed sample, each heart was subjected to a graded series of ethanol solutions beginning with 20% ethanol for 24 h, then 50%, 70%, and 90% ethanol for 1 day each. Following this stage, hearts were immersed either in phosphotungstic acid (PTA) 1.5% in 90% ethanol or iodine potassium iodide (I_2KI) 1.5% in 90% ethanol. This technique was successfully used by other colleagues in our laboratory in order to stain rodent neural tissue and visualize it by micro-CT scan.²⁵ Higher concentrations of iodine and PTA were previously shown to cause soft tissue shrinkage but could shorten time required for staining

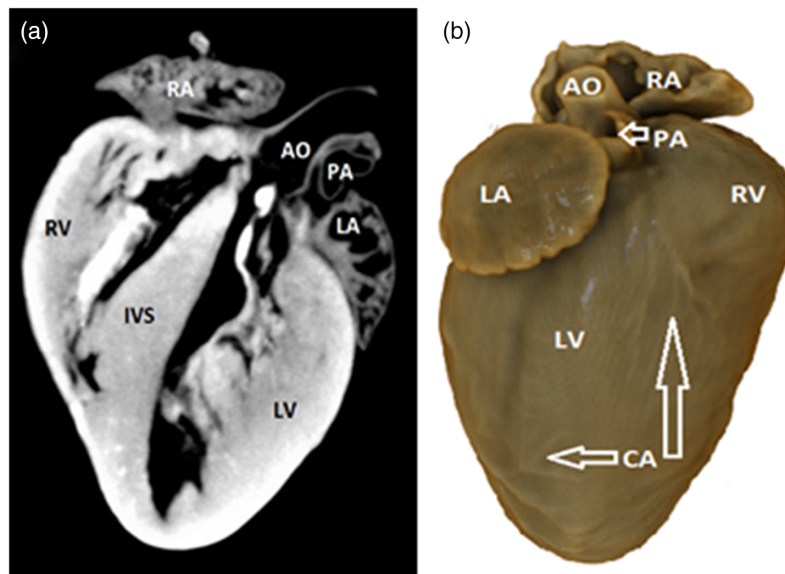


Fig. 1 (a) Long axis section of a mouse heart; (b) an example of a 3D volume-rendered mouse heart. AO, aorta; PA, pulmonary artery; RA, right atrium; LA, left atrium; RV, right ventricle; IVS, interventricular septum; LV, left ventricle; CA, coronary artery.

agent diffusion in the tissue.^{3,26} Stained hearts were imaged in different stages in order to assess the staining quality with micro-CT scan images. Samples were washed in absolute ethanol and then placed on the CT scanner.

2.3 Data Acquisition

A commercial Caliper Quantum FX micro-CT scanner was used to scan the mice hearts. Tissue specimens were removed from the staining solution, washed in ethanol 90%, and placed on the stationary loading dock of the Caliper Quantum FX, between the rotating system of x-ray source and detector. The scanning time was pre-set at 3 min with the field of view 10 mm in diameter and the chosen mode of fine quality. Other setup parameters of the Caliper Quantum FX machine include voltage: 90 kv, capture: small, CT: 200 μ A, and live: 80 μ A. The maximum resolution achievable by this setting is 20 μ m/voxel. Increasing scanning time will enhance imaging resolution; however, we believed this setting was adequate for the purpose of this study.

The resultant images were stored as a DICOM series in coronal, axial, and sagittal views. The 3D renderings (Fig. 1) presented here were created by Drishti (Australian National University, Australia) and ImageJ (National Institutes of Health, USA) software, which are open-source software.

2.4 Data Analysis

Qualitative comparison of image acquisition and soft tissue stain absorption was visualized with two commercially available software applications namely ImageJ and Drishti (both are open source). Raw DICOM data were imported as image sequences, and myocardial staining quality and tissue contrast penetration were assessed visually in three different axes (x , y , and z).

Quantitative staining agent tissue penetration is assessed with grayscale intensity. Grayscale intensity is one in which the value of each pixel in a single sample represents an amount of light, that is, it carries only intensity information. In an 8-bit image, the intensity values of 0 to 255 are displayed, with 0 being dark (no light) and 255 being white (all light). Cardiac structure comprises of various types of soft tissue, but myocardium makes up the bulk of cardiac structure and it is expected that staining gray value intensity is similar once all the hearts are completely stained. Uniform staining of myocardium is the main goal as the underlying tissue structure is uniform.

To compare iodine- and PTA-enhanced tissue imaging, a “region of interest” (ROI) as a line from epicardial to endocardial border in the mid-left ventricle (LV) free wall is chosen. In this study, left ventricular free wall in mid-LV cavity was chosen as it is the thickest section of the cardiac structure with visually differing staining agent penetration through the mid-myocardial tissue. In our study samples, the left ventricular thickness ranged from 0.9 to 1.8 mm with more than 50% of samples having LV free wall thickness of <1.5 mm. Therefore, values from 0.1 to 1.5 mm from the edge in all samples were included and values >1.5 mm LV thickness were excluded. We defined adequate staining as being when the lower quartile was at least 85% of the staining intensity at the edge.

The epicardial edge was defined as the outermost layer of the LV free wall with the highest intensity value and the values of outer most pixels lower than that were disregarded as image acquisition noise. An average of three intensity values at 0.1-mm distance interval from the epicardial edge and its ratio to the epicardial edge intensity value are analyzed. We compared median staining intensity between PTA and iodine at 0.1-mm intervals from the edge using the Mann Whitney test with correction for multiple comparisons.

3 Results

A total of 60 mouse hearts from different generations and strains were studied in two staining protocols using iodine and PTA. One PTA-stained sample was imaged at 14 days, 3 PTA-stained samples were imaged at 25 days, and 30 PTA-stained samples were imaged at 187 and 780 days. All iodine-stained samples were imaged at 3 days.

Gross examination and visual comparison of these data highlight the tremendous soft tissue enhancement achieved by the iodine staining protocol in a short time. However, distinct intensity variations are observed between stained and non-stained areas of samples in the PTA group in the first few months post-staining. Figure 1 shows the 3D tissue geometry of a mouse heart reconstructed from micro-CT images stained with PTA.

3.1 PTA-Enhanced Micro-CT Scan Images

A timeline of axial plane of a PTA-stained heart sample is shown in Fig. 2. This figure depicts the overall penetration of PTA contrast agent in the myocardial tissue. The intensity level of the gray-scale image shown increases quicker in the epicardium and endocardium than the mid-myocardium; since the intensity is related to the amount of contrast agent accumulation, this indicates the epicardium and endocardium accumulate the contrast agent faster than the myocardium. This is due to the fact that the epicardium and endocardium are in direct contact with the contrast agent and thus stain quicker than the middle section suggesting that contrast agent reached this area primarily by diffusion through the surface layers.

Figure 3 demonstrates the plot histogram of gray value intensity through the selected sample line (yellow line, ROI) from epicardial to endocardial border of the left ventricular free wall. Gray value intensity is highest in the epicardial layer then in the endocardial and mid-ventricular myocardial layers, respectively. This initially results in a biphasic histogram appearance with the higher gray values in the superficial layers and the lowest intensity in the mid-ventricular wall. As time passes, PTA penetration extends through the mid-myocardial layers and creates a more homogenous histogram.

3.2 Iodine-Enhanced Micro-CT Scan Images

On the other hand, Fig. 4 shows a 2D section of a mouse heart stained in the IKI2 1.5% in 90% ethanol solution and scanned after 72 h. It is clear that iodine diffuses through the myocardial layer faster and more evenly. The plot histogram of gray values in the sample line confirms balanced opacification of the myocardial soft tissue with IKI2.

Iodine staining was also performed on a PTA partially stained mouse heart in order to assess the CT scan image quality of superimposed staining agents. The result is illustrated in Fig. 5 with a partially stained PTA-enhanced heart after 165 days [Fig. 5(a)] and the same heart perfectly stained by IKI2 after 72 hours [Fig. 5(b)].

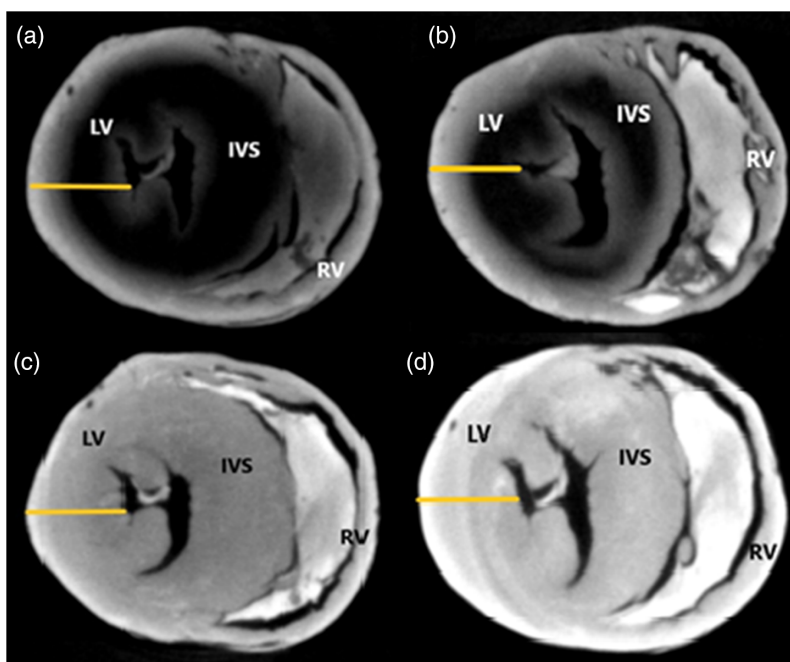


Fig. 2 The same individual mouse heart stained with PTA 1.5% in ethanol 90% and scanned in various duration post-staining: (a) 14 days, (b) 25 days, (c) 187 days, and (d) 780 days. Yellow line indicated the ROI in mid-LV free wall. LV, left ventricle; RV, right ventricle; IVS, interventricular septum.

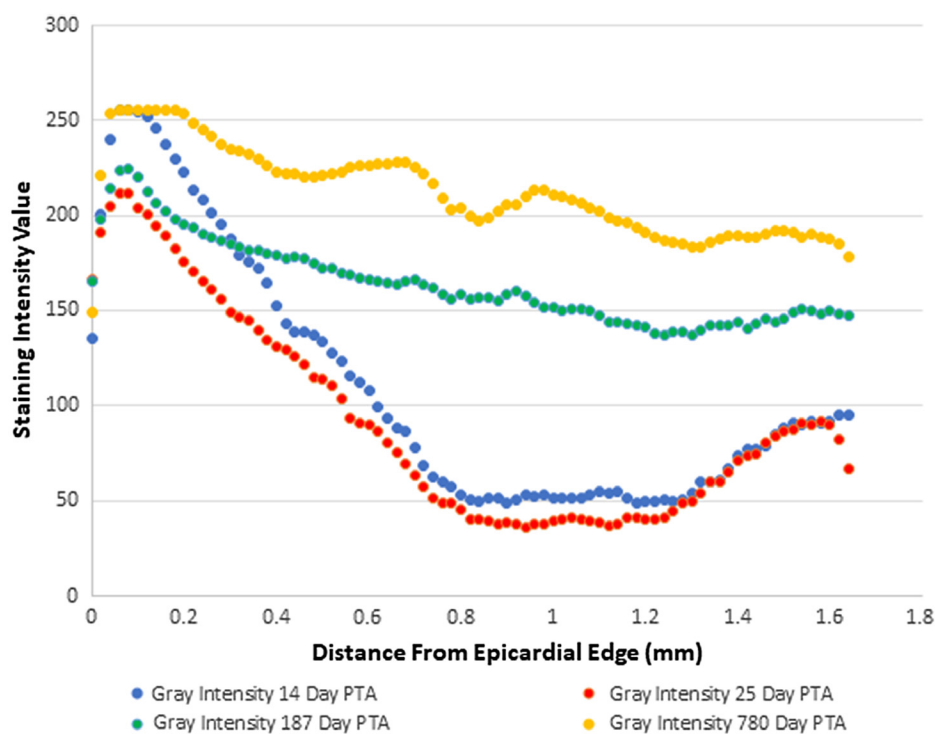


Fig. 3 PTA staining intensity value plot of ROI in heart samples shown in Fig. 2; x axis shows the distance from the epicardial edge to endocardial edge and y axis shows the staining intensity value in an 8-bit image presentation.

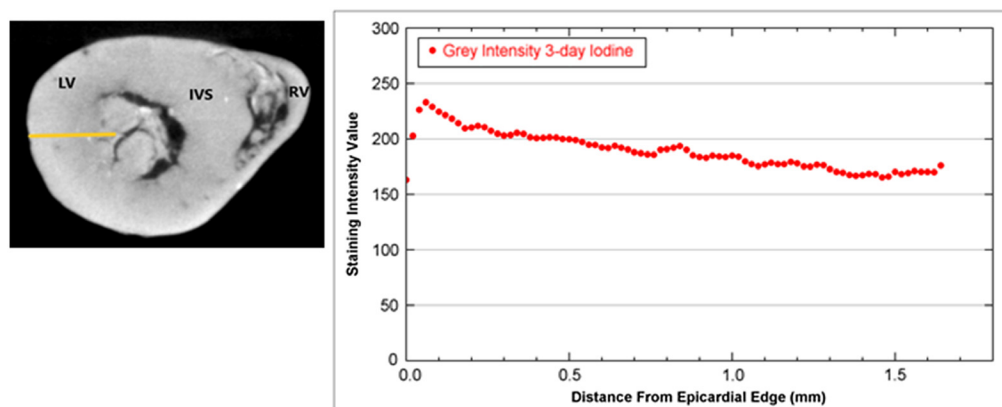


Fig. 4 A mouse heart stained with I_2kl 1.5% in 90% ethanol 3 days after staining with the staining intensity plot of the ROI (yellow line). LV, left ventricle; RV, right ventricle; IVS, interventricular septum.

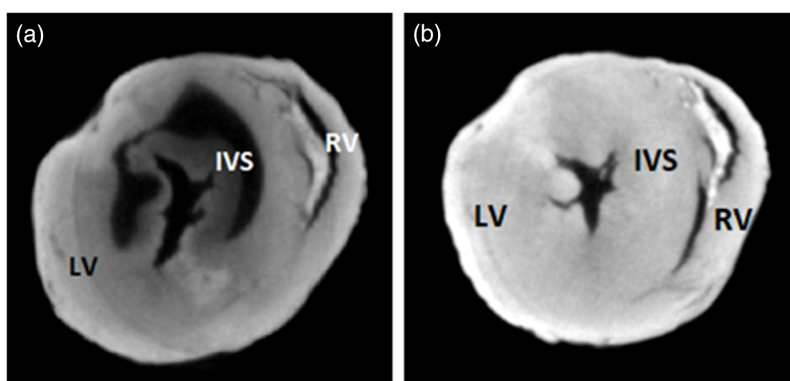


Fig. 5 (a) 165 days post-staining with PTA 1.5% in ethanol 90%. (b) Same heart stained by I_2kl 1.5% in 90% ethanol and imaged after 3 days.

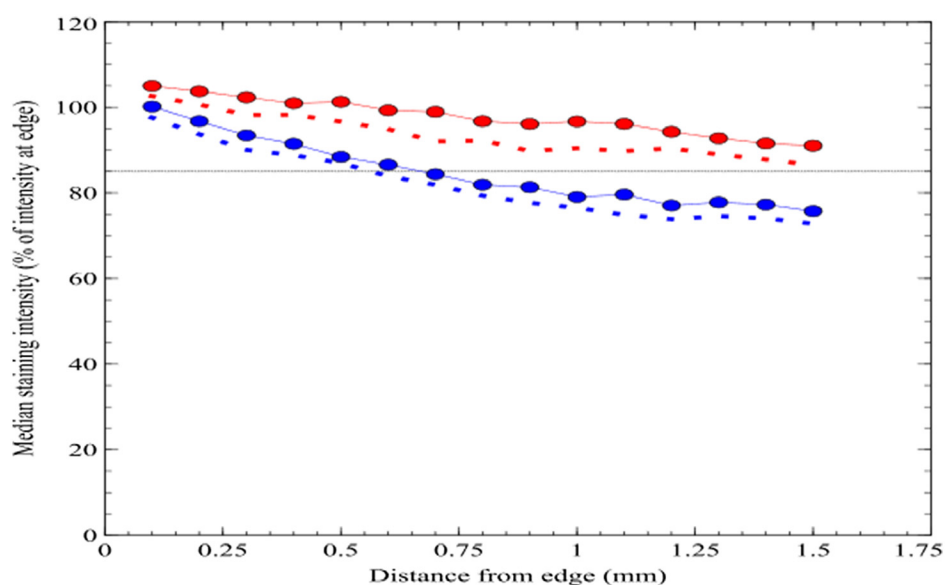


Fig. 6 Median staining intensity (as % of intensity at edge, circles) and lower 90% confidence limit (dotted lines) for staining with PTA (780 days, blue) and iodine (3 days, red).

3.3 Comparison of 3-Day Iodine Staining and 780 PTA Staining Results

To objectively compare iodine and PTA staining results, we analyzed 30 mouse hearts stained with each contrast agent, 3-day iodine staining versus 780-day PTA staining. Figure 6 depicts the median staining intensity (as percentage of epicardial edge intensity) with lower quartile for both staining agents. The 3-day iodine staining is associated with strongly higher staining intensity percentage throughout the myocardial thickness. Median staining intensity with iodine was higher than with PTA from 0.1- to 1.5-mm distance from the edge with p value <0.01 (or lower) throughout.

Moreover, iodine staining shows a homogenous pattern of diffusion with all median values up to 1.5 mm from epicardial edge above 85% of epicardial edge intensity value, whereas PTA-stained heart samples have lower than 85% of epicardial edge intensity from 0.75- to 1.5-mm distance to epicardial border (Fig. 6).

4 Discussion

In this study, we have demonstrated that non-destructive contrast-enhanced micro-CT scan technique can provide high spatial resolution 3D volume imaging of soft tissues with detailed anatomical structure. We compared two commercially available and commonly used contrast agents for visualization of *ex-vivo* small animal soft tissues. Here, we have shown that inorganic iodine staining is more effective and efficient compared to PTA in staining of post-natal mouse hearts and should be the agent of choice. Iodine allows the researcher to be able to investigate the sample in a shorter time duration.

PTA is a large inorganic polar molecule, and its binding appears to be selective for positively charged molecular groups. It binds protein compounds including fibrin, amino acids, acid glycosaminoglycans such as collagen.^{27,28} Iodine, on the other hand, is an inorganic non-polar contrast agent, which binds to lipids and carbohydrates and is about 20 times smaller than ionic PTA.^{27,29} Therefore, shorter staining time for iodine is likely due to its smaller molecule size and polarity differences.

Metscher¹³ applied similar staining methods in micro-CT scanning of chick embryonic tissues and was able to successfully demonstrate shorter staining time for both iodine 10% and PTA 3% reagents (staining time from 30 min to overnight). The significant longer duration required to stain the whole mouse heart in our study is most likely due to lower concentrations of staining agents and the different stages of development and sample size between two studies. Because of the small size and limited tissue density of embryonic tissues, contrast agent diffuses out into the tissue rapidly.³⁰ We studied post-natal whole heart mouse samples with higher tissue density, which required longer staining time depending on the agent used.

In another experiment, Degenhardt et al.³ imaged whole mouse embryos using various concentration of Lugol solution (12.5% to 25%). Saturated 25% Lugol solution was able to penetrate mouse embryos in 48 h with good soft tissue enhancement. However, this caused more tissue shrinkage and distortion as opposed to isotonic Lugol solution. Vickerton et al.³¹ also compared various I2KI concentrations from (2%, 6%, 10%, and 20%) for contrast-enhanced micro-CT scanning of murine tissues and showed significant tissue shrinkage and deformation using higher iodine concentrations. We opted to use less concentrated iodine solution (IKI2 1.5%) to minimize tissue shrinkage as we planned to compare CT scan obtained images with histological sectioning later. Also, samples stained in Degenhardt et al. study were embryonic tissues as opposed to our samples being post-natal mouse hearts.

High-resolution imaging achieved with micro-CT allows for a detailed 3D geometry, such as fine structures of blood vessels, valvular structure, and pectinate muscles in the right atrial appendage. The acquired images enabled us to segment cardiac chambers and large blood vessels and detect structural cardiac abnormalities. This image quality could be achieved using both staining mediums, as long as complete contrast penetration in soft issue has occurred.

In conclusion, using micro-CT, we were able to show that it is technically easy to do 3D visualization of the whole mouse heart with contrast staining without having to dissect and section them. Using this technique, we could acquire phenotypic information, which provides a

new, efficient way to identify cardiac anomalies. Our data demonstrated that iodine staining is easier and quicker to facilitate contrast enhancement of mouse cardiac tissue compared to PTA. Most importantly, stained samples are preserved for future histopathology and genotyping experiments.

Disclosures

Authors have no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work.

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