

*Senior Thesis on the Methodology of Formalin Fixed Paraffin Embedded
Cardiac Tissue*

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Cardiac Tissue Preservation
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ABSTRACT

This lab analyzed the effectiveness of Formalin-fixation and paraffin embedding in terms of preserving cardiac tissue. There are many ways that cardiac tissue can be tested and analyzed post mortem. The storage method of cardiac tissue can cause variation in analysis results. It is becoming important that tissue can be analyzed via mass spectrometry imaging in order to visualize the spatial density of various lipids and biomarkers in the samples. If cardiac tissue is going to be stored via formalin-fixation and paraffin embedding the sample needs to be washed in an antigen removal bath to allow for accurate quantification.

INTRODUCTION

Formalin-fixed paraffin embedded (FFPE) is a form of tissue preservation and preparation. This form of tissue preservation and preparation allows for tissues to be collected in a variety of formats and aids in the examination, experimental research, and diagnostic and drug developments. This is a common method of storing biopsies from patients in the clinical setting. Samples stored in FFPE have been found to have no significant differences between samples stored for 11-12 years versus samples stored for less than a year. This allows clinicians to store, utilize, and compare samples from the same patient very easily. Samples can also be used for further examination, experimentation, diagnosis, or drug development methodologies [1,2].

Formal-fixed paraffin embedding can be used to preserve any biopsy or whole organ tissue. This method can be used for human or animal samples. In both scenarios the tissue can be used in several application types. Usually FFPE is used in oncology, hematology, or immunology applications. It is also common that samples can be taken for comparison between healthy and unhealthy donors. One disadvantage of using FFPE is that the proteins present in the tissue are often denatured in the formaldehyde fixation

process. This crosslinking can be seen in Figure 1. This denaturing makes it nearly impossible for the antibodies developed to detect them to find the proteins. Techniques called antigen retrieval can be used to recover DNA, RNA, and other specialized heavy proteins from the sample [3,4,5].

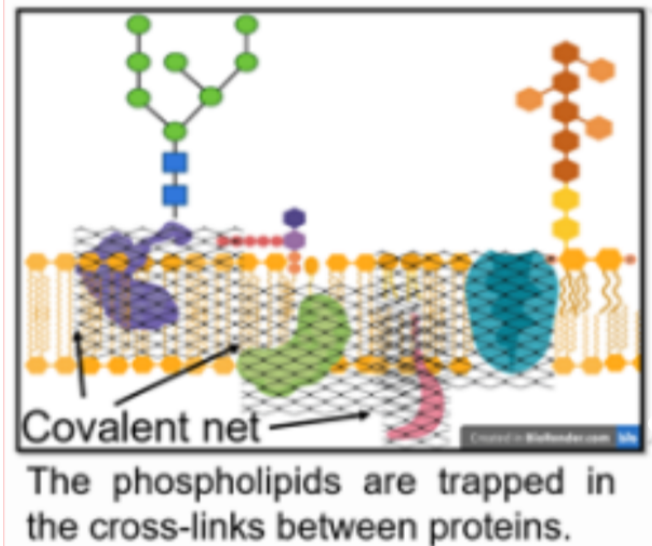


Figure 1. Shows the crosslinking of the covalent bonds formed during the paraffin embedding process. These mask the protein and lipids making them not appear on the spectrum of MALDI-MSI in most cases

FFPE methods are used as an alternative to freezing tissue. There are many advantages to using FFPE over freezing tissue. It is more cost-effective to store FFPE than frozen tissue since freezers are expensive and require maintenance and power. FFPE tissue is also much more durable than frozen tissue. Frozen tissue deteriorates at room temperature and is fragile to handle, whereas FFPE can be handled at room temperature and is not fragile due to the embedding process. Frozen tissue can often only be sampled once whereas FFPE can be used for multiple studies [6].

Since FFPE is a common form of embedding and has so many advantages, it is vital to determine a way to obtain usable Matrix-Assisted Laser Desorption Ionization - Mass Spectrometry Imaging (MALDI-MSI) images. MALDI-MSI is important because it allows the user to visualize the spatial density of lipids in a sample. MALDI-MSI is a way to visualize the molecular features of tissues

based on mass detection. A matrix solution is applied to a slide with tissue. This slide is then put into a mass spectrometer where a laser is shot at the sample. The matrix absorbs the laser wavelength and ionizes the matrix and sample. The matrix is coated in a homogenous manner that allows for accurate sensitivity and intensity data to be recorded. This data is then put into a plot that shows which masses were recorded in the highest intensities. These masses can be characterized to determine the material characteristics of the sample. In cases where the formaldehyde is not broken down the protein masses of the sample will not be read making it very challenging to identify the sample [7,8,9,10].

METHODS

For the FFPE process, the tissue sample is preserved by cross-linking proteins and then it is embedded in a paraffin wax block that allows for ease in cutting slices appropriate in size to mount on a microscope slide for examination. For this lab the tissues were FFPE by researchers at Purdue University. For their methodology, short axis cardiac infarction samples were acquired from murine models. Immediately after excision, the tissue is immersed in a solution of 10% neutral-buffered formaldehyde. This immersion process occurs for 24 hours. The tissue hardens during this process. The tissue dehydrated using 70% ethanol washes. After these washes the sample is embedded into paraffin. It is important that the sample is properly dehydrated and given the 24 hours to be fixed otherwise the tissue will not be preserved. These samples were then sent by mail to the University of Tennessee, where various preparation methods were used to prepare the sample for MALDI-MSI [10,11].

After the samples arrived at University of Tennessee, they were then cut at 5 μ m using a microtome. A pipet tip, paintbrush, and flat tipped tweezers were used to grab the rolled sample from the top with the roll facing in toward the tweezers. The rolled side of the samples was then placed in a 40°C water bath at a 60° angle to the water to allow the sample to unroll. A slide was then held under the water and under the sample and then lifted up, so the sample sat on the slide. Slides were placed in a variety of environments to determine the best

drying method. These included a 65°C oven for an hour and a half, a -80°C freezer for 24 hours, a 37°C oven for 24 hours, and a desiccator for 24 hours. For MALDI-MSI analysis several methods were used to treat the slides prior to imaging. These included applying matrix directly, washing twice with xylene for 3 minutes, and then twice with water for 3 minutes, and washing twice with xylene for 3 minutes, and then ammonium formate three times, for 30 seconds each time which is a type of antigen retrieval wash. We also tried the same method of the xylene and ammonium formate wash but with citric acid instead of ammonium formate. After washing the slides, the samples were dried in a desiccator before applying matrix. Two matrices were used: 9-Aminoacridine Hydrochloride Monohydrate in negative mode and alpha-Cyano-4-hydroxycinnamic acid in positive mode. This methodology order can be seen in Figure 2 [12,13,14,15].



Figure 2. Step methodology of microtome, the three potential drying methods and the two potential washing methods to get a finalized slide.

Once matrices were applied, samples were placed in the mass spectrometer. Both positive and negative mode were both attempted to determine if there was a specific mode that provided better spectrum data. Mass and intensity data was acquired simultaneously. This data was then

processed in HDImaging software to compare the various peaks and peak intensities present.

RESULTS AND DISCUSSION

FFPE was effective in preserving the cardiac tissue samples. Samples were best stored after sectioning in an oven at 37°C for 24 hours or until dry. These samples had the least amount of bubbles and folds in the wax compared to other post-cutting storage methods. Samples without folds or bubbles were able to have the entire sample read by the mass spectrometer. It was also determined an antigen removal wash revealed the most accurate mass and intensities. Performing MALDI-MSI without using antigen retrieval provided only one peak. Samples were still able to be detected even without the wash. Figure 3 shows that after antigen retrieval in both positive and negative mode ions were detected in the cardiac tissue using MALDI- MSI.

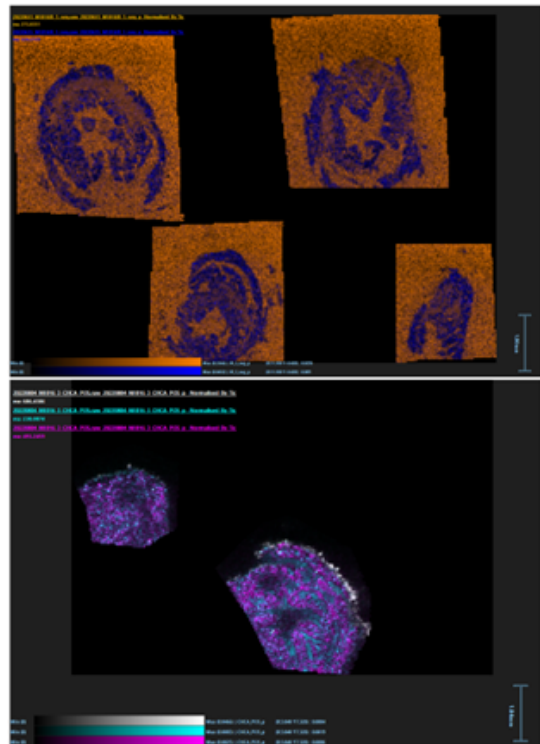


Figure 3. The top image shows FFPE cardiac tissue after MALDI-MSI in negative mode. The bottom image shows FFPE cardiac tissue after MALDI-MSI in positive mode.

Distinct lipid peaks were found at high intensities in washed samples. In positive mode with DHB matrix applied on the same myocardial infarction cardiac sample, one slide was analyzed with no wash and another was analyzed after antigen washing technique. The spectrum data from this can be seen in figure 4 [16,17,18].

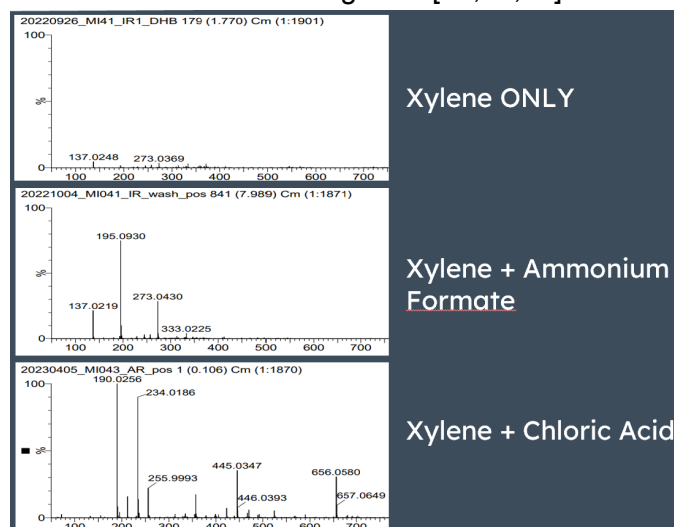


Figure 4. Mass spectrum of sample MI041 with DHB matrix in positive ion mode. The top spectrum is after no antigen retrieval wash, the middle spectrum is following the antigen removal wash with ammonium formate, and the bottom is following the antigen removal wash with chloric acid.

Overall, it was shown that the MADI-MSI can be used to characterize short-axis sections of FFPE cardiac tissue. It is much more useful after antigen wash. Figure 5 shows the image of the tissue at a known cardiac biomarker peak of 299.13 m/z. It is evident that the fissure that went through antigen washing, seen on top, had a higher intensity.

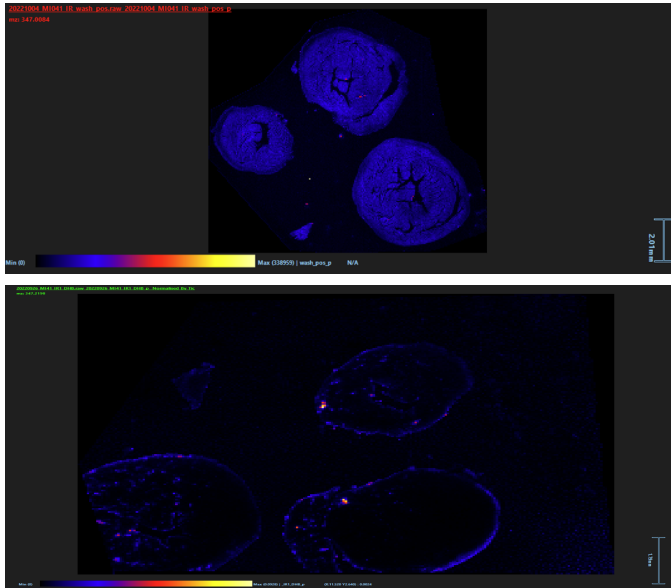


Figure 5. Top image is peak 299.13 m/z on a cardiac sample imaged with DHB in positive mode after antigen washing. The bottom image is peak 268.80 on a cardiac sample imaged with 9AA in negative mode after antigen washing.

It was also observed that negative mode mass spectrometry did not present as many peaks and had lower peak intensity at expected cardiac biomarker peaks. Figure 6 demonstrates this technique.

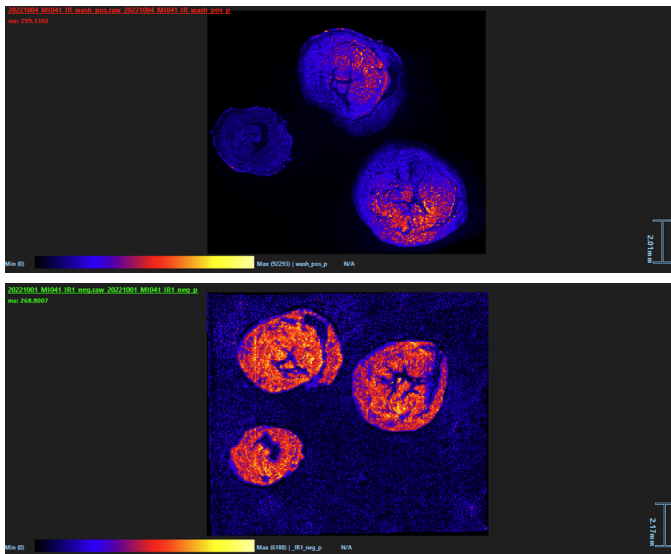


Figure 6. The top image is peak 347.008 m/z on a cardiac sample imaged with DHB in positive mode after antigen wash. The bottom image is peak 347.21 with DHB in positive mode with no antigen washing.

The top sample imaged in positive mode had a higher intensity than the negative mode. It also had higher intensities in the tissue regions as

seen in the images. The top image has a completely black background, meaning the signal was only picked up by tissue regions. The negative mode picked up signals on areas without tissue, demonstrating that the negative mode was not as accurate.

These results all demonstrate that tissue should be prepared in a 37°C oven for 24 hours or until dry, then antigen washed prior to matrix application, and then MALDI-MSI is performed in positive mode to get the high intensity mass peaks with the most accuracy [19,20].

CONCLUSION

It is necessary to wash the FFPE samples before performing MALDI-MSI in order to break the cross links of the formalin to get accurate mass results. Images retrieved from the MALDI-MSI analysis demonstrated clear areas of varying ions which represents the effectiveness of identifying different areas of cardiac tissue. In the future, other washing techniques and matrices could be used in order to better determine which method is best for cardiac tissue preserved in this way. This washing and drying method also took several hours and lots of expensive equipment and chemicals to perform. This process could be improved to shorten the time of the process or make it more cost effective.

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