Focused Ultrasound Extraction (FUSE) for Formalin-Fixed, Paraffin Embedded (FFPE) DNA

Isabelle Mehochko

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Biomedical Engineering

Eli Vlaisavljevich
Hal Holmes
Irving Coy-Allen

May 3, 2023
Blacksburg, VA

Keywords: Focused Ultrasound, DNA, DNA Extraction, FFPE

Focused Ultrasound Extraction (FUSE) for Formalin-Fixed, Paraffin Embedded (FFPE) DNA Extraction

Isabelle Mehochko

ABSTRACT

Formalin-fixed, paraffin embedded (FFPE) tissue is the most abundant, accessible, and versatile tissue sample type available for genetic research and clinical applications. However, FFPE DNA extraction presents unique challenges and requires lengthy incubation periods, which can be impractical for certain applications. Here, we propose the use of focused ultrasound extraction (FUSE) technology for improved DNA extraction from FFPE tissue. FUSE generates a dense bubble cloud of acoustic cavitation capable of ablating tissue into an acellular lysate. FUSE treatment was applied to de-paraffinized porcine pancreas FFPE scrolls, followed by heated incubation for formaldehyde-induced DNA-protein crosslink reversal. When applied for 30 minutes, FUSE was found to successfully extract DNA from FFPE tissue as defined by increased DNA yield and improved purity ratios compared to conventional methods. DNA extracted via FUSE showed comparable fragmentation to conventional methods, and three out of four samples successfully amplified via PCR, indicating suitability for downstream analysis. These findings suggest that FUSE has the potential to increase the efficiency and effectiveness of DNA extraction from FFPE tissue. Further development and optimization of this protocol could develop a streamlined, easy to use extraction method that would simplify FFPE DNA extraction methods and address the primary time constraints which currently make FFPE DNA extraction time-consuming and impracticable for high-throughput applications.

Focused Ultrasound Extraction (FUSE) for Formalin-Fixed, Paraffin Embedded (FFPE) DNA Extraction

Isabelle Mehochko

GENERAL AUDIENCE ABSTRACT

Formalin-fixed, paraffin embedding (FFPE) has historically been the most popular method of biological tissue preservation, as it allows tissue to remain shelf stable for decades. As such, FFPE tissue is the most abundant, accessible, and versatile tissue sample type available for genetic research applications. Here, we propose the use of focused ultrasound extraction (FUSE) technology for improved DNA extraction from FFPE tissue. FUSE treatment applies rapid, focused ultrasound waves to tissue, resulting in the mechanical breakdown of cells and subsequent release of DNA. FUSE treatment was applied to pig pancreatic FFPE samples. When applied for 30 minutes, FUSE was found to successfully extract DNA from FFPE tissue as defined by increased DNA yield and improved purity compared to conventional methods. Three out of four DNA samples extracted via FUSE were successfully amplified, and DNA fragment lengths were comparable between FUSE and conventional methods, showing that FUSE did not fragment DNA beyond useful fragment lengths. These findings suggest that FUSE has the potential to increase the efficiency and effectiveness of DNA extraction from FFPE tissue. Further development and optimization of this protocol could develop a streamlined, easy to use extraction method that would simplify FFPE DNA extraction methods and address the primary time constraints which currently make FFPE DNA extraction time-consuming and impracticable for high-throughput applications.

ACKNOWLEDGEMENTS

There are so many people who have offered invaluable support throughout my time in this lab and on this project. First and foremost, I'd like to thank Eli Vlaisavljevich for offering me a position in the lab as an undergrad despite being in the middle of a pandemic, and especially for being willing to be my graduate advisor at such late notice. Thank you for being such a patient and encouraging mentor as I've found my way on this project, I could not have done this without your support and insight.

My biggest source of day-to-day support has been Alexia Stettinius, whose knowledge and experience I have leaned on at every turn. I can't express how thankful I am for your mentorship, insights, and constant willingness to help. Hal Holmes has also been an invaluable contributor, thank you for sharing your wealth of knowledge. I think my biggest takeaway from this project came from watching Alexia, Hal, and Eli's optimism and problem solving at work; I was constantly coming to them feeling like I had hit a dead end, and they consistently came back with a myriad of suggestions and solutions to explore, demonstrating both a deep knowledge of the field and a creative problem-solving mindset which I strive to develop and emulate.

Dr. Coy Allen and his team have been tremendously helpful, particularly Imran who patiently handled my frequent questions and sample requests and served as a crucial source of expertise on genetic concepts. I'd also like to thank Margaret Nagai-Singer and Tamalika Paul for their help with sample acquisition and dealing with the constantly changing needs of the project.

The rest of the FUSE team has also been wonderfully supportive, especially Qian Zhang, who helped me run gels on what seemed like an insane number of samples.

Beyond technical support, I want to thank my family and friends for their infinite support. I'd like to thank my parents for encouraging me to stay at Tech and pursue my master's even though it was such a last-minute decision. Thank you to my mom for our long phone calls and shared venting, I love and appreciate you so much. My siblings and the rest of my family have been wonderful, even if they don't realize it; I'd particularly like to thank Seraphine for helping me edit my abstract. And of course, thank you to all my friends who constantly listened to my project-related woes and kept me sane. I know my favorite memories from this year will be board game nights with Noah, Kailee, and Ryan, line dancing with Britt and Ty, and of course spending hours talking, laughing, and exploring Tennessee with Caitlin. Thank you for making this year a great one, I can't wait to see what everyone does next and the impact you will make.

Table of Contents

Chapter 1: Introduction

1.1 FFPE DNA Extraction Background

As genetic technology continues to expand and evolve, we have seen a greater emphasis placed on genetic biomarker discovery and validation, personalized medicine, and disease diagnosis via genetic analysis [1]. Fresh-frozen tissue has traditionally been considered ideal for these applications, but there are often substantial barriers making it difficult for researchers to access tissue directly from patients. The process of obtaining tissue is long and requires immense coordination and cooperation by all patients, surgeons, and pathologists involved, as well as being heavily regulated by the IRB to help ensure patient safety [2]. This becomes particularly difficult when investigating rare diseases, where these barriers are confounded by small patient populations, variable timeframes for disease progression, and incomplete knowledge of the disease pathophysiology [3].

Many researchers are turning to formalin-fixed, paraffin embedded (FFPE) tissue, as it offers a solution to many of these barriers to fresh-frozen tissue access. For decades, it has been standard procedure to fix tissue in formalin to preserve its chemical state, then embed it in paraffin to fix it mechanically in space. This facilitates easy analysis and assessment by pathologists and allows the tissue to be shelf stable for years [4]. Thus, many hospitals and biobanks have an abundance of FFPE tissue currently available, making it easily accessible and often much cheaper than its fresh-frozen counterparts. Many of these samples can be de-identified, allowing researchers to streamline or even bypass the IRB approval process. In the US, these specimens must be retained for 10 years following diagnosis, meaning that long-term, retrospective data may be available. Additionally, the vast selection of samples and the long-term nature of their storage increases researchers' chances of finding specimens representative of rare diseases [2]. Working with FFPE

tissue also has significant implications for low- and middle-income countries (LMICs), where FFPE is often the only tissue available due to the impracticality of storing and transporting fresh-frozen tissue samples [5]. The ability to efficiently extract DNA from FFPE tissue could be an access point for personalized medicine in LMICs.

Despite its abundance and ease of access, FFPE tissue presents unique challenges for DNA extraction. The formaldehyde in formalin is known to form crosslinks between proteins and nucleic acids, which must be reversed to obtain functional nucleic acid and minimize DNA degradation [2], [6]. It is well established that heat is required to reverse formaldehyde-induced crosslinks [4], [7]. A majority of today's commercially available FFPE DNA extraction protocols reverse these crosslinks via a heated incubation period, ranging from 2 hours to overnight [8], [9].

1.2 FUSE Background

In previous studies, our group has developed Focused Ultrasound Extraction (FUSE) technology. FUSE has been shown to extract more DNA in less time than conventional methods for various tissue types, including salmon [10], leaf [11], and timber tissue [12]. FUSE is a new application of the focused ultrasound technology used in histotripsy, an emerging non-invasive medical therapy being developed for clinical tissue ablation applications.

In FUSE, a focused ultrasound transducer sends high pressure, low duty cycle ultrasound pulses into the tissue. The convergence of these pulses at the transducer's focal point causes a significant drop in pressure, generating a dense bubble cloud. Pulses are sent at a high pulse repetition frequency (PRF), causing rapid, repeated expansion and collapse of these bubble clouds; this places the tissue in the focal region under high stress, resulting in mechanical cell lysis [10]. The

released DNA can be purified for downstream analysis from the resulting acellular lysate [10], [11].

1.3 Hypothesis

Here, we propose the application of this FUSE technology towards FFPE DNA extraction. It is hypothesized that FUSE can be used to extract nucleic acids of sufficient quality for downstream analysis from FFPE tissue. The long-term vision of this project is to develop and optimize a protocol in which FUSE is used to lyse FFPE tissue, which can be followed by DNA purification and various downstream DNA analysis techniques. This study aims to establish initial feasibility of FUSE for this application, which can lead to further protocol optimization in the future. As a secondary goal, we hope to gain insights regarding what niche FUSE may be able to fill in the future by potentially enhancing speed, efficacy, or quality of DNA yield from FFPE tissue, which can provide guidance for future investigations and optimizations. We further hypothesize that FUSE can achieve DNA extraction in less time than conventional methods and allow for a more streamlined process for FFPE DNA extraction.

Chapter 2: FUSE Parameter Identification for FFPE Applications

2.1 Introduction

Previous studies have established how FUSE can improve the efficacy and efficiency of DNA extraction from various tissue types [10]–[12]. Here, we extend the application of FUSE to FFPE DNA extraction, with a vision of FUSE being utilized for medical research and clinical applications. FFPE tissue is widely used for various research and clinical purposes, including biomarker discovery and validation, personalized medicine, and other diagnostic applications. This investigation presents a novel approach to addressing challenges associated with DNA extraction from FFPE tissue.

In this chapter, initial FUSE experiments were conducted to test the feasibility of FUSE and inform which acoustic parameters should be investigated in subsequent studies. As outlined in prior FUSE experiments, common parameters for FUSE include transducer frequency, peak negative pressure, pulse repetition frequency (PRF), and treatment time.

Transducer frequency refers to the ultrasound frequency of each pulse and is dictated by the individual elements which make up the transducer. To ensure consistency with prior FUSE investigations, a 500kHz transducer was employed in this study to match the transducers used in previous studies [10], [11]. Cavitation generation requires a focal peak negative pressure which exceeds the medium's intrinsic threshold. Given that the mammalian tissue employed in this study is expected to be mechanically similar to the salmon tissue used in a previous study, a peak negative pressure of ~40MPa was selected to align with the values used in prior salmon investigations [10].

By adjusting the treatment time and PRF, we will systematically investigate the effects of FUSE dose on DNA yield. One method of minimizing treatment time while maintaining FUSE treatment dose would be to increase the PRF. However, higher PRFs come with additional implications and reduced bubble cloud predictability which require careful consideration and attention. Higher PRFs are likely to result in shielding effects, whereby residual bubbles from one pulse seed cavitation in the next pulse. By seeding cavitation in front of the sample instead of on the sample, shielding causes a decrease in the peak negative pressure experienced by the tissue, reducing ablation efficiency [13]. Thus, PRF and treatment time must be appropriately balanced to achieve effective and efficient cell lysis.

The primary vision for this project is for FUSE to improve the cell lysis portion of the FFPE DNA extraction process. In many protocols on the market today, cell lysis and formaldehyde-induced crosslink reversal is achieved concurrently via the lengthy heated incubation period. Here, we aim to achieve cell lysis via FUSE, but it is unknown whether FUSE can also reverse these crosslinks. As such, FUSE will be investigated with and without a heated incubation period to determine whether the heated incubation is necessary for adequate crosslink reversal and DNA extraction.

The goal of this parameter exploration is to determine baseline FUSE and incubation parameters that consistently yield positive results and identify parameters which warrant a more in-depth investigation in the following sections.

2.2 Materials & Methods

2.2.1 Samples

Each sample is a 50μm thick scroll taken from Pan02 mouse pancreatic tumor FFPE blocks. Tissue was fixed in 10% neutral buffered formalin for 24 hours before being embedded in paraffin.

Samples were stored under ambient conditions for roughly 2.5 years prior to utilization in this study. Samples were assigned to treatment groups randomly.

2.2.2 QIAGEN QIAamp FFPE DNA Extraction Kit

The QIAGEN QIAamp DNA FFPE Tissue kit was used for this study. For control samples, the QIAamp protocol was followed as recommended by the manufacturer. For FUSE treated samples, the QIAamp protocol was modified as follows: a FUSE treatment period was added prior to the heated incubation, FUSE groups were treated in 2x lysis buffer (Proteinase K, Buffer ATL, and Buffer AL) volume, and heated incubation was either shortened or eliminated. The general protocols can be seen in Figure 2-1.

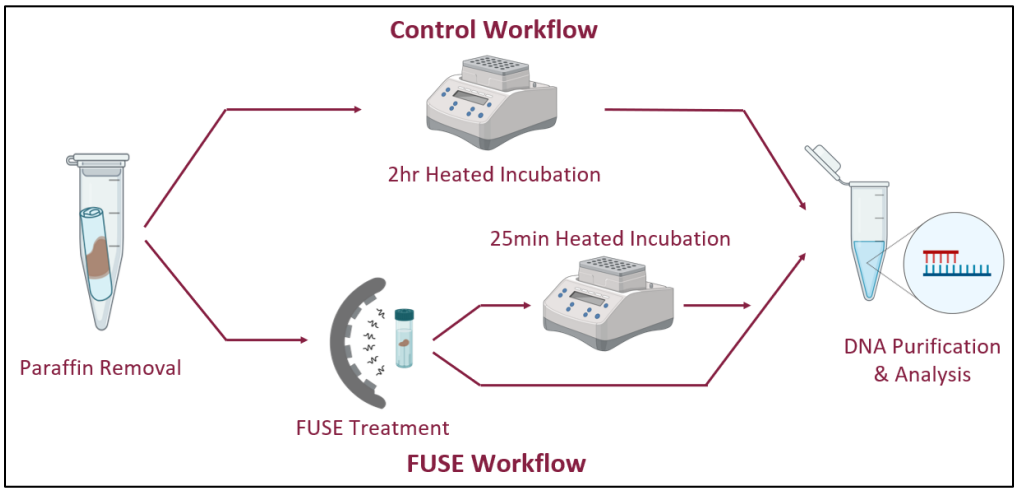

Figure 2-1: Experimental workflow for Control (top track) FUSE (bottom track)

2.2.3 Sample Preparation and Sample Holder

Each sample underwent a paraffin removal procedure as outlined by the QIAGEN QIAamp DNA FFPE Tissue protocol. This involved dissolving the paraffin with xylene, removing the bulk xylene, washing with EtOH to remove excess xylene, removing the bulk EtOH, and finally

incubating the samples uncovered at 35°C until all excess EtOH had evaporated (roughly 10-15 minutes).

After paraffin was removed and samples were resuspended, samples in FUSE treatment groups were transferred out of the 1.5 microcentrifuge tubes and into a treatment tube for FUSE treatment. Treatment tubes were 3.5cm in length of clear Tygon PVC, chosen for its acoustic permeability which caused minimal attenuation of ultrasound waves (1/4in. inner diameter, 3/8in. outer diameter; Tygon PVC E-1000, McMaster-Carr 5229K58). These tubes were secured via a custom fixture attached to a motorized positioning system, which allowed for precise placement of the sample directly into the FUSE treatment focal zone. The FUSE treatment tubes and sample holder can be seen in Figure 2-2B, and the custom fixture can be seen in Figure 2-2C.

2.2.4 FUSE Pulse Generation

All FUSE treatment groups in this study were treated with a custom-made 32-element 500kHz ultrasound transducer with a geometric focal point 75mm from its center, an aperture size of 150mm, and an effective f-number of 0.58 [14]. This transducer was driven by a custom high-voltage pulser connected to a field-programmable gate array (FPGA) board (Altera DE0-Nano Terasic Technology, Dover, DE, USA), which generated short single cycle ultrasound pulses for histotripsy applications. A schematic of this setup can be seen in Figure 2-2A.

The acoustic pressure output of the transducer was measured with a custom-built fiber-optic probe hydrophone (FOPH), which had been calibrated with a reference hydrophone (Onda HNR-0500) [15]. These acoustic pressure measurements were performed in degassed water at the focal point of the transducer, which was located using a 3D beam scan. Because cavitation at the hydrophone's fiber tip would disallow pressure measurements, acoustic output was only measured at pressures

below the cavitation threshold. Thus, the focal pressures of each individual element were measured and summated to estimate higher pressures induced by the entire transducer. An estimated peak negative pressure of 40 MPa was applied to each sample treated with FUSE.

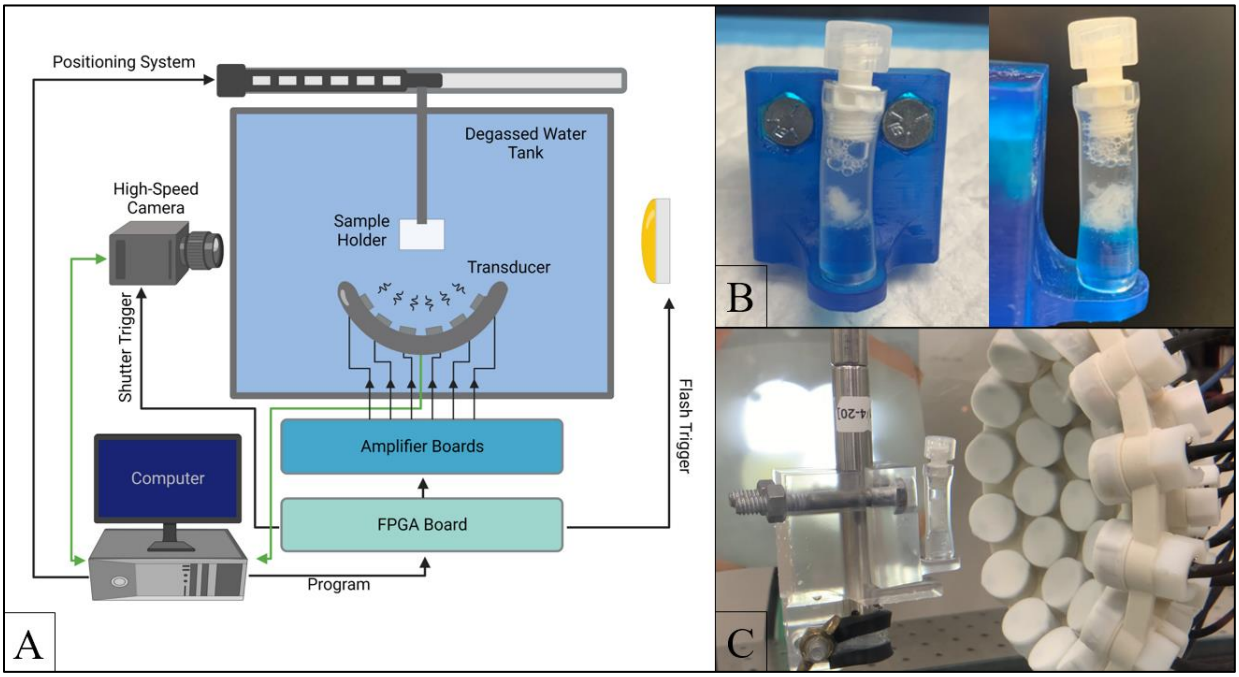

Figure 2-2: FUSE treatment setup. (A) shows a birds-eye schematic of how the robotic positioning system holds the sample in the focal zone of the transducer, which is driven by the FPGA board and amplifier. (B) shows the FFPE sample (post paraffin removal) suspended in lysis buffer in the treatment tube and custom sample holder. (C) shows the sample being held in the transducer's focal zone during FUSE treatment.

2.2.5 Treatment Parameters

The first parameters investigated were FUSE treatment time, PRF, and incubation. Treatment times of 3, 5, and 15 minutes were investigated at PRFs of 500Hz and 1kHz. Because heat is the most common method of formaldehyde-induced crosslink reversal, these treatment groups were repeated both with and without a 25-minute 90°C incubation period following FUSE treatment [4], [7].

The next set of experiments took a closer look at PRF to determine if there is a strong PRF effect. PRFs of 250Hz, 500Hz, and 1kHz were investigated, and FUSE dose was controlled at 450,000 pulses (corresponding to treatment times of 30, 15, and 7.5 minutes, respectively). Samples underwent a 25-minute 90°C incubation period following FUSE treatment.

2.2.6 DNA Analysis

All samples were analyzed with a Nanodrop One (ThermoFisher, Waltham, MA, USA) spectrophotometer. DNA yield was reported as the quantity of DNA released in ng per µL of elution buffer, as measured by the NanoDrop.

2.3 Results & Discussion

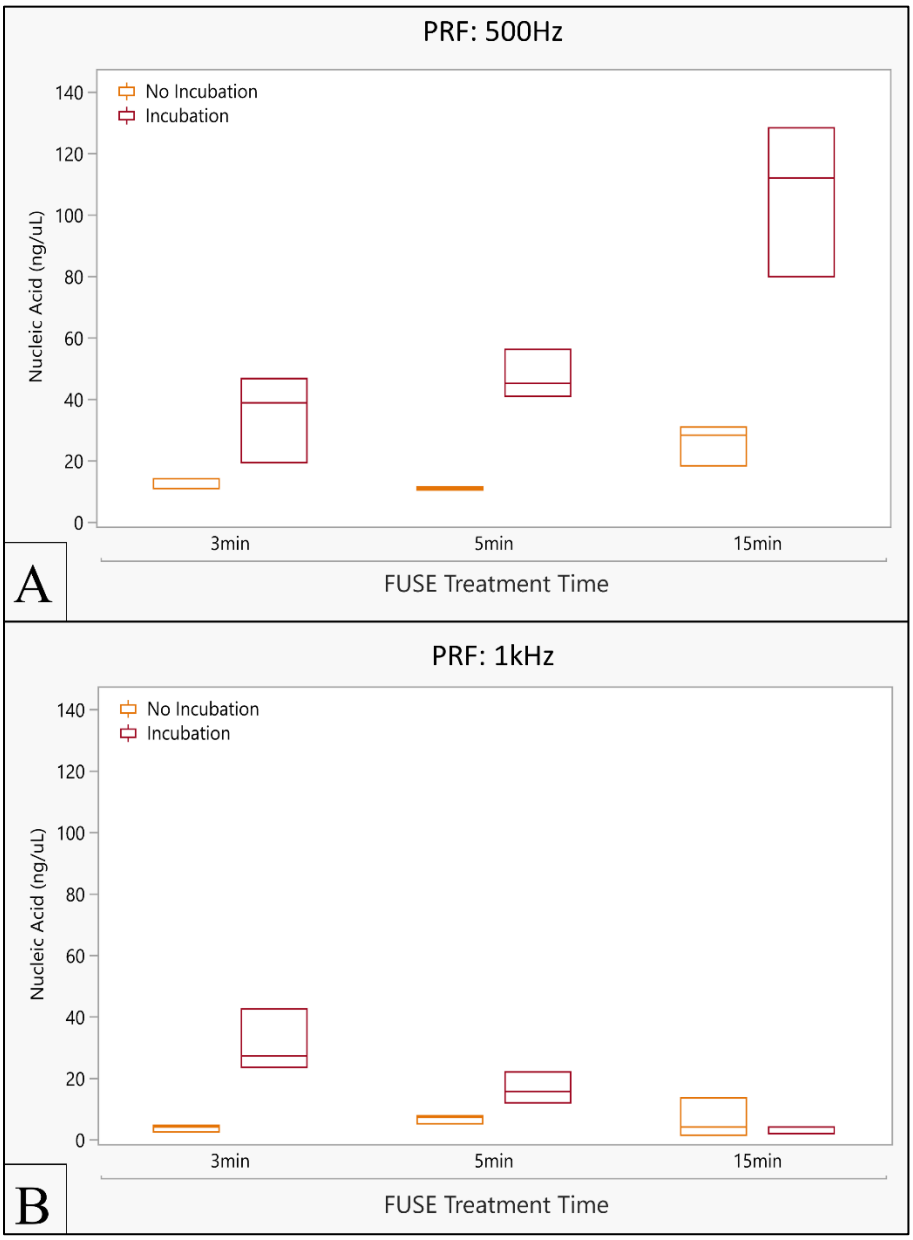

Figure 2-3 DNA yield for FUSE treatment times of 3, 5, and 15 minutes, with and without incubation (25 minutes at 90C), at PRFs of 500Hz (A) and 1kHz (B); n=3 for all groups.

The 500Hz trials (figure 2-3A) show DNA yield consistently increasing with FUSE treatment time and with the addition of a heated incubation period. The 1kHz trials (figure 2-3B), however, show only a slight increase in DNA yield with treatment time without incubation, and the incubation groups experience a decrease in DNA yield as treatment time is increased. This is an unexpected

result, given that doubling the PRF also doubles the dose, which is expected to lead to more comprehensive cell lysis and thus increased DNA yield. This could be due to the limitations of high PRFs listed above, such as shielding effects and pre-focal cavitation. This could also be an instance of overtreating, which could cause DNA degradation or breakdown into fragments which are too short to be read by the NanoDrop. However, prior literature suggests that NanoDrop spectrophotometry can accurately measure DNA fragments as small as 150 base pairs (bps) [16]; thus, the sample fragmentation would have to be extremely drastic, substantially more so than observed in previous studies, to cause such a reduction in measured DNA yield [11].

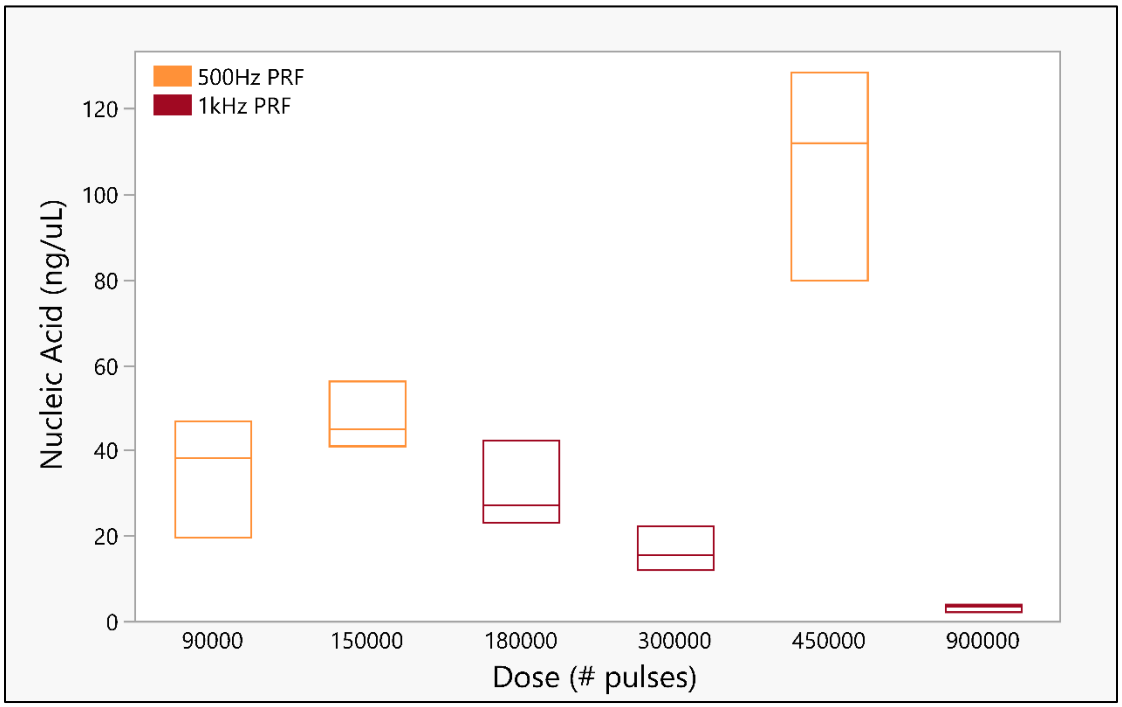

Figure 2-4 DNA yield graphed against dose (dose is treated ordinally for ease of interpretation), for various FUSE treatment times and PRFs, with incubation; n=3 for all groups.

To better visualize and comprehend this unexpected result, the data has been rearranged in figure 2-4 such that dose is on the x-axis and only the incubation groups are shown. This view suggests that there is no main dose effect, rather that there may be some interaction effect between dose,

PRF, and incubation. To further investigate this, the next set of experiments controlled for dose to enable for a more in-depth analysis of any PRF effect.

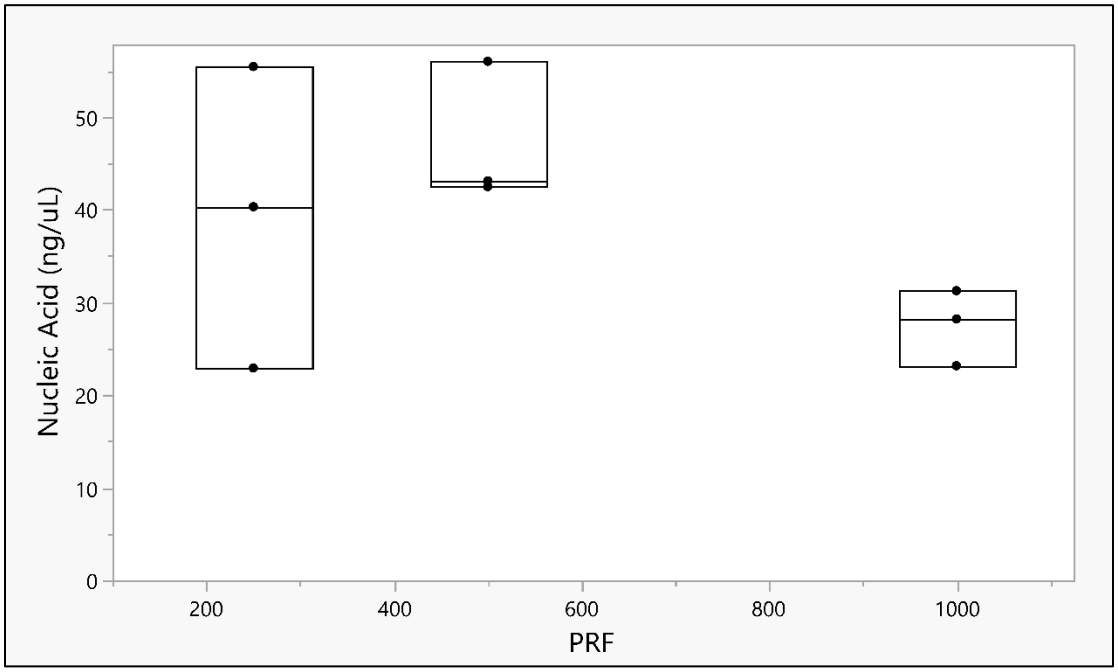

Figure 2-5: DNA yield for FUSE dose of 450,000 pulses at PRFs of 250Hz, 500Hz, and 1kHz, with incubation; n=3 for all groups.

When controlling for dose (figure 2-5), 1kHz PRF appears to yield slightly less DNA than 500Hz. However, there is still not a strong or consistent trend between PRF and DNA yield. The cause of this reduction in DNA yield at 1kHz is still unclear, as overtreatment and shielding are both feasible causes. This deserves further investigation as this trend could have significant implications for FUSE, particularly if the cause is found to be overtreatment.

2.4 Conclusion

Based on the results shown in Figure 2-3, it was determined that including the 25-minute heated incubation period greatly improves DNA yield, and thus incubation was included in all subsequent experiments. Additionally, Figure 2-3 suggests that a PRF of 500 Hz may be preferable to 1 kHz for this application, which is corroborated by the results of the PRF experiments shown in Figure

2-5. Thus, a PRF of 500Hz was used for the remainder of this study. Lastly, there appears to be a positive correlation between FUSE treatment time and DNA yield at a 500Hz PRF, seen in Figure 2-3. As such, treatment times of 15 minutes and 30 minutes were chosen for investigation in the reminder of this study. The results of these initial parameter exploration trials are encouraging and suggest the viability of employing FUSE technology to extract DNA from FFPE tissue. Our results here demonstrate that FUSE can be utilized to retrieve 50% of the DNA obtained by conventional methods in less than half the time. Additionally, these experiments have provided a valuable baseline for selecting the appropriate parameters in further trials.

Chapter 3: Efficacy of FUSE for FFPE Applications

3.1 Introduction

There are many barriers to FFPE DNA extraction: paraffin must be removed, the tissue must be emulsified and cells lysed to physically release the DNA, and the crosslinks must be reversed in order to chemically release the DNA. In previous studies, FUSE has been shown capable of breaking down various tissue types to physically release DNA [10][11][12]. Additionally, the previous chapter established baseline FUSE parameters which are capable of extracting DNA from FFPE tissue. Thus, it is hypothesized that FUSE can effectively and efficiently break down FFPE tissue to physically release the DNA.

The presence of the crosslinks is an area of novelty in this study, as there is no precedent indicating if and how FUSE will affect these crosslinks. Many interactions may occur between DNA and formaldehyde upon tissue fixation, with the most common reactions resulting in aminal linkages and hemiaminal thioether linkages [7], [17]. These crosslinks are commonly reversed by heat, often in combination with proteinase K treatment. However, the reported optimal incubation protocol varies vastly between sources. Reviewing literature in this area reveals claims of suitable crosslink-reversal temperatures ranging from 55°C to 95°C [7], [17]–[19]. This range is likely due to protocol and specimen differences, as the substantial diversity between FFPE specimens makes it impossible for one protocol to be optimal for all specimen types and FFPE preparation techniques [8], [20]. Because our primary objective is to determine whether FUSE has a direct effect on DNA yield, only a few incubation options will be investigated: the full 2-hour incubation period recommended in the QIAGEN QIAamp DNA FFPE Tissue protocol (1 hour at 56°C followed by 1 hour at 90°C), and a shortened 90°C 25-minute incubation period. These incubation options were chosen to explore multiple potential applications of FUSE within this field. It is

possible that FUSE could be used to achieve satisfactory DNA yield in a shorter amount of time, or that FUSE could be added to the existing 2-hour protocol to achieve enhanced DNA yield and/or quality. By exploring both possibilities, we hope to gain a better understanding of how this technology could fit into existing protocols and its potential for future applications.

Here, we delve deeper into the efficacy and potential applications of FUSE for FFPE extraction. While the previous chapter explored initial feasibility and parameter planning, this chapter aims to identify whether FUSE treatment significantly influences DNA yield, and how treatment time and/or incubation may play a role. To achieve this, samples which have been lysed with FUSE will be directly compared to a control group which was processed without FUSE. Following the cell lysis period, each sample will undergo a heated incubation period to facilitate crosslink reversal. Finally, all samples will undergo DNA purification and analysis.

3.2 Materials & Methods

3.2.1 Samples

Sample are 50µm thick scrolls taken from porcine pancreatic FFPE blocks. Pancreases were taken from two large (130lbs) pigs, and three tissue sections were taken from each pancreas, resulting in a total of six FFPE blocks. Tissue sections were fixed in 10% neutral buffered formalin for 24 hours before being embedded in paraffin. Samples were stored under ambient conditions for roughly 2.5 years prior to utilization in this study. Each treatment group was designated three samples from pig 1, and three samples from pig 2.

3.2.2 QIAGEN QIAamp FFPE DNA Extraction Kit

The QIAGEN QIAamp DNA FFPE Tissue kit was used for this study. For control samples, the QIAamp protocol was followed as recommended by the manufacturer, with some samples receiving a shortened incubation time (detailed in the *Treatment Groups* section). For FUSE treated samples, the QIAamp protocol was modified as follows: a FUSE treatment period was added prior to the heated incubation, FUSE groups were treated in 2x lysis buffer (Proteinase K, Buffer ATL, and Buffer AL) volume, and various heated incubation options were investigated. The modified protocols can be seen in Figure 3-1.

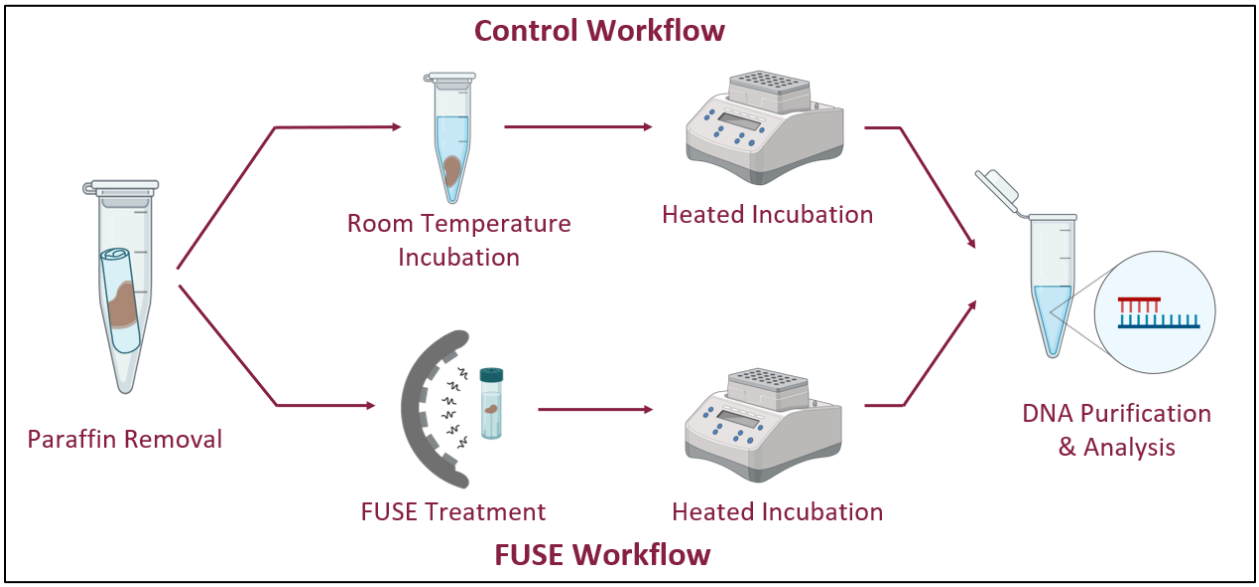

Figure 3-1: Experimental workflow for Control (top track) and FUSE (bottom track)

3.2.3 Sample Preparation, Sample Holder, and FUSE Pulse Generation

Sample preparation, the sample holder, and FUSE pulse generation were set up and performed as described in Chapter 2.

3.2.4 Treatment Groups

Treatment times of 15 and 30 minutes were investigated at 500Hz PRF. Two separate incubation periods were investigated: the full 2-hour incubation (1 hour at 56°C followed by 1 hour at 90°C), as recommended by the QIAGEN protocol, and a shortened 25-minute incubation period (90°C). Incubation was performed in a thermomixer (Eppendorf Thermomixer 5350 Mixer) set to 500 rpm. To determine the effect of FUSE on DNA quality and yield, each treatment combination was performed with and without FUSE. The Control groups sat at room temperature in the lysis buffer while the corresponding FUSE groups underwent FUSE treatment.

3.2.5 DNA Quantity & Quality

To determine suitability for downstream analysis, DNA quality must be assessed by evaluating DNA quantity, degree of fragmentation, and purity [17].

All samples were analyzed with a Nanodrop One (ThermoFisher, Waltham, MA, USA) spectrophotometer. DNA yield was reported as the quantity of DNA released in ng per µL of elution buffer, as measured by the NanoDrop. Purity was evaluated based on the A260/A230 and A260/A280 absorption ratios reported by the NanoDrop. These ratios are commonly used to assess the purity of nucleic acids, which absorb light at a wavelength of 260nm. The 260/230 ratio is used to assess the presence of organic compounds which typically absorb light at 230nm, including carbohydrates, phenol, Guanidine HCL, EDTA, and guanidine thiocyanate. Similarly, the 260/280 ratio assesses the presence of compounds such as proteins or phenols which typically absorb light at 280nm. 260/230 ratios of 1.9-2.2 are considered acceptable, with 2 being ideal. 260/280 ratios of 1.7-2.0 are acceptable, and 1.8 is considered ideal [17], [21].

The DNA fragmentation of each sample was analyzed via gel electrophoresis. Samples were normalized to a concentration of 20 ng DNA/µL. 15 µL of normalized sample volume was mixed with 3 µL of gel loading dye (6x) and loaded into a 1% agarose gel. Electrophoresis was run for 1 hour at 100V.

3.2.6 PCR

One representative sample from each treatment group was amplified via PCR. Samples with a DNA concentration closest to the mean DNA concentration were chosen to be the representative sample for each treatment group. Human primer 18s and universal primer 18s were used for amplification. Standard PCR protocol was used: reagents (125L GoTaq, 85L water, 10L primer, and 30L purified DNA sample) were mixed and centrifuged in PCR tubes, PCR was performed per thermocycler and primer parameters, and amplified DNA was evaluated via agarose gel electrophoresis followed by ethidium bromide staining. Gel electrophoresis was performed in a standard 1% agarose gel at 150V for 45 minutes.

3.2.7 Statistical Analysis

Statistical analysis of DNA yield was performed in JMP (Version 16.0.0. SAS Institute Inc., Cary, NC, 1989–2023). The direct effect of FUSE was analyzed by comparing each FUSE group to its corresponding control group with a single-tailed student's t-test. All parameter interaction and main effects on DNA yield were assessed with three-way ANOVA. P-values under 0.05 were considered indicative of statistical significance.

3.3 Results & Discussion

3.3.1 DNA Quality & Quantity

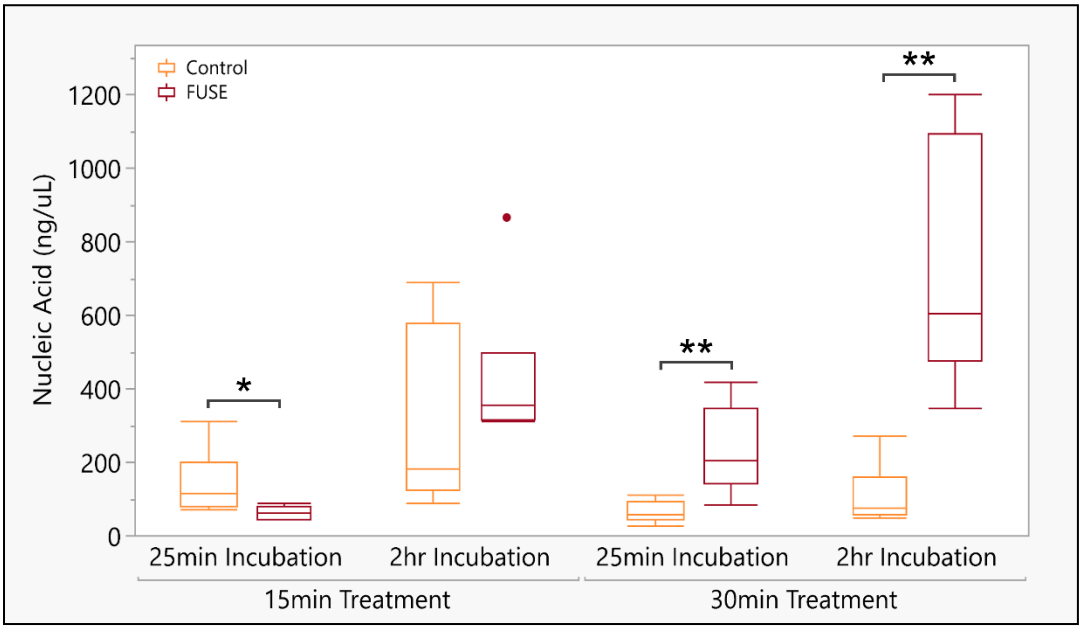

Figure 3-2: DNA yield with and without FUSE treatment (500Hz PRF) for 15 and 30 minutes, with either 25-minute (90°C) or 2-hour (1 hour at 56°C, 1 hour at 90°C) heated incubation periods following treatment. Annotated based on one-tailed t-tests (* for $p<0.05$, ** for $p<0.01$); n=6 for all groups.

FUSE was shown to cause a significant increase in DNA yield when samples were treated for 30 minutes. 15-minute FUSE treatments, however, did not experience an increase in DNA yield, with the 25-minute incubation group demonstrating a slight decrease in yield with the addition of FUSE (figure 3-2).

Table 3-1: Full factorial three-way ANOVA effect test results, ordered in ascending p-value. Calculations performed in JMP.

Source	Sum of Squares	F Ratio	Prob > F
Incubation	841605.42	26.8405	<.0001***
Treatment	514153.47	16.3974	0.0002***
Treatment Time*Treatment	404784.96	12.9094	0.0009***
Treatment*Incubation	319922.64	10.2030	0.0027**
Treatment Time*Treatment*Incubation	42544.32	1.3568	0.2510
Treatment Time	27367.78	0.8728	0.3558
Treatment Time*Incubation	46.92	0.0015	0.9693

The ANOVA effect test shown in Table 3-1 indicates that the length of heated incubation has the strongest effect on DNA yield, followed by treatment (i.e. FUSE vs Control) and the interaction between treatment and treatment time. This confirms that FUSE has a significant effect on DNA yield but highlights an interaction effect with treatment time which deserves additional investigation in future studies.

Table 3-2: Mean absorbance ratios for all treatment groups, n=6. Cells are shaded green to denote values within ±2% of the acceptable range for the corresponding absorbance ratio [17], [21]. Asterisks denote values which are significantly greater than their counterparts (* for $p<0.05$, ** for $p<0.01$) as determined by one-tailed t-tests.

		15min FUSE Treatment		30min FUSE Treatment	
		25min inc	2hr inc	25min inc	2hr inc
A260/ A230	Control	1.118	1.774	1.116	1.146
	FUSE	1.291	2.081	1.972 *	2.206 **
A260/ A280	Control	1.986	1.968	1.951	1.909
	FUSE	1.939	1.987	1.952	1.993

As denoted by the shaded cells in table 3-2, all groups had a mean 260/280 ratio within the acceptable range. In contrast, only the 30-minute treatment time FUSE groups and the 15-minute treatment, 2-hour incubation FUSE groups fell within the acceptable range for the 260/230 ratio,

indicating that the Control groups and the 15-minute treatment, 25-minute incubation FUSE group may have significant quantities of contaminants which absorb light at 230nm, such as carbohydrates, phenol, Guanidine HCL, EDTA, and guanidine thiocyanate [17], [21]. Single-tailed t-tests found the 30-minute FUSE treatments to have significantly increased 260/230 ratios than corresponding control groups, while all other FUSE groups showed no significant differences in 260/230 ratio compared to the controls.

3.3.2 Fragmentation & PCR

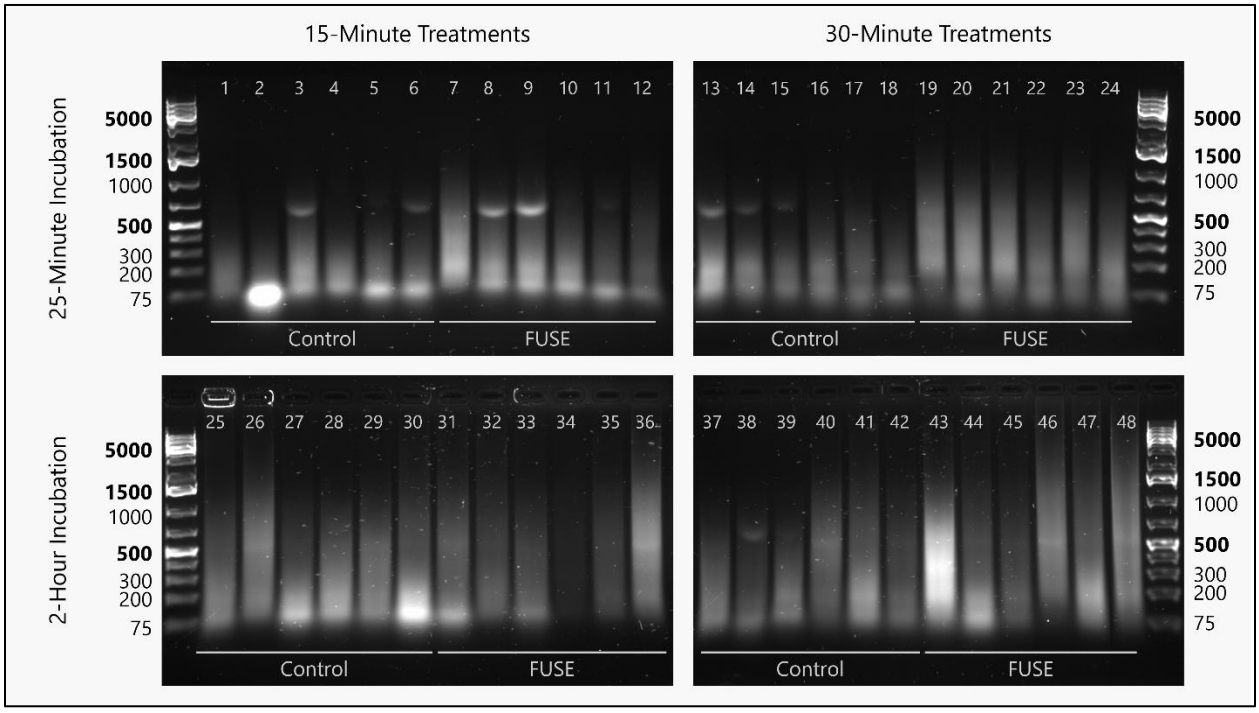

Figure 3-3: Gel electrophoresis lanes for each sample, with annotated ladder (Image has been edited slightly for consistency and easier comprehension. Unedited version of the images can be found in Appendix A).

Gel electrophoresis results (figure 3-3) indicate that the FUSE and control groups experienced comparable fragmentation in most treatment combinations, showing that FUSE did not cause

significantly more fragmentation than conventional methods. Several bands of around 700bps are visible in the 25-minute incubation groups (shown in the top row), which are largely absent in the 2-hour incubation groups. This suggests that longer heated incubation times results in the breakdown of these 700bp fragments. Visually, FUSE groups appear to have a wider range of fragment sizes, especially the FUSE 30-minute treatment group with 25-minute incubation (lanes 19-24). This differs from the trend of FUSE causing more fragmentation than conventional methods seen in previous FUSE studies [10]–[12], a difference which can likely be attributed to the inevitable fragmentation which accompanies formalin fixation [22]. Specifically, we are seeing longer DNA fragments in the 15-minute FUSE treatment groups than previously seen in the leaf study groups treated under the same parameters [11].

Several samples show a bright band at the very bottom of the lane (such as in lanes 2 and 30), which can be indicative of RNA contamination. This is likely due to a lack of RNase treatment in the protocol and suggests that RNase should be used in the future to avoid RNA contamination. Alternatively, this posits that FUSE may be effective for RNA extraction from FFPE tissue, an application which could be explored more in depth in future studies.

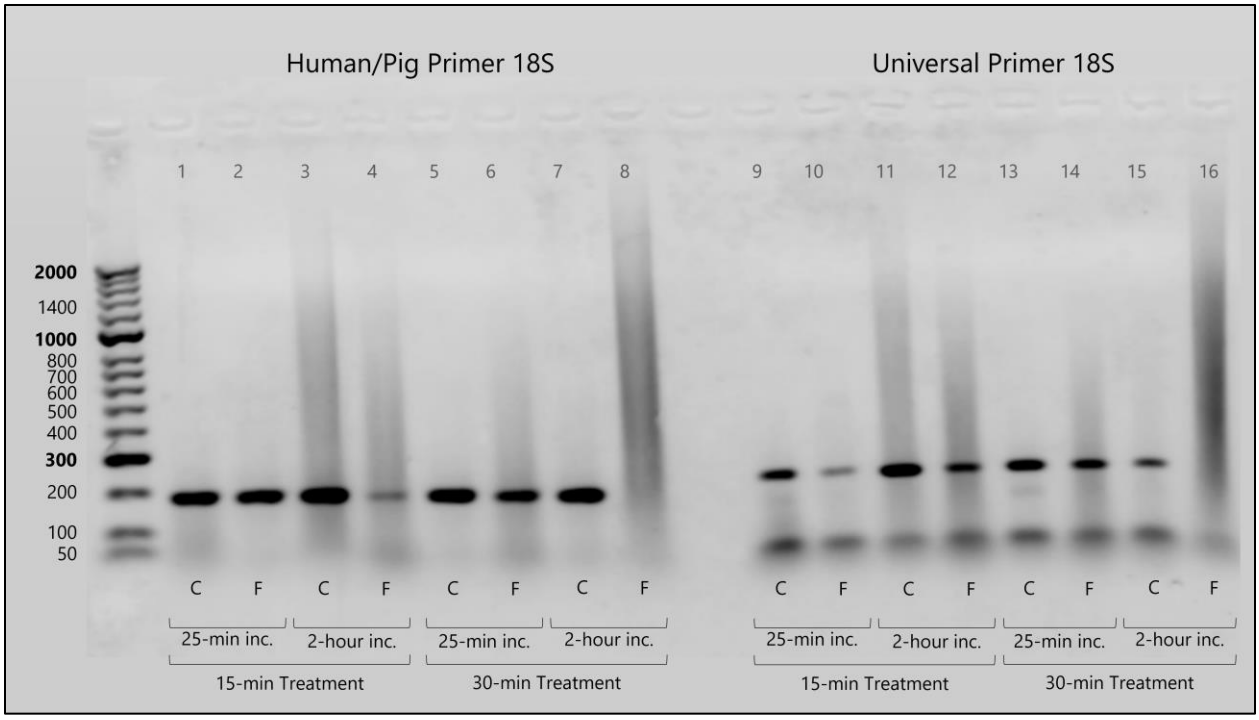

Figure 3-4: Gel electrophoresis for representative samples following amplification via PCR, with annotated ladder (Controls are labeled C, FUSE samples are labeled F).

Figure 3-4 shows the gels run after amplification of eight representative samples via PCR. All samples successfully amplified with both primer sets, as indicated by a distinct band in the 200bp region, except for the 30-minute FUSE treatment with 2-hour incubation (lanes 8 and 16). This is promising, as three out of four samples treated with FUSE successfully amplified, indicating FUSE did not fragment the DNA beyond usable lengths or release inhibitors which prevented amplification. The failure of one FUSE sample to amplify could be due to contamination or excessive fragmentation. However, neither of these seem likely, as this treatment group had purity ratios within the ideal range (Table 3-2) and showed comparable fragmentation (this sample corresponds with lane 46 in Figure 3-3) to other groups which successfully amplified. Due to time constraints, we were only able to amplify one representative sample from each treatment group, meaning that the sample size is small. Thus, further investigation must be done to determine the reproducibility of these results and potentially identify the cause of the failed amplification.

In addition to the bands at 200bps, the universal primers resulted in faint bands around 50bps; these are suspected to be the result of primer dimers, or non-specific amplification [23]. Some lanes also showed noticeable smearing. It was found that the samples in the smeared lanes had higher DNA concentrations (greater than 200ng/µL) than the samples in the unsmeared lanes. Thus, these smears are likely due to the lack of concentration normalization prior to amplification. Lanes 4 and 10 have very weak bands, which suggest delayed amplification or limited quantities of the target gene sequence. It is unclear why these samples may have experienced delayed amplification; thus, further investigation is required to determine if this is a replicable trend or if these faint bands are an irregularity.

Due to the nature of PCR and gel electrophoresis, it is highly probable that the observed 200bp bands represent the targeted gene segment, but without sequencing this cannot be confirmed with certainty. Thus, fully confirming the success of the amplification would require sequencing of these samples.

3.4 Conclusion

When applied for 30 minutes, FUSE was found to extract DNA of higher quantities, better purity scores, and comparable fragmentation compared to conventional methods. The 15-minute FUSE treatment resulted in comparable or less DNA yield, with comparable purity scores and fragmentation. These results show that FUSE can be used to effectively extract DNA from FFPE tissue with similar quality as conventional methods. Three of four treatment groups were successfully amplified via PCR, showing that FUSE can produce DNA samples suitable for PCR and other forms of downstream analysis.

Chapter 4: Conclusions

4.1 Key Takeaways

The results of this thesis demonstrate promising potential for the use of FUSE for FFPE DNA extraction. FUSE was found to be capable of releasing DNA from FFPE tissue, which can then be followed with heated incubation and DNA purification to obtain functional DNA for downstream analysis. In these trials, 30-minute FUSE treatments resulted in DNA of significantly higher quantity, improved purity, and comparable fragmentation than the conventional protocol. These results held true across both incubation time periods, indicating that FUSE can be used with the abridged 25-minute incubation period to extract DNA in half the processing time.

While 30-minute FUSE treatments resulted in higher quantities of DNA than conventional methods, 15-minute FUSE treatments resulted in comparable or reduced quantities of DNA, with no significant differences in purity ratios. This highlights a tradeoff between speed and DNA quality, which must be carefully considered when choosing a DNA extraction method.

Additionally, three out of four samples treated with FUSE were successfully amplified via PCR, showing that FUSE can extract amplifiable DNA. Further investigation will have to be done to determine the replicability of these findings, as they are limited by small sample size.

The most unexpected outcome is the reduction in DNA yield at 1kHz PRF as seen in chapter 2, a result that requires additional exploration. This behavior will be especially interesting if it is determined to be caused by overtreating or extensive fragmentation, as this will have strong implications for future FUSE studies and our understanding of how focused ultrasound interacts with nucleic acids. Several options have been suggested to mitigate the potential overtreating issue. One possibility is the development and employment of a filtration system during treatment, which

allows liberated DNA to exit the focal zone to avoid overtreatment while intact cells and nuclei will continue to be treated.

4.2 Limitations & Future Work

This work represents the first investigation of FUSE for FFPE DNA extraction. As an initial feasibility and parameter study, this work has limitations that will need to be further investigated in future studies. For instance, there is much work to be done to optimize the FUSE procedure. PRF, treatment time, peak negative pressure, incubation time, and incubation temperature all require further investigation to optimize and refine this methodology. PRF deserves particular attention based on the results of this study, as indicated above. Incubation time and temperature also received limited analysis in this study. Based on the fragmentation results (figure 3-3) which suggest that increased heated incubation time has a notable effect on fragmentation, incubation can likely be fine-tuned in future studies to determine optimal incubation time and temperature for specific applications without causing excessive fragmentation.

Different FUSE setup options also deserve exploration. This study is potentially limited by its use of a prototype FUSE setup that was developed for previous feasibility studies with other tissue types (salmon, timber). Our finding that heated incubation is required to achieve effective DNA extraction from FFPE tissue presents several possibilities for novel setup options which could be explored. The most straightforward improvement to the FUSE set-up used in this study could be to perform FUSE treatment in a heated medium. In this way, the heated incubation and FUSE treatment could be performed partially or fully concurrently, thus increasing the protocol's efficiency. Alternatively, other focused ultrasound options exist besides the non-thermal, low duty cycle type used here and in histotripsy. For instance, HIFU [24], [25] and boiling histotripsy [26], [27] are two popular types of focused ultrasound which heat the medium being treated, and thus

could be explored for this application to perform FUSE treatment and the heated incubation concurrently.

Another consideration could be to develop a version of the FUSE workflow which eliminates the paraffin removal step. Typically, the first step in any FFPE DNA extraction process is manual paraffin removal using xylene and EtOH. However, it has been suggested that this step could be skipped, as the paraffin will melt during the heated incubation period and solidify separately from the lysate when cooled [17]. If this paraffin melting effect can be replicated parallel to tissue lysis in the FUSE workflow, manual paraffin removal could be skipped, further reducing processing time by roughly 30 minutes to an hour.

4.3 Long-Term Vision

An ideal FFPE DNA extraction workflow would consist of a simple benchtop device in which an FFPE sample could be de-paraffinized, lysed, and its crosslinks reversed all in one simple step, resulting in a crude DNA extract which can be used as is or further purified for improved quality [17]. Members of our research team are currently developing portable and low-cost FUSE systems which can be optimized for DNA extraction from various tissue types and used in laboratory settings. The ultimate goal is to develop a sample preparation system that can be integrated with existing protocols to address the primary time constraints which currently make FFPE DNA extraction so impracticable for large-scale and high-throughput applications. The development of this efficient and effective technology for DNA extraction from FFPE tissue holds immense promise for advancing precision medicine and personalized therapies, paving the way for more personalized, precise, and impactful medical treatments and interventions.

References

[1] F. S. Ou, S. Michiels, Y. Shyr, A. A. Adjei, and A. L. Oberg, "Biomarker Discovery and Validation: Statistical Considerations," *Journal of Thoracic Oncology*, vol. 16, no. 4, 2021, doi: 10.1016/j.jtho.2021.01.1616.

[2] K. Potluri, A. Mahas, M. N. Kent, S. Naik, and M. Markey, "Genomic DNA extraction methods using formalin-fixed paraffin-embedded tissue," *Anal Biochem*, vol. 486, pp. 17–23, Jul. 2015, doi: 10.1016/J.AB.2015.06.029.

[3] B. E. Bax, "Biomarkers in Rare Diseases," *Int J Mol Sci*, vol. 22, no. 2, pp. 1–4, Jan. 2021, doi: 10.3390/IJMS22020673.

[4] H. Do and A. Dobrovic, "Sequence Artifacts in DNA from Formalin-Fixed Tissues: Causes and Strategies for Minimization," 2014, doi: 10.1373/clinchem.2014.223040.

[5] B. S. van Deventer, L. du Toit-Prinsloo, and C. van Niekerk, "Practical tips to using formalin-fixed paraffin-embedded tissue archives for molecular diagnostics in a South African setting," *Afr J Lab Med*, vol. 11, no. 1, p. 1587, 2022, doi: 10.4102/AJLM.V11I1.1587.

[6] C. Williams *et al.*, "A High Frequency of Sequence Alterations Is Due to Formalin Fixation of Archival Specimens," *Am J Pathol*, vol. 155, no. 5, p. 1467, 1999, doi: 10.1016/S0002-9440(10)65461-2.

[7] J. Kennedy-Darling and L. M. Smith, "Measuring the Formaldehyde Protein−DNA Cross-Link Reversal Rate," 2014, doi: 10.1021/ac501354y.

[8] Z. Frazer *et al.*, "Effect of Different Proteinase K Digest Protocols and Deparaffinization Methods on Yield and Integrity of DNA Extracted From Formalin-fixed, Paraffin-embedded Tissue," *J Histochem Cytochem*, vol. 68, no. 3, pp. 171–184, Mar. 2020, doi: 10.1369/0022155420906234.

[9] S. J. Mcdonough *et al.*, "Use of FFPE-derived DNA in next generation sequencing: DNA extraction methods," *PLoS One*, vol. 14, no. 4, Apr. 2019, doi: 10.1371/JOURNAL.PONE.0211400.

[10] H. R. Holmes *et al.*, "Focused ultrasound extraction (FUSE) for the rapid extraction of DNA from tissue matrices," *Methods Ecol Evol*, vol. 11, no. 12, pp. 1599–1608, Oct. 2020, doi: 10.1111/2041-210X.13505.

[11] A. Stettinius *et al.*, "DNA release from plant tissue using focused ultrasound extraction (FUSE)," *Appl Plant Sci*, p. e11510, Jan. 2023, doi: 10.1002/APS3.11510.

[12] A. Stettinius *et al.*, "Focused Ultrasound Extraction (FUSE) for the Rapid Release of Timber DNA," in *Society for Wildlife Forensic Science Conference*, Ashland, Oregon, Nov. 2022.

[13] Y. A. Pishchalnikov, J. A. Mcateer, and M. R. Bailey, "Acoustic Shielding by Cavitation Bubbles in Shock Wave Lithotripsy (SWL)," in *AIP Conference Proceedings*, 2006, p. 322. doi: 10.1063/1.2210369.

[14] Y. Kim, A. D. Maxwell, T. L. Hall, Z. Xu, K. W. Lin, and C. A. Cain, "Rapid prototyping fabrication of focused ultrasound transducers," *IEEE Trans Ultrason Ferroelectr Freq Control*, vol. 61, no. 9, pp. 1559–1574, 2014, doi: 10.1109/TUFFC.2014.3070.

[15] J. E. Parsons, C. A. Cain, and J. B. Fowlkes, "Cost-effective assembly of a basic fiber-optic hydrophone for measurement of high-amplitude therapeutic ultrasound fields," *J Acoust Soc Am*, vol. 119, no. 3, p. 1432, Feb. 2006, doi: 10.1121/1.2166708.

[16] T. Sedlackova, G. Repiska, P. Celec, T. Szemes, and G. Minarik, "Fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods," *Biol Proced Online*, vol. 15, no. 1, p. 5, 2013, doi: 10.1186/1480-9222-15-5.

[17] D. Lenze, H.-H. Müller, and M. Hummel, "Considerations for the use of formalin-fixed and paraffin-embedded tissue specimens for clonality analysis", doi: 10.1007/s12308-012-0138-8.

[18] N. Einaga *et al.*, "Assessment of the quality of DNA from various formalin-fixed paraffin-embedded (FFPE) tissues and the use of this DNA for next-generation sequencing (NGS) with no artifactual mutation," *PLoS One*, vol. 12, no. 5, p. e0176280, May 2017, doi: 10.1371/JOURNAL.PONE.0176280.

[19] M. T. P. Gilbert *et al.*, "The Isolation of Nucleic Acids from Fixed, Paraffin-Embedded Tissues–Which Methods Are Useful When?," *PLoS One*, vol. 2, no. 6, p. e537, 2007, doi: 10.1371/JOURNAL.PONE.0000537.

[20] L. Atanesyan, M. J. Steenkamer, A. Horstman, C. B. Moelans, J. P. Schouten, and S. P. Savola, "Optimal Fixation Conditions and DNA Extraction Methods for MLPA Analysis on FFPE Tissue-Derived DNA," *Am J Clin Pathol*, vol. 147, no. 1, pp. 60–68, Jan. 2017.

[21] W. W. Wilfinger, K. Mackey, and P. Chomczynski, “Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity,” *Biotechniques*, vol. 22, no. 3, pp. 474–481, 1997, doi: 10.2144/97223ST01.

[22] H. Singh, B. Narayan, A. B. Urs, S. Kumar Polipalli, and S. Kumar, “A novel approach for extracting DNA from formalin-fixed paraffin-embedded tissue using microwave,” *Med J Armed Forces India*, vol. 76, no. 3, p. 307, Jul. 2020, doi: 10.1016/J.MJAFI.2019.02.007.

[23] J. Brownie *et al.*, “The elimination of primer-dimer accumulation in PCR,” *Nucleic Acids Res*, vol. 25, no. 16, 1997, Accessed: Apr. 20, 2023. [Online]. Available: https://academic.oup.com/nar/article/25/16/3235/1455518

[24] T. D. Khokhlova and J. H. Hwang, “HIFU for Palliative Treatment of Pancreatic Cancer,” *Adv Exp Med Biol*, vol. 880, pp. 83–95, 2016, doi: 10.1007/978-3-319-22536-4_5.

[25] G. ter Haar and C. Coussios, “High intensity focused ultrasound: physical principles and devices,” *Int J Hyperthermia*, vol. 23, no. 2, pp. 89–104, 2007, doi: 10.1080/02656730601186138.

[26] T. D. Khokhlova, M. S. Canney, V. A. Khokhlova, O. A. Sapozhnikov, L. A. Crum, and M. R. Bailey, “Controlled tissue emulsification produced by high intensity focused ultrasound shock waves and millisecond boiling,” *J Acoust Soc Am*, vol. 130, no. 5, p. 3498, Nov. 2011, doi: 10.1121/1.3626152.

[27] T. D. Khokhlova, Y. A. Haider, A. D. Maxwell, W. Kreider, M. R. Bailey, and V. A. Khokhlova, “Dependence of Boiling Histotripsy Treatment Efficiency on HIFU Frequency

and Focal Pressure Levels," *Ultrasound Med Biol*, vol. 43, no. 9, pp. 1975–1985, Sep. 2017, doi: 10.1016/J.ULTRASMEDBIO.2017.04.030.

Appendix A: Unedited Gel Electrophoresis Images (annotated)