EVALUATION OF A FIELD HISTOLOGY TECHNIQUE AND ITS USE IN HISTOLOGICAL ANALYSES OF MUMMIFIED TISSUES FROM DAKHLEH OASIS, EGYPT

by

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ABSTRACT

Use of paleohistology to identify histological structures in mummified tissues can allow insight into pathological conditions such as parasites, cirrhosis and lung scarring. However, increasing concerns in archaeological sciences include restrictions on removal of human remains from their site of origin for scientific study. In the case of mummified remains, the use of a field paleohistology kit may be deemed useful for 'point-of-care' pathological assessment of preserved tissues. This study evaluates field paleohistology protocols for mummified soft tissue based on techniques used modern medical field missions. The application of this technique alleviates the need for export or removal of remains from the site for analysis. Samples from the Kellis 1 Cemetery (c. 60BC – AD100) and Deir abu Metta Christian church (c. 4th century AD) in the Dakhleh Oasis, Egypt were processed using field paleohistology techniques in both a laboratory and field setting. Two processes of soft tissue preservation were identified in this sample; anthropogenic or 'artificial', and spontaneous or 'natural' mummification. In cases of artificial mummification, the use of resin, both on internal and external surfaces of the body, caused difficulty in the rehydration process and visualization of the cellular structures. In cases of natural mummification, the technique was more successful in rehydration, slide mounting, imaging, and detection of cellular structures. Results also showed some tissue samples to be unsuitable for this method due to variable preservation and loss of tissue integrity during processing (e.g., liver). However, consistent quality microscope slides and digital images were obtained from samples of skin, muscle, lung, and liver indicating this point-of-care field method is a viable option for paleohistological field analyses and identification of pathological conditions in mummified human remains.

Dedicated to my family
for their continual love and support

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CHAPTER 1: INTRODUCTION

Since the early 1900s (e.g. Ruffer, 1909) the scientific community has been interested in the microscopic examination of mummies. The use of paleohistology to study histological structures in mummified tissues can now provide greater insight into diseases affecting past populations. For example, the presence of parasites in the liver and intestinal tract such as hookworms (Allison et al., 1974) and schistosomes (Adamson, 1976) can help determine how cultural practices like hygiene, sanitation and nutrition were impacted by the environment (Bouchet et al., 2003). Other examples of non-osteological pathological conditions that can be found histologically are cirrhosis of the liver (Reyman et al., 1977) and lung scarring caused by pneumonia (Aufderheide, 2000) or sand pneumoconiosis (Tapp et al., 1975).

With the enactment of Law No. 117 in 1982, the Egyptian Law on the Protection of Antiquities, the removal of all archaeological material (including human remains) found within the Republic of Egypt is regulated cultural property. As such, any removal of antiquities must have written permission from the Egyptian Ministry of State for Antiquities, formerly the Egyptian Supreme Council of Antiquities. Because of these restrictions, the use of a field paleohistology kit may be deemed useful for 'point-of-care' assessment of preserved tissue, where all analyses can be conducted in the field and researchers only leave with only digital images of the samples.

The objective of this thesis is to assess the quality of a medical field technique for paleohistology of mummified tissues with the goal of carrying out differential diagnosis of any pathological condition(s) found in lung and liver tissue from Kellis 1 and Deir abu Metta Christian Church in Dakhleh Oasis, Egypt (Figure 1).

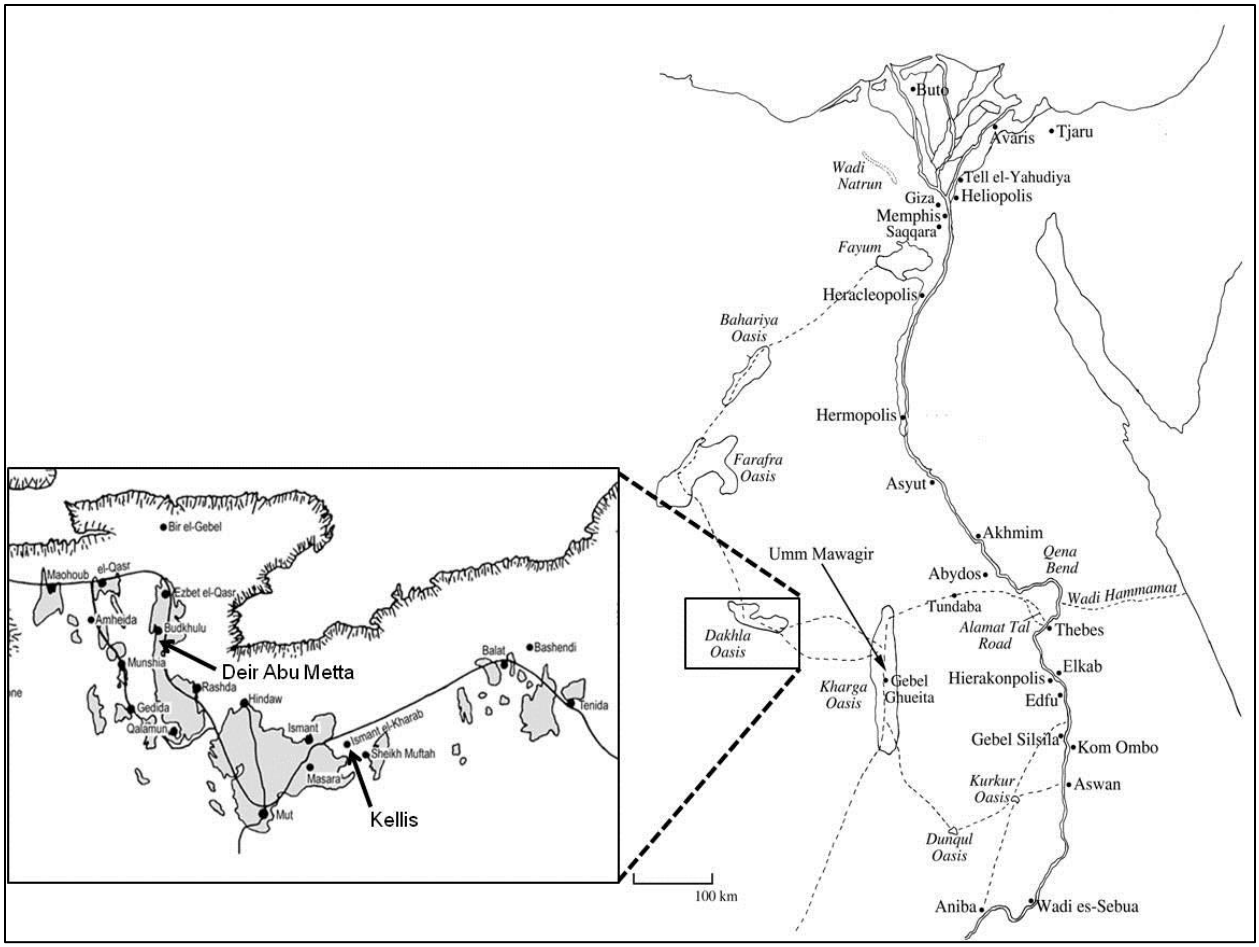

Figure 1: Map of Egypt and Dakhleh Oasis showing location of Kellis and Deir Abu Metta

From a clinical point of view, a differential diagnosis is described as "the methods by which we consider the possible causes of a patient's clinical findings before making final diagnoses" (Richardson et al., 2000). In paleopathology, a differential diagnosis poses the question, "What are all the possible causes of a pathological condition and which one is most likely the cause?" (Ortner, 2003). Unlike modern medical diagnoses, paleopathological diagnoses are limited to any remains left by the individual, either skeletal or mummified soft tissue and cannot aim for an exact diagnosis but rather multiple possible causes of the pathology. Not all archaeological pathological conditions are expressed in bone, a tissue more commonly

preserved through time. Differential diagnosis, for this study, is defined as a process of comparing histological characteristics of known diseases with those shared by an archaeological specimen, in the anticipation of identifying the condition.

Chapter two will focus on the development of a paleohistological field methodology in both the lab and field settings. Fieldwork was completed on site in the Dakhleh Oasis, Egypt. Step-by-step instructions are provided chronicling the advantages and disadvantages as well as feasibility of using a modified microwave technique, portable microtome and basic microscope computer software for identification of mummified tissues in a remote location. As a control, the same processes were followed under ideal conditions in the laboratory at the University of Central Florida. Chapter three will discuss lung and liver diseases found in these samples as well as completing a differential diagnosis for any abnormal histological sections found using the paleohistological field methodology. Chapter four summarizes the conclusions from both chapter two and three and suggests future directions for this research.

CHAPTER 2: EVALUATION OF A POINT-OF-CARE MICROSCOPIC TECHNIQUE FOR HISTOLOGICAL ANALYSIS OF REHYDRATED MUMMIFIED TISSUES

Introduction

Paleohistology has been defined as "the study of histological structures found in prehistoric skeletal remains and mummified tissues" (Mekota et al., 2005). Mummified tissues, even small fragments from canopic jars (Walker et al., 1987), have been successfully rehydrated, fixed and processed for histopathologic assessment in many previous studies (Shaw, 1938; Sandison, 1955; Cockburn et al., 1975; Zimmerman, 1977; Grupe et al., 1997; Ciranni and Fornciari, 2004). These studies have used standard histological laboratory processing, the same used for fresh tissue, with the addition of rehydration when working with mummified remains. However, there are many challenges associated with conducting these analyses in the field because of limitations of removing samples from the country of origin. The objective of this study is to evaluate the medical field technique as presented by Calhoun et al. (2011) for use in the field on mummified tissues with the goal of overcoming restrictions on removal of samples and allow for continued analysis of environment and disease interactions in mummified tissues.

Overview of Mummification

Mummy, as defined by Aufderheide (2003, p 41) is a "physically preserved corpse or tissue that resembles its living morphology but resists further decay for a prolonged postmortem

interval." The initial processes that follow death of a living organism are mainly enzymatic actions that break down proteins, fats and other various structures in the body, which lead to tissue softening and eventually liquefaction or gasification (Gill-King, 1997; Aufderheide, 2003). One of the earliest effects of decomposition is the breakdown of the cell membrane, causing cells to detach from one another and in some cases secreting self-digesting enzymes which cause further membrane breakdown (Gill-King, 1997). This self-destruction or autolysis begins immediately after death but can be inhibited or increased due to environmental substances or factors (Aufderheide, 2003). The mechanisms for mummification require knowledge of these factors that can influence the decay process; factors such as moisture level, pH, and temperature (Aufderheide, 2003).

There are two main forms of mummification: natural or spontaneous and artificial or anthropogenic. Spontaneous or natural mummification is caused by the effects of the environment the individual was placed in where as anthropogenic or artificial mummification is caused by the effects of intentional human intervention on the individual (Aufderheide, 2000). The most common form of both mummification types is the drying of body tissues. A majority of these mummified individuals are found in arid deserts, including Egypt, China, Peru, northern Chile and even deserts in the southwestern USA (Sledzik and Micozzi, 1997; Aufderheide, 2003). Predynastic Egyptian mummies as well as those from the Roman Period buried in shallow desert soil were commonly spontaneously mummified (Aufderheide, 2003). It is suggested that removal of water from the skin's surface through porous clothing or through the porous sandy soil accelerates the body's desiccation rate, promoting spontaneous or natural mummification (Aufderheide, 2003). In many cases in arid regions, dehydration of the outer surface gives the skin a hard leathery appearance, like a thick shell over the body (Galloway, 1997).

Salt can also be used in a similar way, binding with humidity in the air and moisture in the surrounding area causing a dehydrating effect. The removal of water from the body can inhibit the growth of bacteria and harden tissues (Rosendahl, 2010). Artificial mummification in Pharaonic Egypt employed the use of natron, a hydrophilic localized mineral used to cover the deceased's corpse for about six weeks to achieve an anthropogenic form of mummification (Aufderheide, 2003).

In addition to natron, resin was the next most important material used in anthropogenic mummification (Ikram and Dodson, 1998). Resins or tree saps were used to coat chest and abdominal cavities and were applied to the skin as glue for bandages and wrappings following evisceration and desiccation with natron (Aufderheide, 2003). Most resins were extracted from fir, pine or cedar trees, from the areas now known as Lebanon and Syria, and were used in their melted liquid state (Ikram and Dodson, 1998). It is not certain whether this was done to prevent rehydration or was used as a deodorizer and antiseptic to further prolong the preservation of the body (Ikram and Dodson, 1998; Aufderheide, 2003). It is important to note that bitumen, a term commonly used interchangeably with resin, is a thick liquid pitch that originates from the mixture of petroleum and minerals in the area of the Dead Sea (Ikram and Dodson, 1998). Although not commonly used, bitumen can only be distinguished from resin through chemical analysis (Ikram and Dodson, 1998).

Mummification Types Found in the Kellis 1 Cemetery

Two processes of soft tissue preservation have been identified at the Kellis 1 cemetery in the Dakhleh Oasis, Egypt: anthropogenic or artificial mummification (AM), and spontaneous or natural mummification (SM) due to the high temperatures and dry sandy climate (Aufderheide et al., 1999). Of the 169 inhumations recovered from the Kellis 1 cemetery, only 50 were mummified (Aufderheide et al., 1999). The distribution of mummification types at the Kellis 1 cemetery is shown in Figure 2. The use of resin on both the internal and external body surfaces was highly variable with usage ranging from light application to stabilize wrappings, to coatings centimeters thick (Aufderheide et al., 1999). The histological study of mummified tissue can be problematic due to complications from advanced decomposition, style of mummification and extent of resin use. Liquid resin was most likely transparent when new and tends to blacken over time (Ikram and Dodson, 1998). This causes an impermeable barrier around any tissue to which it is applied making rehydration of that tissue impossible.

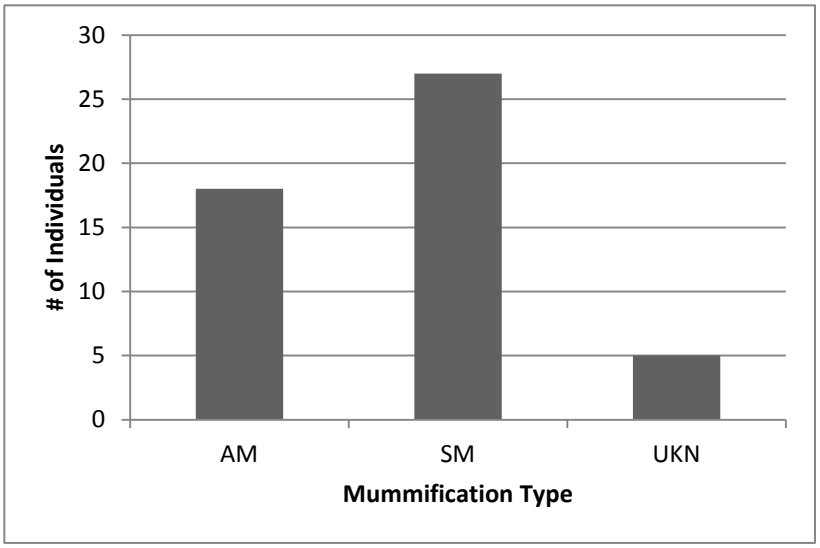

Figure 2: Distribution of mummification types found at the Kellis 1 cemetery site. AM: anthropogenic mummification; SM: spontaneous mummification; UKN: unknown (after Aufderheide et al., 1999)

Some differences and similarities have been noted between mummification practices in Dakhleh and those in Nubia during earlier periods. The absence of natron and reliance on resin has been seen in both Nubian and Dakhleh mummification (Aufderheide et al., 1999). Also, according to Aufderheide et al. (1999), an unusual occurrence of composite mummies was found at Dakhleh. These composite mummies were characterized externally by the presence of torn wrappings of the head, neck, hands and forearms as well as individuals with all wrappings removed and disarticulated bodies (Aufderheide, 2009). These cases were interpreted as evidence of tomb robbers in antiquity and efforts towards reconstruction using wood splints, cords and complete rewrapping is apparent but it is unknown who in particular would have invested such time and money into these reburials (Aufderheide, 2009). The attempts of others to restore these bodies commonly included the application of resin as an adhesive for wrappings and body segments (Aufderheide, 2009). In some cases, the application of resin was so great it suggests there may have been sacred status provided by its treatment (Aufderheide, 2009).

Figure 3 describes the different types of mummification found in the Kellis 1 cemetery and the effect looting may have on resin application.

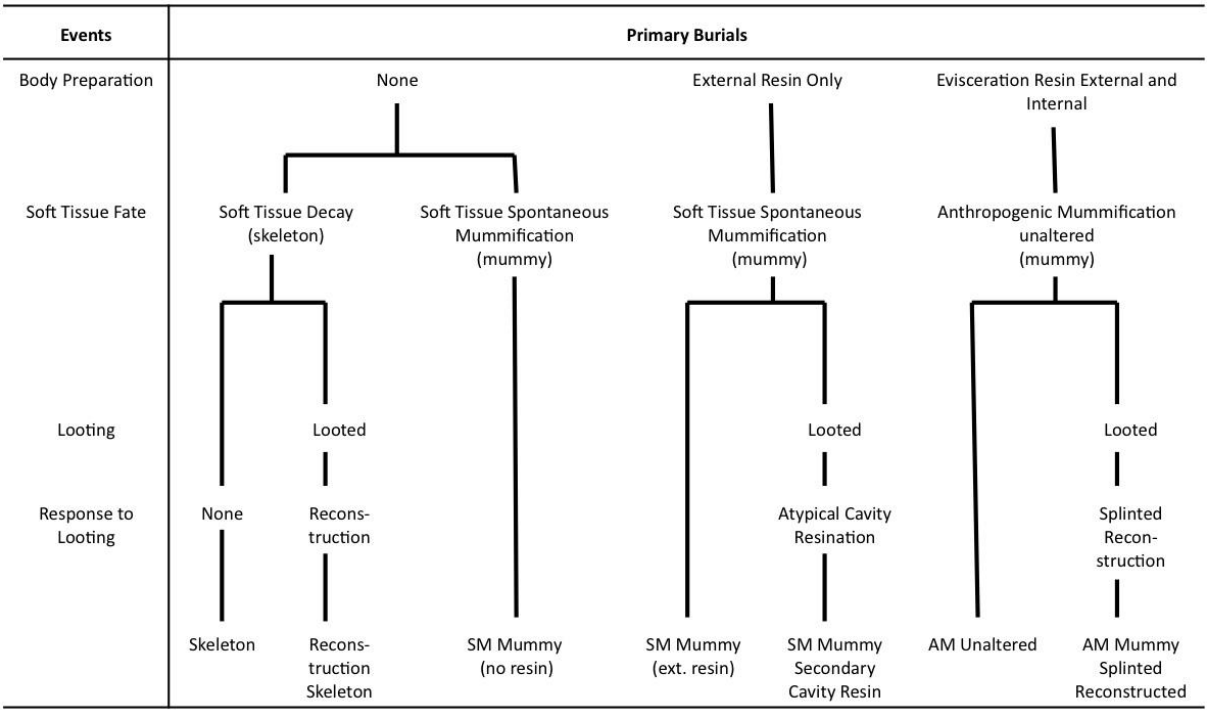

Figure 3: Description of mummification types found at Kellis 1 with effect of looting on resin application. AM: anthropogenic mummification; SM: spontaneous mummification (after Aufderheide, 2009 and field notes courtesy of Dakhleh Oasis Project)

Embalmers during the Late Ptolemaic – Early Roman Period paid less attention to the actual treatment of the body than to its external appearance (Ikram and Dodson, 1998; Aufderheide, 2003). Resin was used liberally with linen bandages while evisceration, removal of the internal organs of the deceased greatly declined (Ikram and Dodson, 1998; Aufderheide, 2003). Transnasal craniotomies or removal of the brain through the nose, was still practiced with liquid resin being introduced into the cranium (Ikram and Dodson, 1998; Aufderheide, 2003).

Overview of Traditional Histological Techniques

Histology is the study of the cellular structure of the tissues of the body and their organization into organs (Junqueira and Carneiro, 2003). Tissues are groupings of similar cells with a common embryonic origin that carry out a particular function. There are four basic tissues: epithelial, connective, muscular and nervous (Junqueira and Carneiro, 2003). With the small size of cells and its components, histology is dependent on the use of microscopes to study these tissues.

The most widely used microscope in the field of histology is the light microscope where a beam of light is transmitted through the tissue (Junqueira and Carneiro, 2003). Given that tissues and organs are too thick for light to pass through they must be cut into thin, translucent sections. This process is achieved through the use of a microtome, a fine cutting instrument that precisely slices the tissue into thin sections averaging between 5 and 25 μm (Kiernan, 1990). Before successful microscopy can be achieved, there are four steps that must be followed to prepare the tissue: fixation, embedding, sectioning and staining.

The goal of fixation is to prevent autolysis or bacterial attack, fix tissues so their volume and shape will not change during processing and to leave tissue as close to its living state as possible (Junqueira and Carneiro, 2003). In chemical fixation, tissues are immersed in a stabilizing solution called a fixative. The most commonly used fixative in histological techniques is a 4% buffered formaldehyde solution but other fixatives can include acetic acid, ethanol, glutaraldehyde, methanol or picric acid (Kiernan, 1990).

After fixation, tissues are usually embedded in a solid medium firm enough to support thin sectioning. Common embedding materials include paraffin and plastic resins (Junqueira and Carneiro, 2003). In preparation for embedding, tissues are dehydrated and cleared. Through dehydration, the fixative and water are removed from the tissue through successive baths in graded alcohols, usually from 60% to 100% (Kiernan, 1990). The dehydrating fluid, in most cases ethanol, is then cleared with a solvent miscible with the embedding medium (Junqueira and Carneiro, 2003). The most common clearing solvent is xylene but toluene, chloroform and benzene can also be used (Kiernan, 1990). Once the tissue is cleared it is placed in the embedding medium, most commonly paraffin wax. The heat of the melted paraffin causes the clearing solvent to evaporate and replaces the spaces within the tissue with paraffin (Junqueira and Carneiro, 2003). Correct orientation of the tissue is the most important step in embedding.

The hard paraffin blocks in which the tissue is suspended, are thin sectioned using a microtome, floated on water and transferred to glass slides to be stained (Junqueira and Carneiro, 2003). Most tissues are colorless and must be stained for microscopy. Stains are usually basic, staining basophilic components of tissue or acidic, staining acidophilic components of tissue (Junqueira and Carneiro, 2003). The combination of hematoxylin, staining acidic molecules shades of blue and eosin, staining basic material shades of red are universally used for routine histological examination (Kiernan, 1990).

Materials and Methods

All tissue samples used in this research came from two sites located in the Dakhleh Oasis, Egypt. The Dakhleh Oasis is one of five major depressions in Egypt's Western Desert. Located about 250 km west of the Nile Valley, the Oasis is a depression approximately 100 m below the surrounding desert, bordered by a large escarpment to the north (Williams, 2008; Wheeler, 2009). Seasonal temperatures in the Dakhleh Oasis range from -4°C - 25°C in winter and 19°C - 50°C in summer with minimal rainfall, averaging 0.3 mm/year (Sutton, 1947; Williams, 2008; Wheeler, 2009). It is an arid, dry desert environment.

The Kellis 1 cemetery (Figure 4), located on the northwest side of the ancient village of Kellis (also known as Ismant el-Kharab), has been dated to the late Ptolemaic and early Roman period (Birrel, 1999). Deir Abu Metta (Figure 5) located south of the village of Budkuhlu and less than 25 kilometers west of Kellis is an early Christian church and burial site dating to around the fourth century AD (Bowen, 2009).

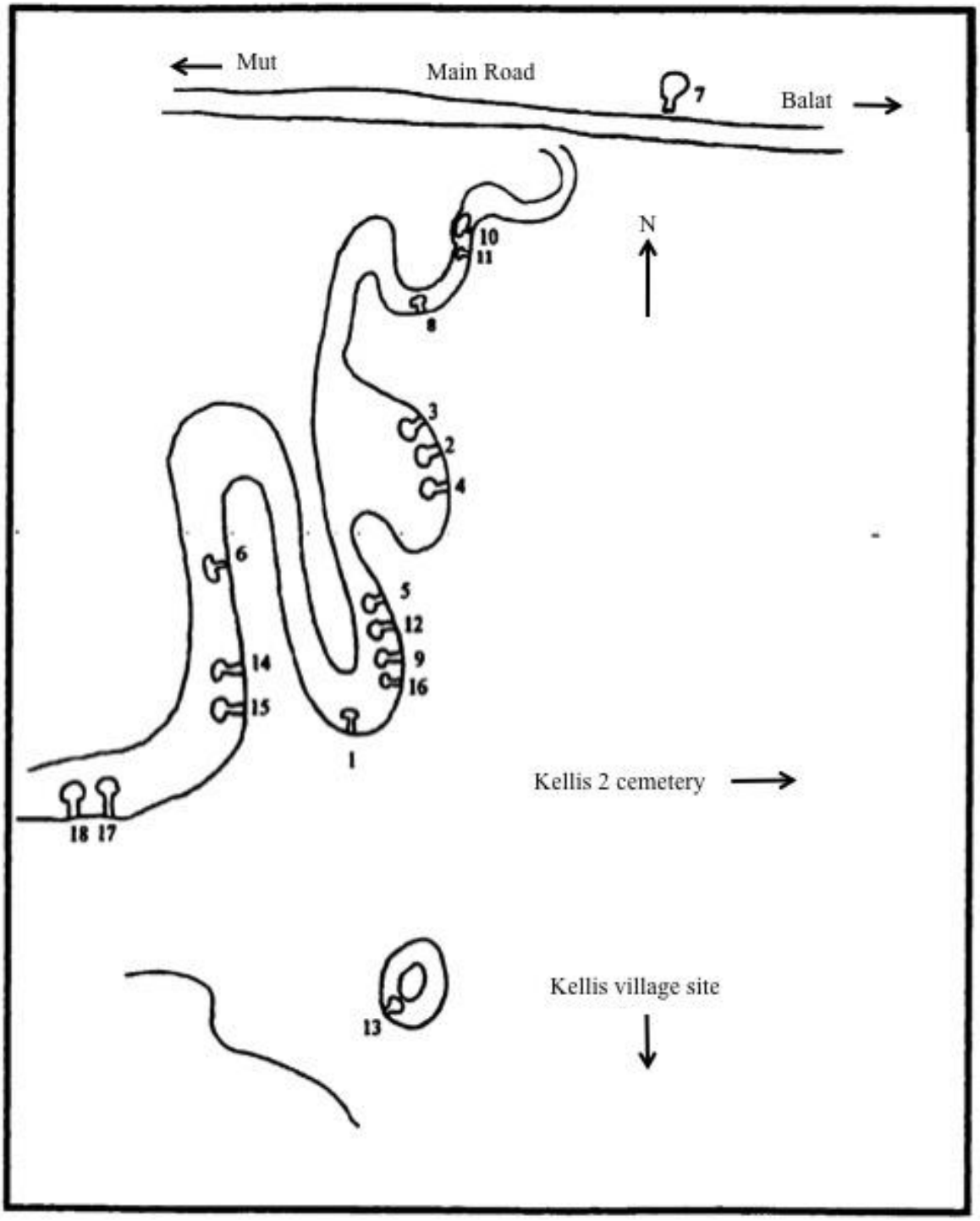

Figure 4: Location of tombs 1 to 18 in Kellis 1 cemetery, Dakhleh Oasis, Egypt. This map is a basic site plan; scale cannot be estimated. Image courtesy of Peter Sheldrick

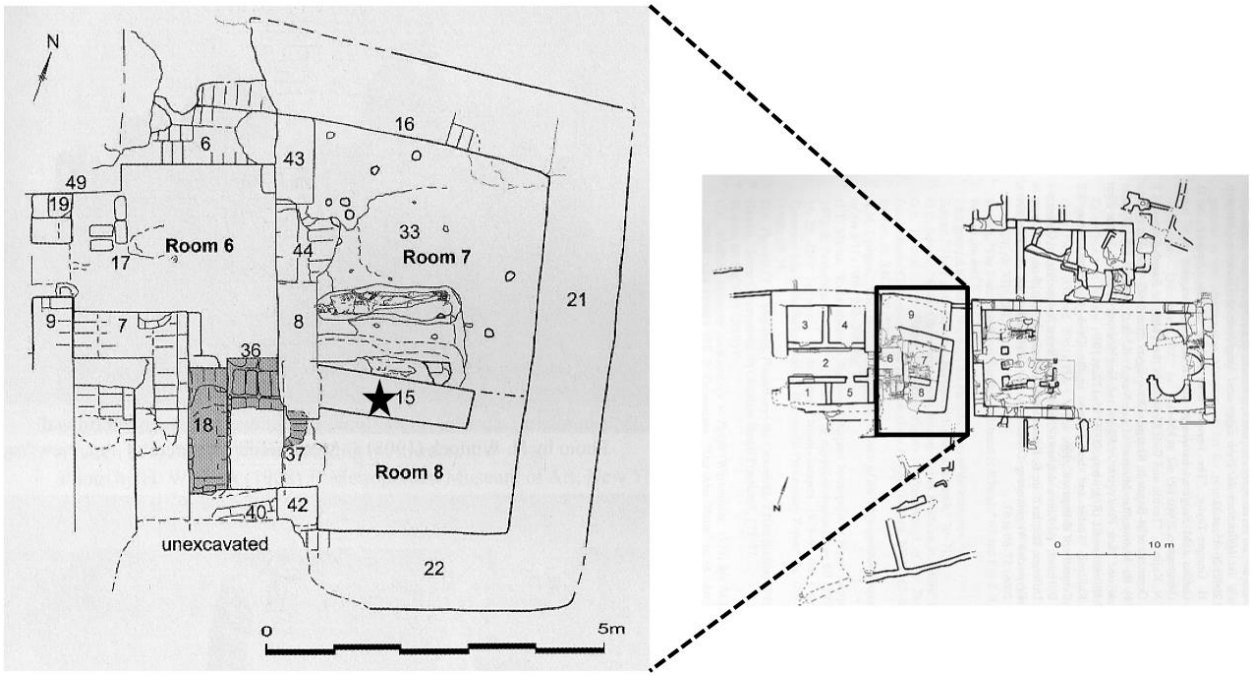

Figure 5: Site map of Deir Abu Metta Christian Church. Expanded view of Trench 5 and starred location of individual 32 in Context 15. Adapted from Bowen, 2009

The human mummy tissue samples used in this thesis come from the Kellis 1 cemetery excavated during the 1992 and 1998 field seasons (Aufderheide et al., 2003) and from Deir Abu Metta Christian Church (excavated in 2009) (Bowen, 2009). For the Kellis 1 material, both external and internal examinations were performed at the time of excavation with tissue samples collected for further examination, Appendix A (Aufderheide et al., 2003). These samples have now become part of the Dakhleh Oasis Project (DOP) Tissue Bank, housed at the University of Central Florida.

The DOP Tissue Bank consists of over 400 samples from 60 individual mummy autopsies performed by various members of the DOP from 1992 to 1998. Samples include all forms of soft tissue as well as linen wrappings, nasal tampons, wooden sticks and resin

scrapings. A listing of the DOP Tissue Bank inventory along with designation of samples selected for this study can be found in Appendix B. A large majority of the individuals that make up the DOP Tissue Bank are either male or of unknown sex (Figure 6) and range in age from 18-24 months to over 55 years. Mummification types found within the DOP Tissue Bank are mainly anthropogenic, Figure 7. Samples were selected at random but were chosen based on potential success, meaning tissues that exhibited unfavorable conditions such as obvious resin coatings, hard brick-like consistency or even thin paper-like consistency were not selected, see Appendix A for images of selected samples.

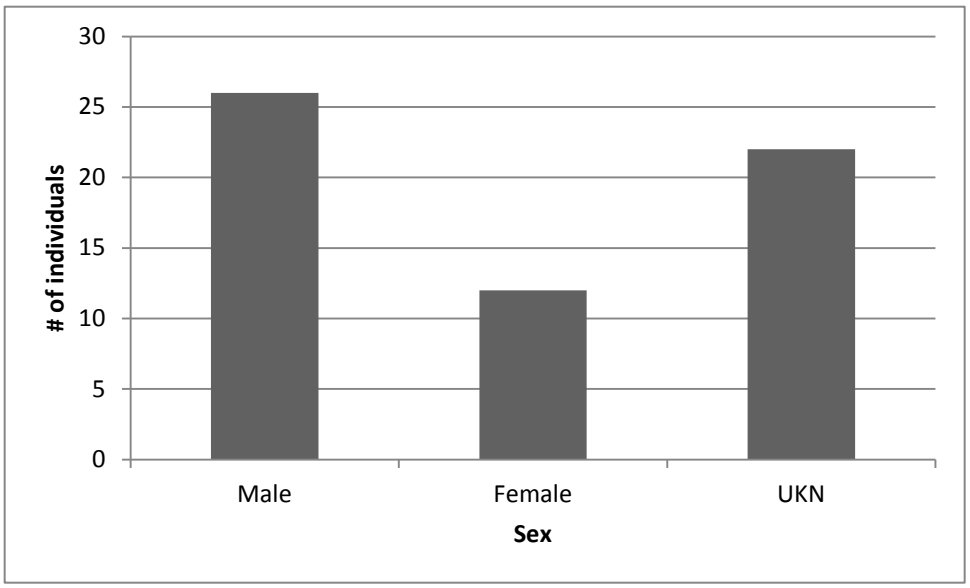

Figure 6: Sex distribution in the Dakhleh Oasis Project Tissue Bank

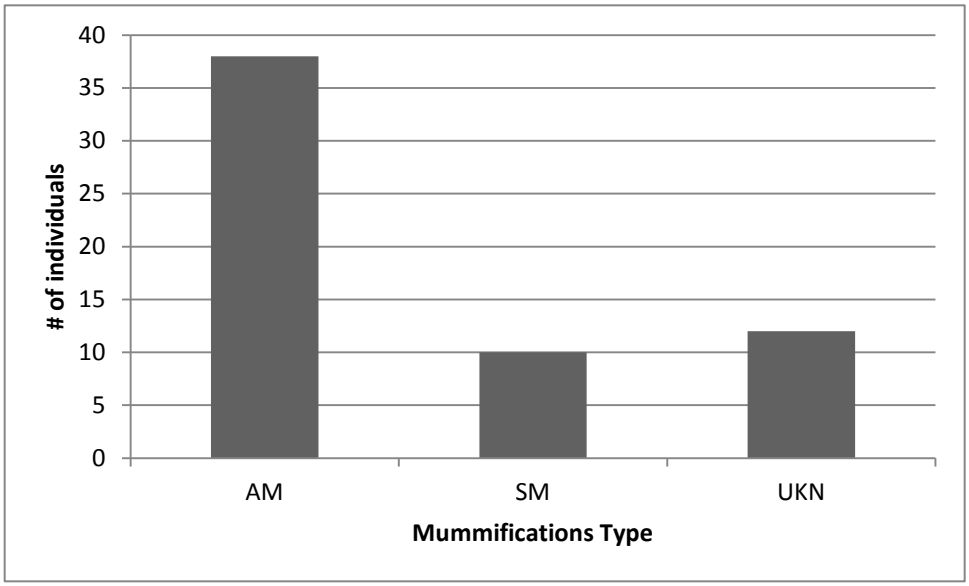

Figure 7: Mummification type distribution in the Dakhleh Oasis Project Tissue Bank

Tissue samples consisting of lung, liver, skin and muscle were selected. Thirteen samples from 12 individuals were used from the DOP Tissue Bank (Appendix B) and two samples from one individual were used from Deir Abu Metta, consisting of lung, liver, skin and muscle (Table 1). All autopsy samples from the DOP Tissue Bank were processed in the laboratory at the University of Central Florida and the two samples from Deir Abu Metta were processed in the field, in the Dakhleh Oasis, Egypt.

All samples were examined using the medical field technique as outlined by Calhoun et al. (2011). This protocol and kit (Table 2) were chosen because of its portability and potential use in remote areas. This protocol, originally created for the processing of fresh soft tissue biopsies, consists of three microwave washes that have been modified to be processed in a portable hot pot, followed by an economical means of paraffin embedding with PVC piping and sectioning and microscopy with field-friendly equipment. It has not been previously tested on mummified tissue and the addition of a rehydration step was needed.

Table 1: Sample Set Demographics

Autopsy #	Body/ Tomb	Date Examined	Sex	Age (yrs)	Sample Type	Preservation Score (0-3)	Mummification SM/AM	Notes on Resin Use
1	A/3	1992	M	12-17	Lung	3	SM	No evidence of resin use either externally or internally
4	E/4	1993	F	20-22	Lung	1	SM	No evidence of application of resin is evident either internally or externally
8	C/3	1993	I	8-11	Liver	2	SM	No resin identified in either cranium, spinal canal or body cavities of skin surface
13	UKN	1993	M	55+	Lung	1	AM	Outside body covered with black material and wrappings
102	B/16	1998	M	9-13	Lung	3	AM	Resin soaked body & nasal perforation; no resin in body cavities or cranium
105	4/16	1998	M		Skin	-	AM	Incomplete paperwork; a minimal amount of resin coated hair is present in the temporal regions
107	C/16	1998	M	7.5-8.5	Liver	-	AM	Skin covered in resin, resin soaked linen introduced into thorax via neck
108	E/16	1998	M	21-25	Liver	-	AM	Body painted with thin layer of black resin, no evidence of resin detected within abdominal cavity
110	E/17	1998	M	30-40	Lung	-	AM	Resin introduced via posterior defect into abdomen & it extended into thoracic cavity through diaphragms
111	D/18	1998	M	20-26	Liver	-	AM	Resin only on skin, no evisceration wound
					Skin	-	AM	
123	UKN	1998	M	50+	Skin	-	AM	Skin stained jet black
129	A/18	1998	M	5.5-6.5	Liver	-	AM	Resin introduced into abdomen via an entry defect in post.; gained access to spinal canal & small amount entered cranial cavity
DAM TR5 32	32/TR5	2012	M	30	Skin	-	SM	
					Muscle	-	SM	

Age was estimated on the basis of dental eruption, long bone length and epiphyseal fusion in sub-adults. In adults alterations in pubic symphysis, cranial suture closure and dental attrition were used. Sex was estimated by external genitalia and pelvic skeletal features. Sex: M=Male, F=Female, I=Indeterminate. SM/AM=Spontaneous mummification/Anthropogenic mummification. Preservation score rated at time of autopsy for tissues present: 0=poor to 3=good. Autopsy reports courtesy of Dakhleh Oasis Project

Table 2: Contents of Field Paleohistology Kit

• 2 500mL plastic bottles • 1 600mL glass beaker • 1 150mL glass beaker • 4 30mL glass beakers • 7 8 dram glass chapped tubes • 1 10mL polypropylene graduated cylinder • 1 plastic coplin jar • 1 electric hot pot • 1 DK-10 sliding microtome • microtome blades • desk lamp, glass carafe, nail polish, Parafilm®, tweezers, scalpel, probe, lab coat, safety glasses, Kimwipes®, plastic dividers	• glass microscope slides • glass cover slips • microscope slide box • 4 2x1 inch PVC molds • portable microscope stand • USB digital microscope • 3.025g TRIS buffer • 1 spray can of canola oil • 1 thermometer • 4 50mL centrifuge tubes • gloves, filtration masks, plastic disposable pipettes, marker, plastic wrap, Paraplast®, rubber bands, paperclips, metal washers

Rehydration

Mummification, whether spontaneous or artificial, can cause tissues to become tough, brittle and challenging to manage. The distribution of mummification type within the sample set is shown in Figure 8. The use of rehydration is crucial to optimize any preserved morphological structures in mummified tissues. According to Mekota and Vermehren (2005) there are 13 known methods of rehydration for mummified tissue ranging from the use of inactivated human blood serum to fabric softener. A good rehydrating solution should penetrate evenly into the brittle tissue, remove the brown color, resulting from decomposition fluids and or the burial environment, and retain tissue stability so it does not crumble when handled (Mekota and Vermehren, 2005). The rehydration fluid chosen for this methodology was a 5% aqueous dimethyl sulfoxide (DMSO) solution consisting of 6.05g TRIS buffer, 900mL distilled water and

38.5mL HCl (Grupe et al., 1997; Mekota and Vermehren, 2005). A half recipe was used in the field as well as a substitution of filtered and boiled water for distilled water. All samples were submerged in the rehydration fluid for 24-36 hours or longer to achieve optimal rehydration (Figure 9). Optimal rehydration was reached when the sample, when gently probed, had a slight give and a puffy or inflated appearance.

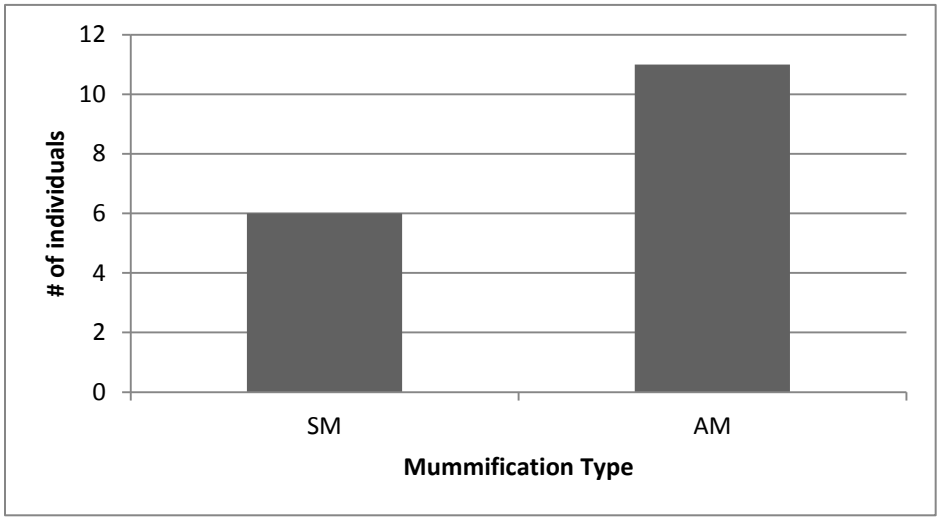

Figure 8: Distribution of mummification type in sample set. SM: spontaneous mummification; AM: anthropogenic mummification

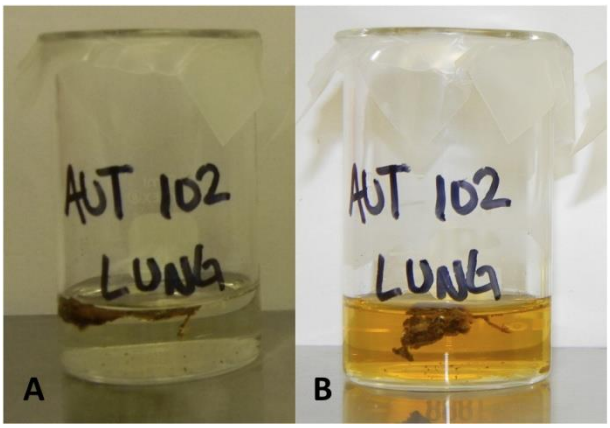

Figure 9: Lung from Autopsy 102 during rehydration in 5% DMSO; A) Lung less than 1 hour in rehydration fluid B) Lung after 36 hours in rehydration fluid; note brown coloration of fluid and inflation of tissue

Fixation

The use of formaldehyde as a fixative is very popular in histology and histopathology because of its ability to form strong bonds and stabilize tissue (Fox and Benton, 1987). The purpose of fixing tissues before dehydration and further processing is to maintain the cells membrane shape while still permitting fluids to pass unrestricted through the tissue (Beebe, 2000). Inadequate fixation or none at all can result in cellular distortion and tissue shrinkage (Beebe, 2000). When tissue shrinkage or collapse occurs, proper dehydration, clearing and infiltration are not possible (Beebe, 2000). Further tissue collapse can then occur during sectioning due to insufficient wax permeation (Beebe, 2000). In the lab, once rehydration was complete, samples were fixed in 10% buffered formalin for at least 24 hours or until needed for embedding. Due to lack of chemical availability, this step was omitted only in the field.

Modified Microwave Washes

Before tissues can be embedded or infiltrated with paraffin, they must be completely fixed with 10% formalin, dehydrated through alcohol washes and cleared with xylene. With traditional histological techniques, this process requires large non-portable expensive automated machinery and overnight processing. Microwave processing allows for a quicker, fewer-step

procedure that can be done with less expensive equipment. In the case of Calhoun et al.'s (2011) field methodology, a laboratory microwave tissue processor is replaced with a standard kitchen hot pot (Figure 10). After sufficient rehydration and fixation, samples were processed using a modified microwave technique as outlined by Calhoun et al., 2011. This process consisted of three 15-minute washes at a consistent temperature between 95°C and 99°C in reagent or ethyl alcohol, isopropyl alcohol and xylene. Tissue samples were suspended in the hot pot using rubber bands and paperclips (Figure 10B).

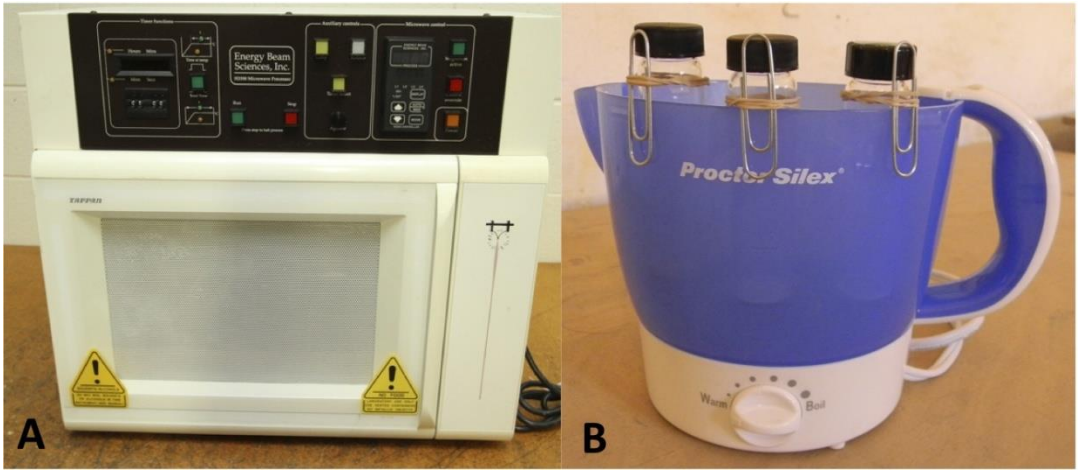

Figure 10: A) Energy Beam® microwave tissue processor used in standard laboratory microwave protocols; B) Standard hot pot with three suspended samples used in field paleohistology protocol

Before tissues can be embedded in paraffin wax, all formalin-based fixative must be removed, followed by dehydration through alcohols and then clearing with xylene (Rohr et al., 2001). Dehydration is achieved through the 15-minute washes of reagent or ethyl alcohol and isopropyl alcohol. The heating of the reagent alcohol rapidly dehydrates the tissue, expelling the formalin fixative from within the tissue (Rohr et al., 2001). When working with rehydrated mummified tissue, the purging of rehydration fluid, in this case DMSO also occurs in this step

(Figure 11). Isopropanol further dehydrates the tissue, releasing any additional water or fixative in preparation for paraffin infiltration (Rohr et al., 2001). As with the previous wash, any extra DMSO is also flushed out (Figure 12). In the field, the only alcohol wash available was isopropyl alcohol. Lastly, the tissue is cleared of all alcohols with a 15-minute wash of xylene (Figure 13). In the field, xylene was replaced with acetone (Lillie, 1965). Standard laboratory microwave tissue processing has been shown to have little significant difference in quality of tissue sections compared to standard overnight procedures (Rohr et al., 2001). Calhoun et al. (2011) also notes little difference with this modified technique.

Figure 11: A) Autopsy 108 liver in ethyl alcohol before microwave wash; B) Autopsy 108 liver in ethyl alcohol after microwave wash; brown coloration due to purging of DMSO and formalin

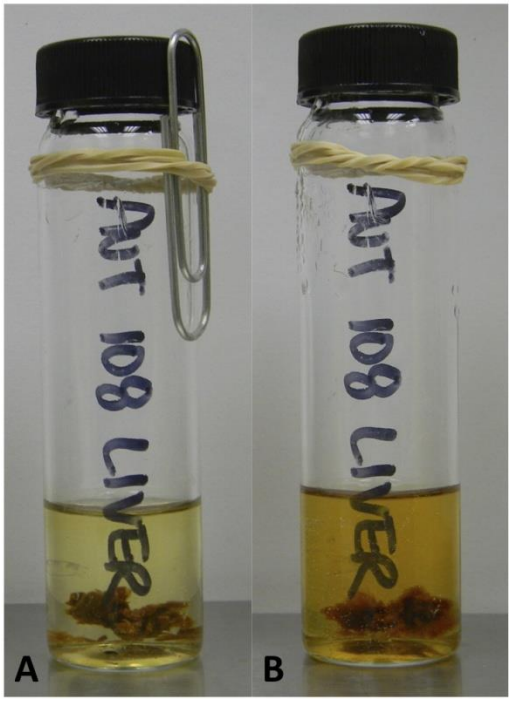

Figure 12: A) Autopsy 108 liver in isopropyl alcohol before microwave wash; B) Autopsy 108 liver in isopropyl alcohol after microwave wash; lighter brown coloration, tissue almost cleared of DMSO and formalin

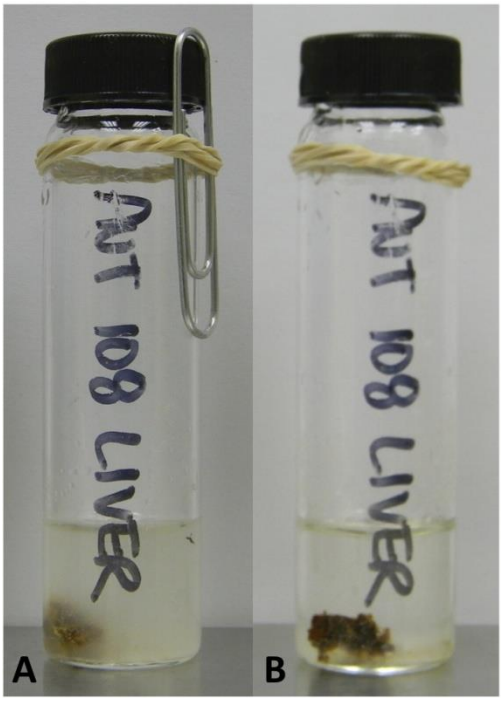

Figure 13: A) Autopsy 108 liver in xylene before microwave wash; B) Autopsy 108 liver in xylene after microwave wash. Fluid remained clear; DMSO, formalin and alcohols have been cleared

Embedding

After samples were dehydrated and cleared, they were embedded in paraffin wax using a 1x2-inch cut polyvinyl chloride (PVC) pipe mold fitted with a crescent shaped inner plastic divider cut from Solo® plastic plates and lubricated with Crisco® original 100% Canola Oil no-stick spray (Figure 14B). The bottom end of the pipe was covered with Glad® Press'n Seal® wrap and reinforced with a rubber band to prevent wax leakage (Figure 14A). Paraplast® was melted in a glass carafe in the same hot pot used for microwave processing (Figure 15). Tissue samples must be kept warm in the hot pot while wax is melting (Figure 15) because melted paraffin will not penetrate a cooled sample and separation of tissue from the block will occur during microtome sectioning (Calhoun et al., 2011).

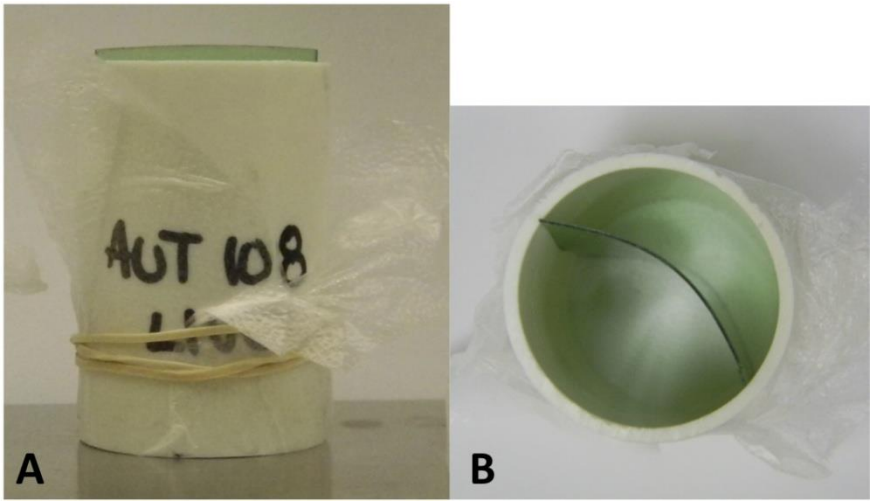

Figure 14: A) PVC mold with Glad® Press'n Seal® wrap; B) PVC mold fitted with plastic divider

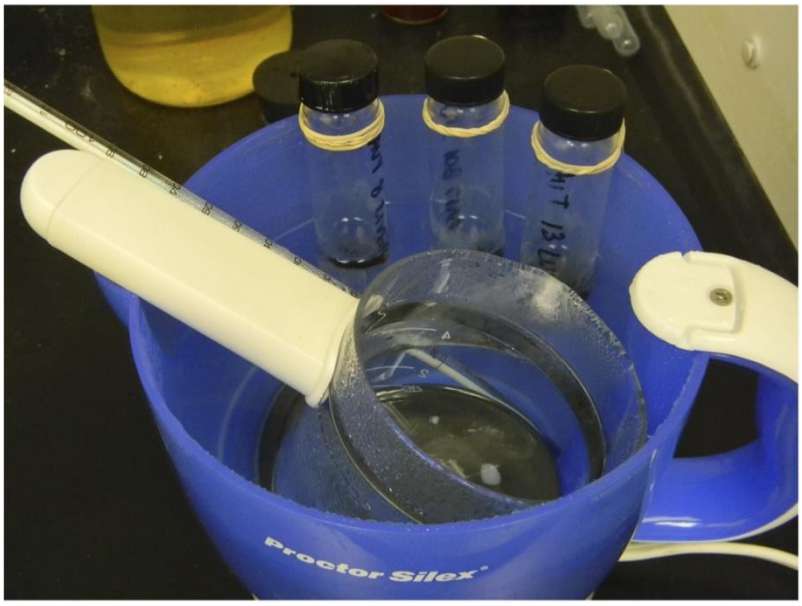

Figure 15: Wax melting in glass carafe with samples, still in xylene, suspended in warm water after microwave washes

Unlike Calhoun et al. (2011), paraffin blocks were not hardened on ice but allowed to 'air-dry' in both lab and field settings. Once paraffin blocks were hardened, they were trimmed with a scalpel to fit the opening of the portable microtome (Figure 16).

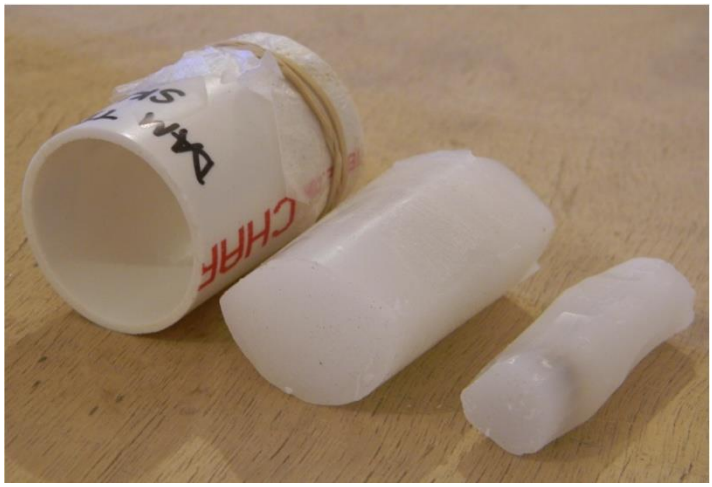

Figure 16: PVC mold and paraffin block before and after trimming

Sectioning

The portable microtome used in both lab and field settings was an Uchida® DK-10 slide microtome (Figure 17). This microtome, originally used for plant microscopy with foam inserts, needed to be altered to work with thicker paraffin molds. The addition of two metal washers measuring up to two inches in diameter was needed to improve blade stability (Figure 17) (Calhoun et al., 2011). The use of this microtome did pose some challenges but with minimal practice generally consistent sections between 10 to 20 μm could be achieved.

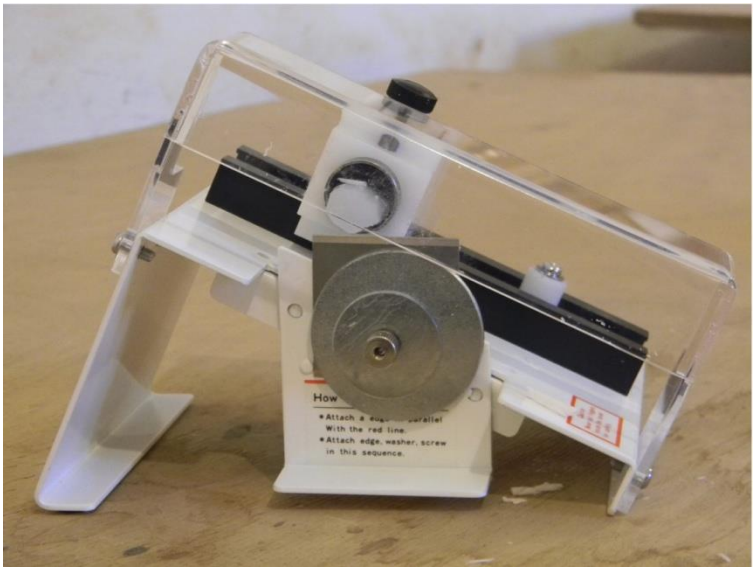

Figure 17: Uchida® DK-10 portable sliding microtome; blade is supported by additional washers for paraffin sectioning

Once sections were made they were floated in a heated water bath (35°C - 40°C) in the hot pot to fully flatten and remove any wrinkles in the wax. In some instances, samples floated out of the wax section, when this occurred, remaining samples were not put in a heated water bath but were alternatively melted on glass slides with a desk lamp. After a heated water bath, 1 to 3 sections were scooped onto each glass slide and allowed to dry for at least 30 minutes or overnight.

Staining and Microscopy

After slides were allowed to dry, they were processed using a modified version of the Harvard hematoxylin-eosin (H&E) staining protocol (Appendix D) (Figure 18). Due to lack of chemical availability, slides were not stained in the field but there is no reason why this protocol would not work in that environment. Deparaffinization was attempted with acetone, however there was no effect. In preparation for microscopy, slides were then cover slipped using Shur/Mount® mounting medium and allowed to dry overnight.

Figure 18: Slides in hematoxylin stain

Field microscopy requires a lightweight, portable, USB-capable microscope. In both lab and field settings a portable microscope stand with a lit stage powered by USB or battery and DP-M01 zipScope® with 200x magnification and 2.0M-pixel camera was used (Figure 19). Software from ProScope® HR was used to examine slides and capture images on a MacBook®.

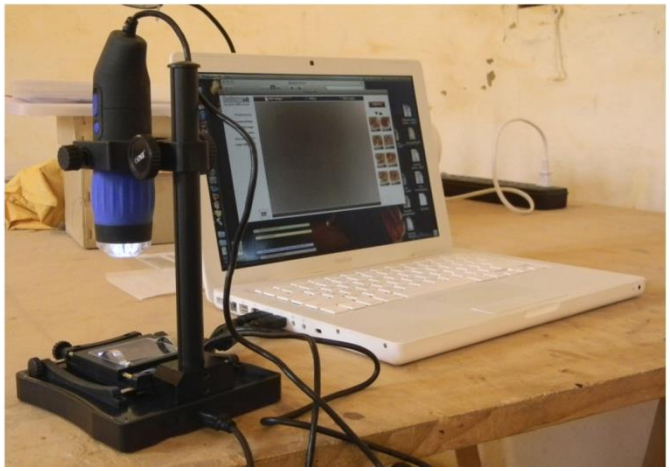

Figure 19: Portable microscope and stand with computer set up

Results

Of the 14 tissue samples processed using the experimental paleohistological field methodology, 32 slides were created, 84% of which exhibited visible cellular structure. A summation of results can be found in Table 3. The remaining 16% did not display visible cellular structure for a number of reasons including poor sample positioning within the paraffin block, tissue folding due to separation of section and slide during staining and transparency of the tissue itself. Insufficient wax infiltration occurred with Autopsy 105; the wax cracked exposing one side of the tissue making sectioning nearly impossible.

Cover slipping slides was deceptively challenging. The mounting medium used, Shur/Mount® was very thick and did not evenly distribute itself under the cover slip. This caused continual bubbling around the samples but did not impede microscopy. Figures 20 through 29 provide a visual representation of the results, highlighting the best microscopic images collected from each sample.

Skin provided the best images in terms of tissue recognition and visible cell structure. Even though fixation in 10% buffered formalin was not possible in the field, tissue type was still recognizable for both Deir Abu Metta skin (Figure 28) and muscle (Figure 29) samples processed. Muscle (Figure 29) did not show specific cell structure but its characteristic striations and smooth appearance was visible.

Table 3: Results of paleohistological field methodology on sample set

Autopsy #	Sample Type	Successful Rehydration	# Slides made	Cellular Structure Visible	Notes
1	Lung	Yes	2	No	Sample was positioned poorly in block, very thin tissue visible
4	Lung	Yes	2	No	Samples folded in on themselves during staining
8	Liver	Yes	5	Yes	Sectioned wonderfully
13	Lung	Yes	1	No	Tissue had very dark almost black appearance after even after microwave washes, light cannot penetrate for microscopy
102	Lung	Yes	4	Yes	Sectioned wonderfully
105	Skin	Yes	-	-	Wax cracked when sectioned, sample has gummy texture, cannot section; too long in DMSO?
107	Liver	Yes	4	Yes	
108	Liver	Yes	2	Yes	First sample lost in methanol, second sample rehydrated well, cirrhosis present in tissue
110	Lung	No	-	-	Lost integrity during rehydration, broke apart
111	Liver	No	-		Dissolved in methanol
	Skin	Yes	5	Yes	
123	Skin	Yes	-	-	Sample has gummy texture, cannot section; too long in DMSO?
129	Liver	No	-	-	After 36 hours in DMSO sample was still hard, yet crumbled when removed
DAM TR5 32	Skin	Yes	1	Yes	Very crumbly and dry in block, flakes away like straw when sectioned
	Muscle	Yes	6	Yes	Straw-like when sectioning, air bubbles around sample in wax, tissue separating in water bath

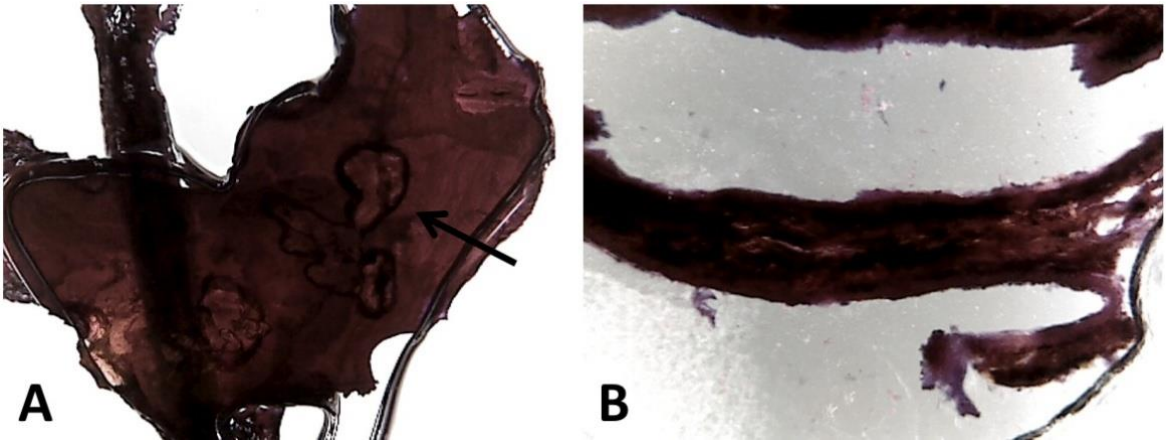

Figure 20: Autopsy 1 lung; A) Possible bronchiole (arrow) but normal alveoli not seen; B) Poor placement of tissue in block showing transverse section of collapsed lung

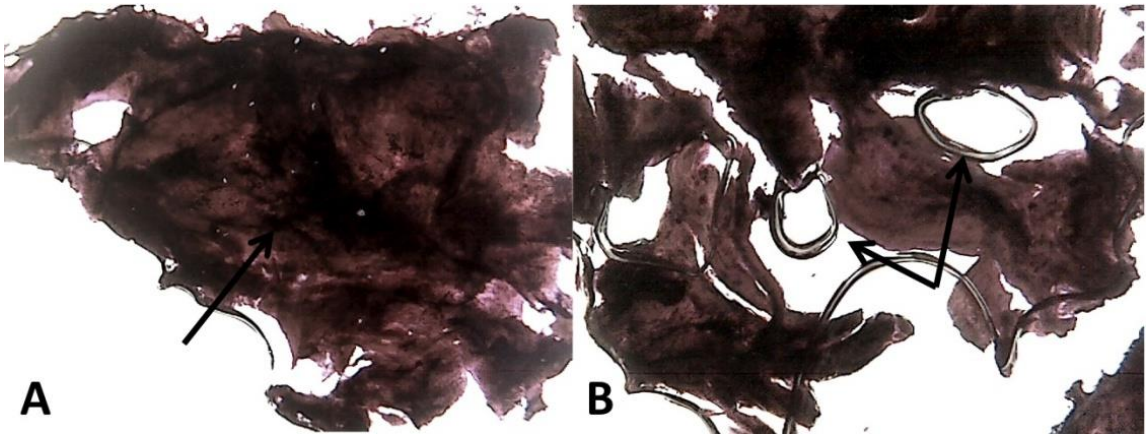

Figure 21: Autopsy 4 lung; A) Cell structure is present but tissue has folded (arrow) in on itself after staining; B) Presence of air bubbles (arrow) from cover slipping

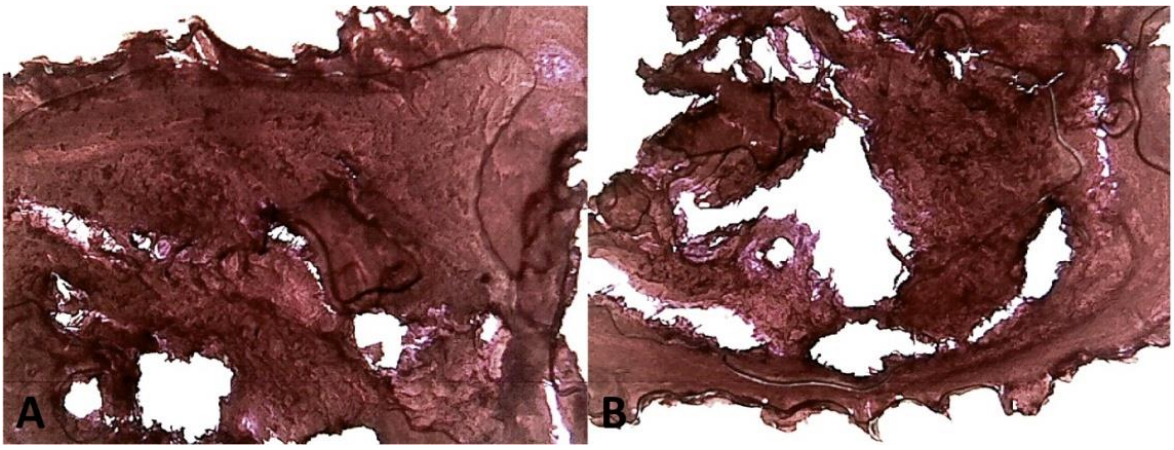

Figure 22: Autopsy 8 liver; both A and B show consistent hepatic cellular structure.

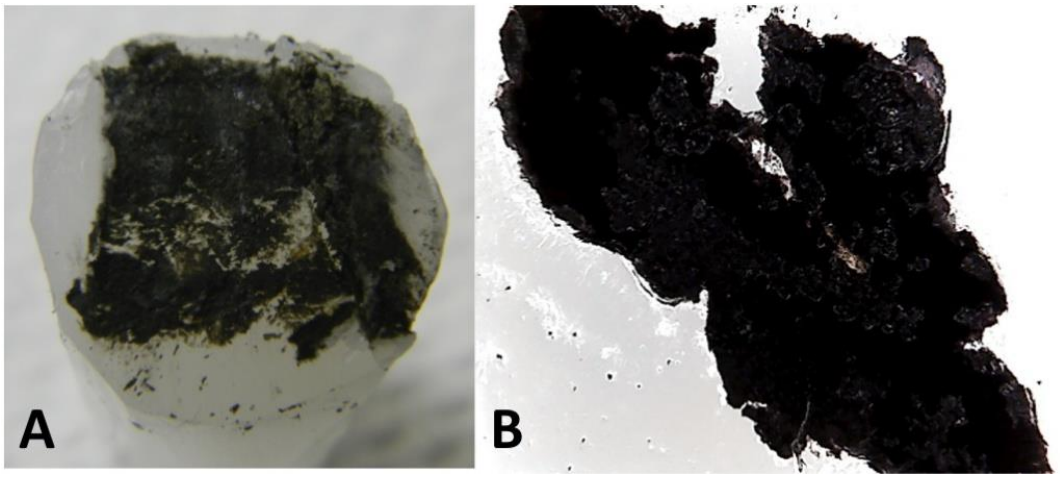

Figure 23: Autopsy 13 lung; A) Block after sectioning, tissue is black in appearance; B) Section of tissue under microscope, no light can penetrate through the already dark coloration

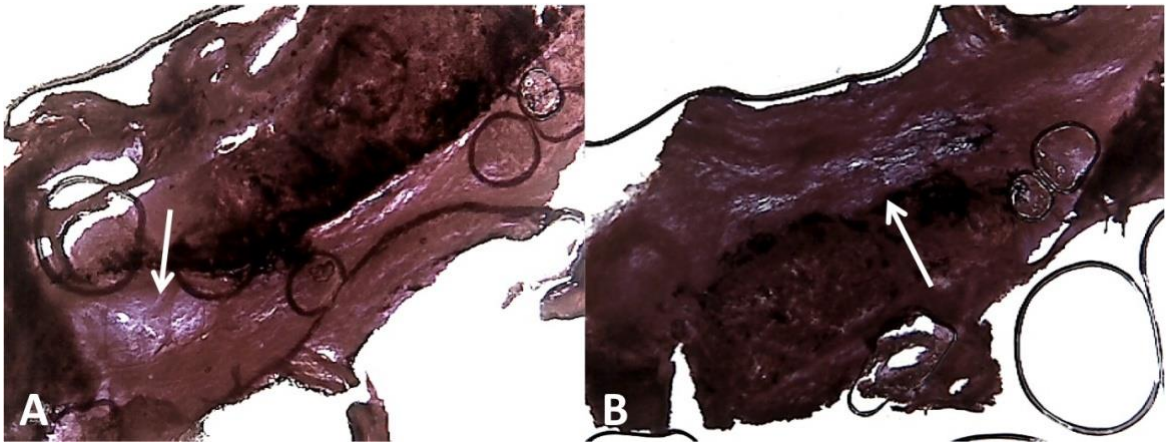

Figure 24: Autopsy 102 lung; both A and B show sections of alveoli (arrows)

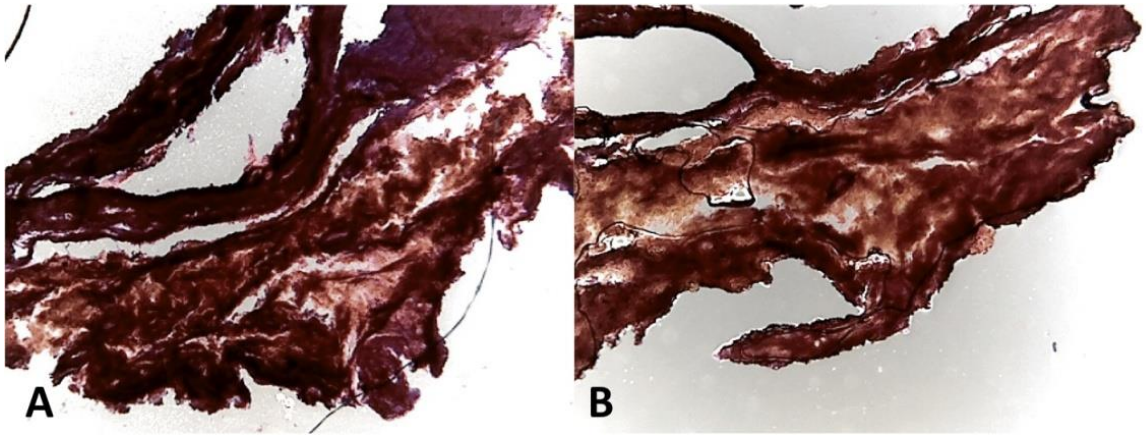

Figure 25: Autopsy 107 liver; A) Hepatic cell structure not clearly visible; B) Cellular structures slightly more visible

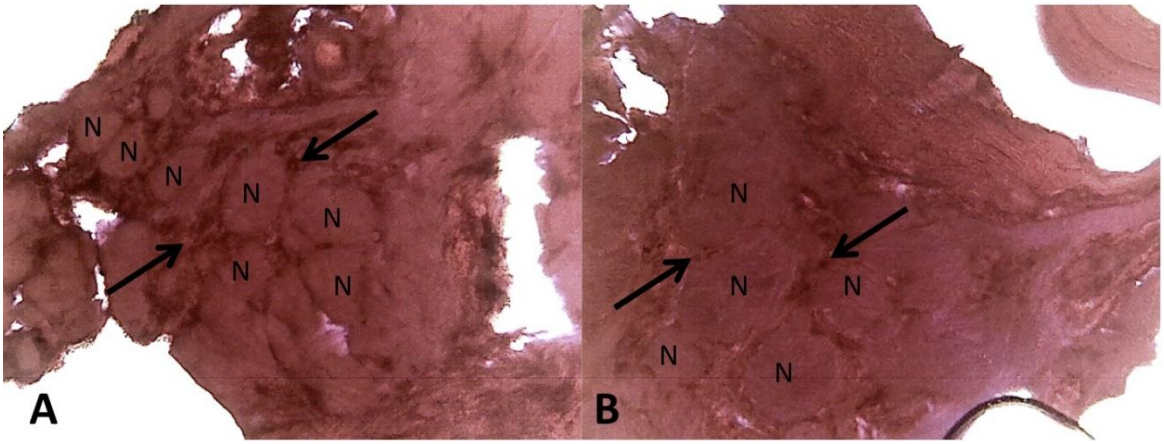

Figure 26: Autopsy 108 liver; both A and B showing nodular regions (N) separated by fibrous tissue (arrow), signs of cirrhosis

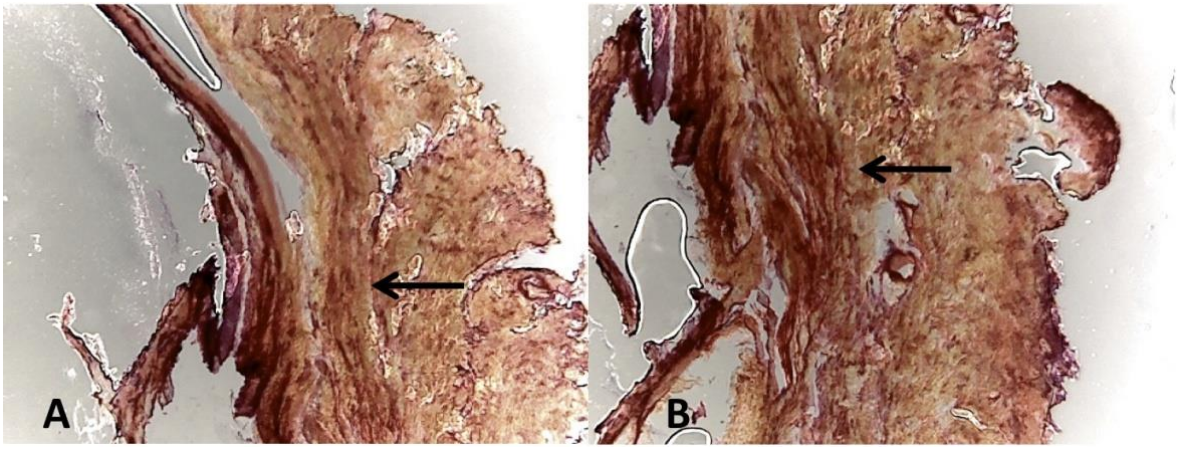

Figure 27: Autopsy 111 skin; both A and B showing parallel layers of subcutaneous region (arrows)

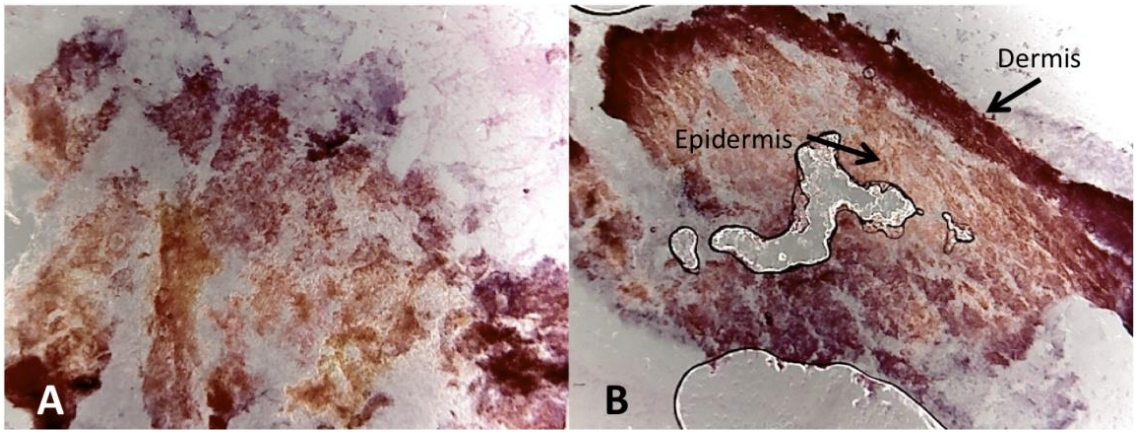

Figure 28: DAM TR5 32 skin; A) Tissue was dry and brittle and would not section completely; B) Characteristic structures of dermis and epidermis still visible

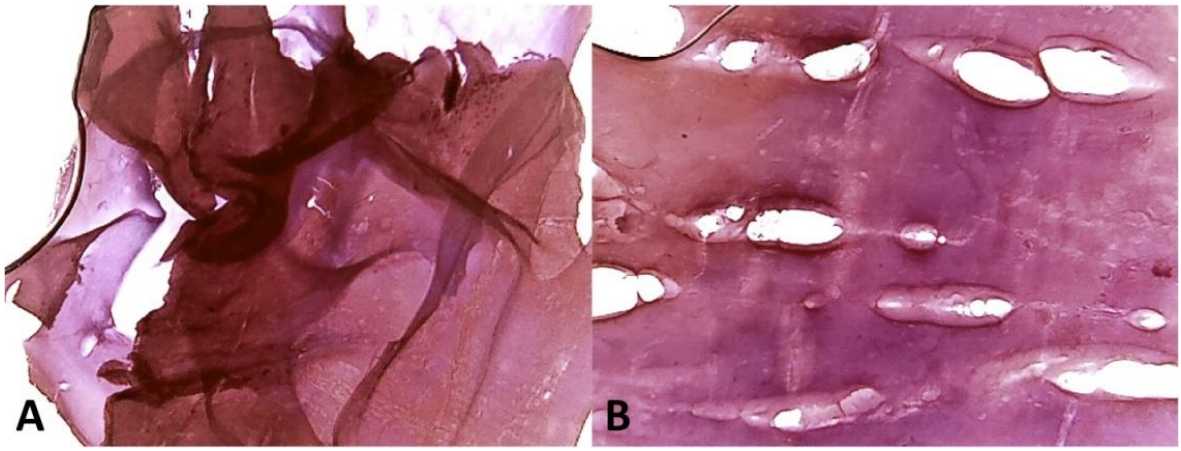

Figure 29: DAM TR5 32 muscle; A) Tissue has folded in on itself during staining; B) Characteristic striations and smooth appearance of muscle tissue

Discussion

Some difficulties were experienced in both the lab and field settings that lead to incomplete preparation of samples. Most of these obstacles can be overcome with either alternative methods or by becoming more familiar with the idiosyncrasies of the field

methodology. A potentially major drawback is the availability of chemicals in the field. As seen in this study, without the use of xylene to de-wax, tissues cannot be stained or clearly seen under the microscope (Figure 30).

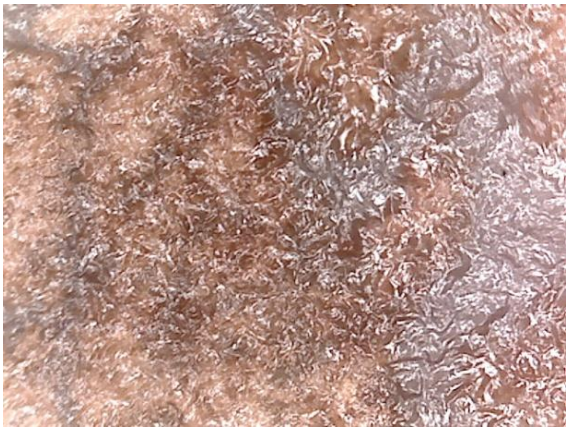

Figure 30: DAM TR5 32 skin without deparaffinization; only the waxy surface can be seen, not the cellular structure underneath

Another concern with this field methodology is the use of harsh high temperature microwave washes for dehydration and clearing on delicate rehydrated mummified tissues. Calhoun et al. (2011) does not explain how this methodology was created or from what techniques it is based. Most rapid tissue processing (Rohr et al., 2001) occurs at much lower temperatures and heats the alcohols just below their boiling point. In Calhoun et al.'s (2011) protocol, all samples are kept at a high boil and at times substantial quantities of the chemicals evaporate out of the glass tubes. To prevent this from occurring, Parafilm® could be used as an additional seal under the cap.

The modifications associated with the microwave washes performed in the field, the use of only an isopropyl alcohol wash and the replacement of xylene with acetone, were replicated in

the lab with Autopsy 102. No differences were noted in the sample and the final wash in acetone showed sufficient clearing of the rehydration fluid as it had in the field. The loss of a second alcohol wash did not seem to affect the adequate removal of DMSO.

With rehydration of artificially mummified tissue, the use of resin, both internally and externally, can cause the affected tissue to take longer in reaching optimal rehydration or potentially not rehydrate at all. This occurred with liver samples from autopsies 108 and 111; after 35 days in 5% DMSO, no improvement was observed. Both samples were placed in methanol to see if what was possibly resin would dissolve and leave the tissue to continue rehydration. After less than 24 hours in methanol, both samples were completely dissolved and lost. Autopsy notes taken by Dr. A. Aufderheide do not account for any resin present in the abdomen of either mummy. It may be possible that resin was administered during artificial mummification and was absorbed by the liver, causing it to not be readily seen on the surface of the organ at the time of autopsy. A second liver sample from autopsy 108 was rehydrated successfully. It is still unclear what inhibited the tissue from rehydrating if not resin.

In some instances, as with lung tissue from autopsy 102, a gritty residue was left after the first alcohol wash (Figure 31). It is unknown what this residue may be, possibly tissue breakdown due to the harsh microwave wash or sediment buildup released from the tissue. Tissue shrinkage was common among all samples after the first alcohol wash; this is likely due to the removal of rehydration fluid within the tissue.

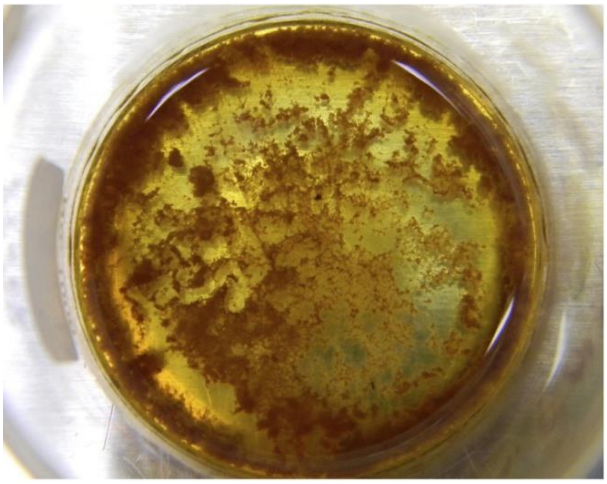

Figure 31: Unknown residue from Autopsy 102 lung after isopropyl alcohol microwave wash

Placement of the tissue within the paraffin block was not an easy task. Best results occurred when the sample was kept toward the bottom of the mold with about a 1.5cm layer of wax beneath it and a longer 'tail' of wax created by filling the entire PVC mold (Figure 32). Allowing paraffin blocks to air-dry also created minor shortcomings. This created a slight concave effect at the top of the block as it hardened because the edges cooled before the middle. This uneven cooling was exacerbated in the field and even caused air bubbles to form (Figure 33), most likely due to the atmospheric temperatures in the Oasis.

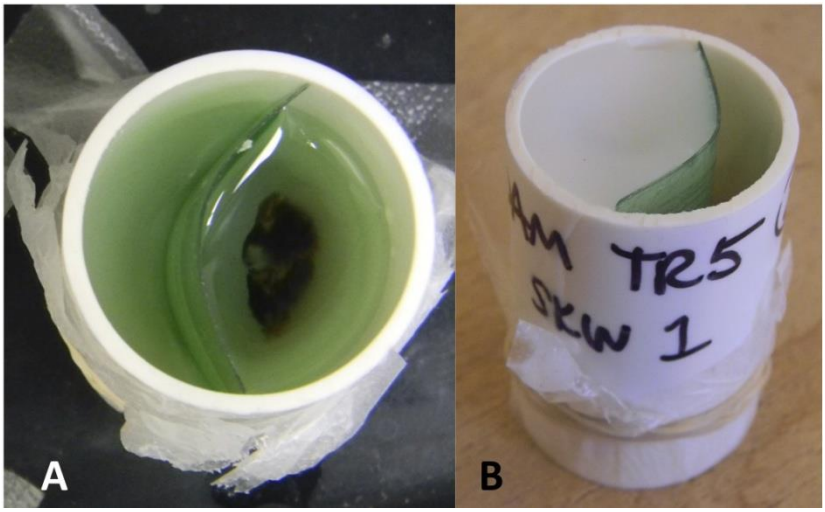

Figure 32: A) Tissue sample in melted wax during embedding; sample kept towards the bottom of the mold; B) Filled wax mold after embedding

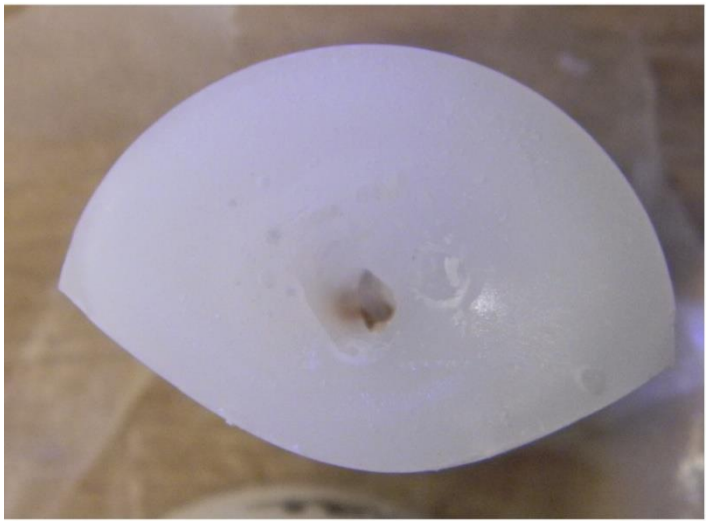

Figure 33: Air bubble formed due to wax hardening too quickly in desert climate

The Uchida® DK-10 microtome is not the ideal machine for paraffin sectioning but as a field friendly, basic microtome it works well enough as long as precautions are taken to achieve best results. As the blade became sticky with residual wax, it continued to move even with the added metal washers. As a result, periodic wipes of xylene were needed to keep the blade clean.

Another issue with this microtome was the progression of the paraffin block itself. As it advanced toward the blade it would reach a point where the housing was fully extended, this necessitated the removal and repositioning of the block, losing its original facing with the blade (Figure 34). By positioning the tissue towards the bottom of the mold during embedding and creating a longer 'tail' to be clamped in the metal housing, there was minimal need to reposition the block once faced. To aid in smoother sectioning, a desk lamp was used to keep the block slightly warmed. Sections tended to curl as they were cut; therefore an angled probe was used to coax the sections out smoothly (Figure 35). Some samples and blocks had too large of a cutting surface for the blade and sections could not be fully removed from the block (Figure 36A) creating a lip of wax (Figure 36B) above the blade that needed to be periodically removed.

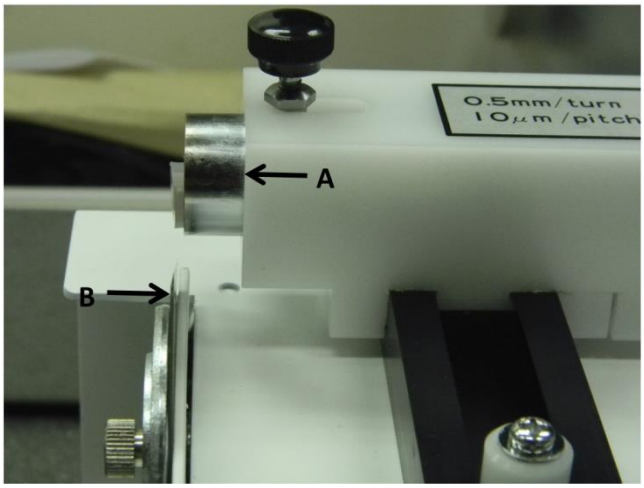

Figure 34: A) Microtome housing fully extended; B) Blade

Figure 35: Author Jennifer Branson sectioning paraffin block; note the use of desk lamp and angled probe

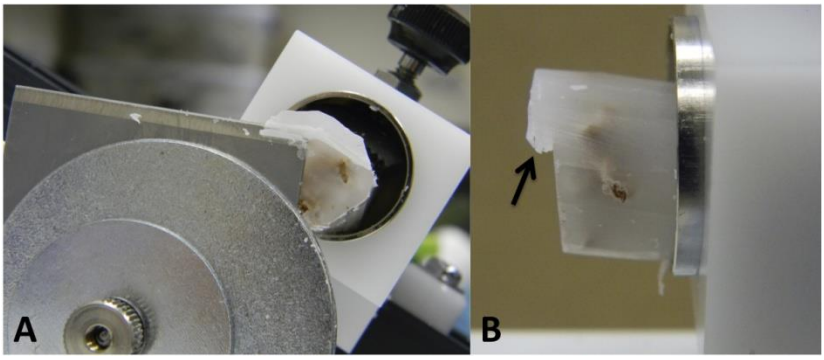

Figure 36: A) Block is too large for blade to fully section; B) Lip created due to poor sectioning (arrow)

During the staining procedure approximately 95% of all samples floated off their slides after deparaffinization. Multiple techniques were employed to control this behavior including further melting of sections onto slides after drying and the use of beakers, petri dishes and coplin jars to prevent tissue mixing. No particular method prevented this from occurring therefore each autopsy tissue type was stained individually. Because the tissue was no longer adhering to the slide it became difficult to reapply the section without tissue overlap occurring. This, however,

did not prevent the samples from being sufficiently stained. It is unknown why the samples floated off their slides, it is not a common problem with fresh tissue histology. There were no occurrences of this problem found in the paleohistology literature.

Conclusion and Future Research

A majority of the items used in this field paleohistology kit should be part of standard laboratory equipment, with the exception of a field microscope, portable microtome and the 'do-it-yourself' PVC molds with plastic plates and plastic wrap. The most expensive of these items is the portable microtome ($229 USD) and field microscope ($108 USD for scope and stand). A listing of the costs for all other items used in the field paleohistology kit can be found in Table 4. Even with the purchase of everything listed in the kit, the total cost is still under $1000 USD. Assuming all other items are supplied, the minimal resources needed in the field are electricity and water.

The basis of Calhoun et al.'s (2011) technique was for immediate biopsy processing but this quick turnaround is not necessary for mummified material. The use of high temperatures in this methodology may not be suitable for all types of tissue. A future study could compare histological integrity between high and low temperature microwave washes or the feasibility of hand processing with graded alcohols. Though this may be a time consuming process, it may yield a higher rate of results.

Table 4: Costs associated with items used in paleohistology field kit

Qty	Description	Cost – USD	Qty	Description	Cost – USD
1L	10% formalin	$19	500mL	Harris hematoxylin stain	$29
1L	HCl	$8.10	500mL	Eosin stain	$18
500mL	Ethyl alcohol	$6.15	4oz	Shur/Mount®	$34
450mL	Xylene	$8	100g	TRIS buffer	$16
500mL	Isopropyl alcohol	$3	450mL	5% aqueous DMSO	$32
2	500mL plastic bottles	$20.95ea	1	600mL glass beaker	$5.40
7	8 dram glass capped tubes	$2ea	1	150mL glass beaker	$3.65
1	10mL polypropylene graduated cylinder	$3.20	4	30mL glass beakers	$3.70ea
1	Plastic coplin jar	$4.95	1	Electric hot pot	$11
1	DK-10 sliding microtome	$229	1 box	Microtome blades	$19.95
1	Dissecting kit	$11.75	1 box	Kimwipes®	$4.15
1 bag	Paraplast®	$15.75	72pkg	Glass microscope slides	$4.95
4	2x1 inch PVC molds	$3.39	100pkg	Glass cover slips	$5
1	Portable microscope stand	$22	1	Microscope box	$4
1	USB digital microscope	$86	1	Spray can of canola oil	$5.75
1	Thermometer	$6	1pkg	Plastic plates	$5
1box	Filtration masks	$2.75	1pkg	Plastic disposable pipettes	$14.50
4	50mL plastic centrifuge tubes	$1ea	Misc	Lab coat, safety glasses, gloves, marker, plastic wrap, rubber bands, paperclips, metal washers, desk lamp, nail polish, glass carafe, Parafilm®	$50

Field-friendly alternatives to xylene may be a mixture of isopropanol and mineral oil or dishwasher soap (Buesa and Peshkov, 2009), but these have not been tested with mummified tissues. To eliminate the use of other harsh chemicals such as hydrochloric acid, alternative rehydration fluids could be further tested. Mekota and Vermehren (2005) rank Solution III, which employs the use of "Comfort" fabric softener, as an all around acceptable rehydration solution for various tissues. Possible alternatives for the 10% buffered formalin are the use of a 70/30 alcohol/xylene solution or 70% alcohol as a fixative (Beebe, 2000) but these have not been tested on rehydrated mummified tissue. These alternatives could be cost effective and safer on the environment.

The sample size used in this study was small and limited to only skin, muscle, lung and liver. Further testing is needed on other tissue types to see if this methodology will continue to provide adequate diagnostic images.

CHAPTER 3: PALEOHISTOLOGICAL EXAMINATION OF LUNG AND LIVER TISSUE SAMPLES FROM KELLIS 1 CEMETERY SITE, DAKHLEH OASIS, EGYPT

Introduction

The development of techniques for histological processing of mummified tissue (Ruffer, 1921; Zimmerman, 1977) has made it possible to evaluate health and disease in ancient populations on a microscopic level. The study of various diseases affecting ancient populations along with knowledge of dietary habits and lifestyles can provide important information about past environments and way of life (Ciranni and Fornaciari, 2004). The objective of this study is to analyze paleohistological samples using a field technique to identify and possibly diagnose and evaluate the etiology of conditions within the Dakhleh Oasis.

Overview of Potential Paleopathological Conditions of the Liver and Lung

Pathological conditions of the liver present in antiquity may include, but are not limited to hepatitis, cirrhosis, fatty change, hemorrhage and the presence parasites such as schistosomes or liver flukes. Paleopathological evidence of cirrhosis has been found in an 800 year old Thule child in northern Alaska (Zimmerman, 1998) and at Kellis 1 in Egypt (Zimmerman and Aufderheide, 2010). Schistosomes have been found in the liver of 2100 year old Japanese mummies (Peng and Wu, 1998) as well as in the rectum of mummies from Kellis 1 in Egypt

(Zimmerman and Aufderheide, 2010). Other parasites, such as liver flukes have also been found in Egyptian mummies from the 12th Dynasty (Curry et al., 1979). An individual's diet and living conditions could dictate a majority of potential lung and liver diseases but the presence of parasites like schistosomes would indicate the individual had contact with standing water containing the parasite (Adamson, 1976; David, 2000). In the Dakhleh Oasis, the most likely origin of shistosomes is the Nile River, 400 kilometers to the east. As this Oasis was along trade routes in antiquity, it is not unlikely that an individual could have visited the Nile Valley, became infected with shistosomes and returned to Dakhleh. Schistosomiasis is still a major cause of liver disease in modern Egypt.

Cirrhosis is a pathologic condition characterized histologically by the presence of nodules in the liver ranging in size from small to large, surrounded by fibrous scar tissue (Wiegand and Berg, 2013). Cirrhosis is a disease that affects the whole liver and therefore impairs liver function. The nodular effect is a result of regeneration stimulated by repetitive liver injury and the fibrotic tissue results from persistent inflammation (Wiegand and Berg, 2013). It is not always possible to prove etiology through histological examination, although in some cases of viral hepatitis, lymphocytes can be found infiltrating the liver. The two main causes of cirrhosis in clinical studies are viral hepatitis (B and C) and alcohol abuse (Wiegand and Berg, 2013). In modern Egypt, hepatitis C virus (HCV) has replaced schistosomiasis as the foremost cause of liver disease (Strickland, 2006).

Causes of paleopathological diagnoses of cirrhosis are scarce. In most instances, it is inferred that cirrhosis is caused by parasites like schistosomes (Millet et al., 1998; Peng and Wu, 1998). Alcoholism is a frequent cause of cirrhosis in modern societies but usually takes several decades of abuse to become apparent (Robbins et al., 2010). Zimmerman and Aufderheide

(2010) note the presence of fibrosis in the liver of an adult male from Kellis 1 and conclude that since there were no parasite ova present, cirrhosis was likely due to alcohol consumption. Hepatitis B virus (HBV) was recently found in a Korean mummy from the 16th century (Bar-Gal et al., 2012) through ancient DNA (aDNA) analysis. Cook (1994) diagnoses possible cirrhosis of the liver as being caused by hepatitis in a naturally mummified male from Dakhleh. In the case of a 16th century Italian mummy (Ciranni and Fornaciari, 2004), various modern methods were used to determine the cause of cirrhosis with no clear results.

Lung, a much more delicately preserved tissue, can still hold traces of scarring caused by the inhalation of dust and sand in the Egyptian environment. Particles of sand as well as scarred fibrous tissue have been found histologically in Egyptian mummies (Tapp et al., 1975; Curry et al., 1979; Tapp, 1979; Cockburn et al., 1998). Pneumonia (Aufderheide, 2000) and even emphysema (Zimmerman, 1998) in lung tissue have also been documented in antiquity.

Pneumoconiosis, the accumulation of inhaled particles in the lung, can be classified histologically as both fibrotic and nonfibrotic (Chong et al., 2006). Some fibrotic forms of pneumoconiosis are silicosis, sand pneumoconiosis and coal worker pneumoconiosis (Chong et al., 2006). Nonfibrotic forms can include siderosis from iron oxide, stannosis from tin oxide and baritosis from barioum sulfate (Chong et al., 2006) but these are not expected to be found in antiquity.

Silicosis is a well recognized lung disease in northeast Africa and the Middle East, often referred to as “desert lung syndrome” (Derbyshire, 2007). The earliest known case was reported by Tapp et al. (1975) in the lungs of a 12th Dynasty Egyptian mummy. Histological features of silicosis include fibrosis or scar tissue throughout the lung, in some instances creating a nodular affect, as well as remnant silica particles (Derbyshire, 2007). More recently, silicosis has been

linked to tuberculosis, causing a deficiency in the immune system and an increase in infection (Derbyshire, 2007).

Anthracosis, the general presence of carbon particles in the lung, can be present with or without the occurrence of fibrotic tissue (Walker et al., 1987). When fibrotic tissue is present with large coal macules, it is defined as coal miner pneumoconiosis (Chong et al., 2006). The dry, sandy and windy environment of the desert can cause sand pneumoconiosis which, when examined under polarized light, will show particles of silica present in the lung (Cook, 1994). Both conditions could lead to pneumonia and even tuberculosis (Cook, 1994).

Materials and Methods

All tissue samples came from the Dakhleh Oasis, Egypt. The Dakhleh Oasis is one of five major depressions in Egypt's Western Desert. Located about 250 km west of the Nile Valley, the Oasis is a depression approximately 100 m below the surrounding desert, bordered by a large escarpment to the north (Williams, 2008; Wheeler, 2009). Seasonal temperatures in the Dakhleh Oasis range from -4°C - 25°C in winter and 19°C - 50°C in summer with minimal rainfall, averaging 0.3 mm/year (Sutton, 1947; Williams, 2008; Wheeler, 2009). It is an arid, dry desert environment.

The Kellis 1 cemetery, located on the northwest side of the ancient village of Kellis (also known as Ismant el-Kharab) (Figure 37), has been dated to the late Ptolemaic and early Roman period (Birrel, 1999). Dakhleh and its villages were along a caravan route through the desert from the Nile to Lybia and became an important trading center (Aufderheide et al., 1999)

Samples of mummified lung and liver tissue were chosen from 10 individuals (Table 5) belonging to the Dakhleh Oasis Project (DOP) Tissue Bank, housed at the University of Central Florida. The autopsies of these individuals were performed between 1992 and 1998 (Aufderheide et al., 2003).

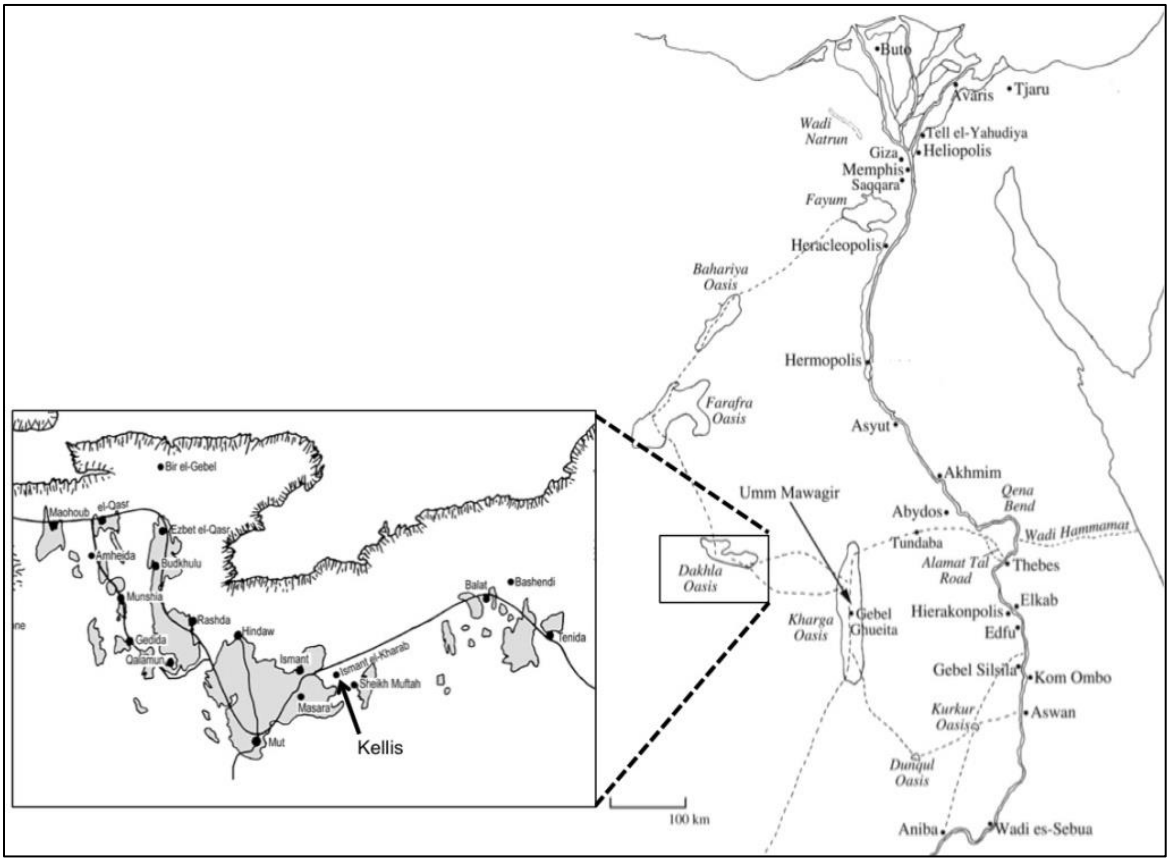

Figure 37: Map showing location of Kellis in Dakhleh Oasis, Egypt

Table 5: Demographics of liver and lung samples from Kellis 1 cemetery, Dakhleh Oasis, Egypt

Autopsy #	Body/ Tomb	Original Date Examined	Sex	Age	Sample Type	Preservation Score (0-3)	Mummification SM/AM	Notes on Resin
1	A/3	1992	M	12-17	Lung	3	SM	No evidence of resin use either externally or internally
4	E/4	1993	F	20-22	Lung	1	SM	No evidence of application of resin is evident either internally or externally
8	C/3	1993	I	8-11	Liver	2	SM	No resin identified in either cranium, spinal canal or body cavities of skin surface
13	UKN	1993	M	55+	Lung	1	AM	Outside body covered with black material and wrappings
102	B/16	1998	M	9-13	Lung	3	AM	Resin soaked body & nasal perforation; no resin in body cavities or cranium
107	C/16	1998	M	7.5-8.5	Liver	-	AM	Skin covered in resin, resin soaked linen introduced into thorax via neck
108	E/16	1998	M	21-25	Liver	-	AM	Body painted with thin layer of black resin, no evidence of resin detected within abdominal cavity
110	E/17	1998	M	30-40	Lung	-	AM	Resin introduced via posterior defect into abdomen & it extended into thoracic cavity through diaphragms
111	D/18	1998	M	20-26	Liver	-	AM	Resin only on skin, no evisceration wound
129	A/18	1998	M	5.5-6.5	Liver	-	AM	Resin introduced into abdomen via an entry defect in post.; gained access to spinal canal & small amount entered cranial cavity

Age was estimated on the basis of dental eruption, long bone length and epiphyseal fusion in sub-adults. In adults alterations in pubic symphysis, cranial suture closure and dental attrition were used. Sex was estimated by external genitalia and pelvic skeletal features. Sex: M=Male, F=Female, I=Indeterminate. SM/AM=Spontaneous mummification/Anthropogenic mummification. Preservation score rated at time of autopsy for tissues present: 0=poor to 3=good. Autopsy reports courtesy of Dakhleh Oasis Project

The DOP Tissue Bank consists of over 400 samples from 60 individual mummy autopsies performed by various members of the DOP from 1992 to 1998. Samples include all forms of soft tissue as well as linen wrappings, nasal tampons, wooden sticks and resin scrapings. A listing of the DOP Tissue Bank inventory along with designation of samples selected for this study can be found in Appendix B. A large majority of the individuals that make up the DOP Tissue Bank are either male or of unknown sex (Figure 38) and range in age from 18-24 months to over 55 years. Mummification types found within the DOP Tissue Bank are mainly anthropogenic, Figure 39. Samples were selected at random but were chosen based on potential success, meaning tissues that exhibited unfavorable conditions such as obvious resin coatings, hard brick-like consistency or even thin paper-like consistency were not selected, see Appendix A for images of selected samples.

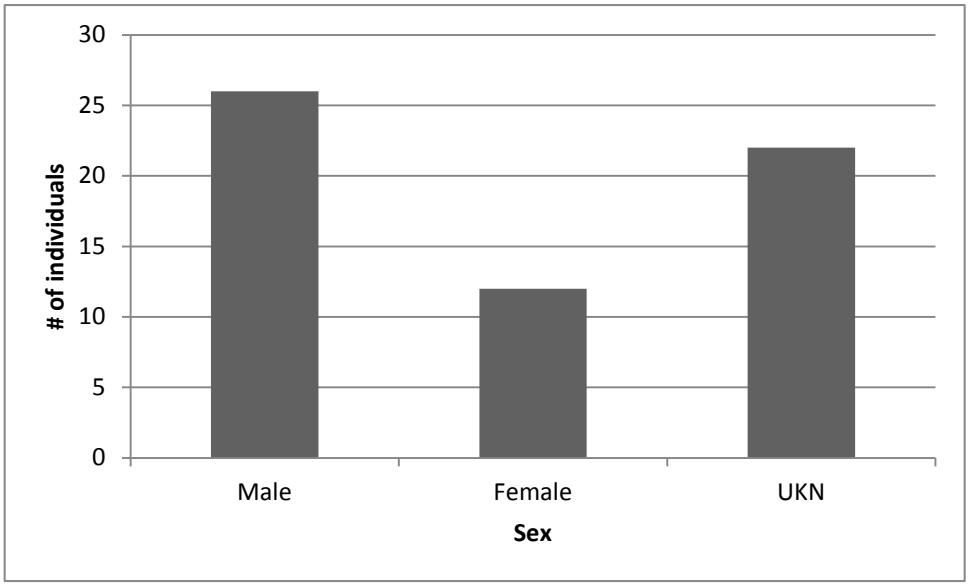

Figure 38: Sex distribution in the Dakhleh Oasis Project Tissue Bank

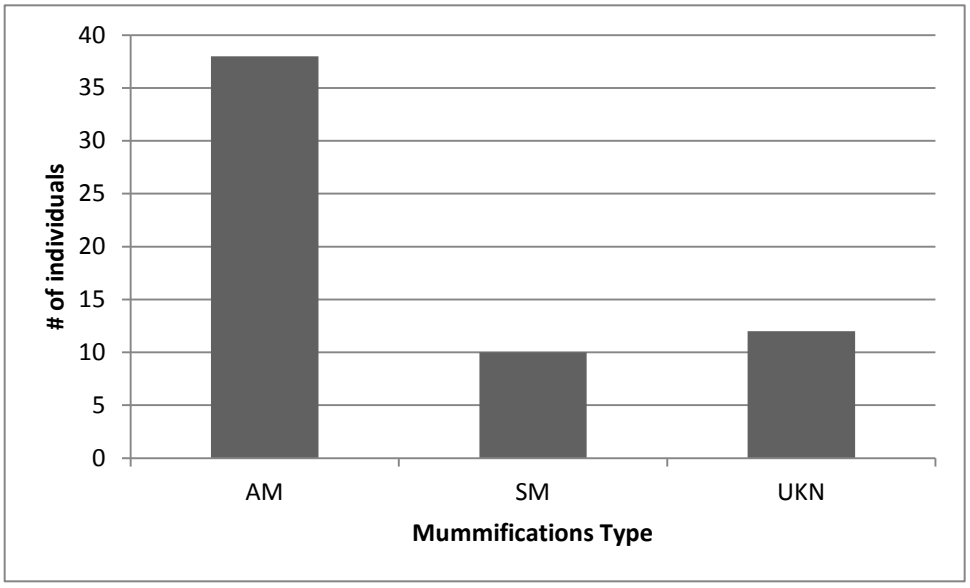

Figure 39: Mummification type distribution in the Dakhleh Oasis Project Tissue Bank

All samples were processed in the laboratory at the University of Central Florida following a field paleohistology protocol (Appendix C). The tissues were rehydrated using a 5% aqueous DMSO solution (Grupe et al., 1997). Depending on the tissue, this process took an average of 24-36 hours.

After samples reached optimal rehydration, they were fixed in 10% buffered formalin for at least 24 hours and dehydrated using a modified microwave technique (Calhoun et al., 2001). This technique consists of three 15 minute washes in ethyl alcohol, isopropyl alcohol and xylene. Samples were placed in glass-capped tubes and heated in a portable electric hot pot at 95°C to 99°C for 15 minutes each.

The processed samples were embedded using a 2x1-inch cut polyvinyl chloride (PVC) pipe fitted with a crescent shaped inner plastic divider cur from Solo® plastic plates and lubricated with Crisco® original 100% Canola Oil no-stick spray. The bottom end of the pipe

was covered with Glad® Press'n Seal® wrap and reinforced with a rubber band to prevent wax leakage. Paraplast® was melted in a hot water bath using a glass carafe and a hot pot. Tissue samples were then suspended within the mold. The paraffin block was then trimmed to fit the opening on a portable microtome. A portable Uchida® DK-10 slide microtome was used for both field and lab sectioning. The blade needed to be reinforced with two additional metal washers measuring up to two inches in diameter to improve stability. Sections were cut between 10 and 20 μm, floated in a heated water bath (35°C to 40°C), mounted on glass slides, dried, heated under a desk lamp and hand stained using a basic cellular hematoxylin and eosin stain (Appendix D). A portable microscope stand and a DP-M01 zipScope® with 200x magnification and a 2.0M pixel camera, both with light sources, was used for microscopy and software from ProScope® HR was used to examine slides and capture images. Analysis and diagnosis of the images was conducted by the author.

Results

Not all samples were successfully rehydrated and processed due to potential resin application, poor sample positioning of the sample within the paraffin block, and tissue folding due to separation of section and slide during staining; Table 6 provides a summary of these results. With tissues that were effectively rehydrated and processed, quality histological microscopic imaging was achieved. No other testing or further histochemical staining was performed to look for other pathological conditions such as bacteria-causing tuberculosis. Of the

10 individuals in this study, 20 slides were successfully produced from seven individuals. Of the 20 slides, 15 provided quality images used for paleohistological analyses.

Table 6: Success of rehydration and histopathologic findings of samples from Kellis 1 cemetery

Autopsy #	Sample Type	Successful Rehydration	# Slides made	Cellular Structure Visible	Disease Identified	Notes
1	Lung	Yes	2	No	-	Sample was positioned poorly in block, very thin tissue visible
4	Lung	Yes	2	No	-	Samples folded in on themselves during staining
8	Liver	Yes	5	Yes	No	Sectioned wonderfully
13	Lung	Yes	1	No	-	Tissue had very dark almost black appearance after even after microwave washes, light cannot penetrate for microscopy
102	Lung	Yes	4	Yes	I	Sectioned wonderfully
107	Liver	Yes	4	Yes	No	
108	Liver	Yes	2	Yes	Cirrhosis	First sample lost in methanol, second sample rehydrated well, cirrhosis present in tissue
110	Lung	No	-	-	-	Lost integrity during rehydration, broke apart
111	Liver	No	-	-	-	Dissolved in methanol
129	Liver	No	-	-	-	After 36 hours in DMSO sample was still hard, yet crumbled when removed

I = indeterminate

Liver tissue from Autopsy 8 showed consistent hepatic cellular structure with no sign of disease or parasite present (Figure 40).

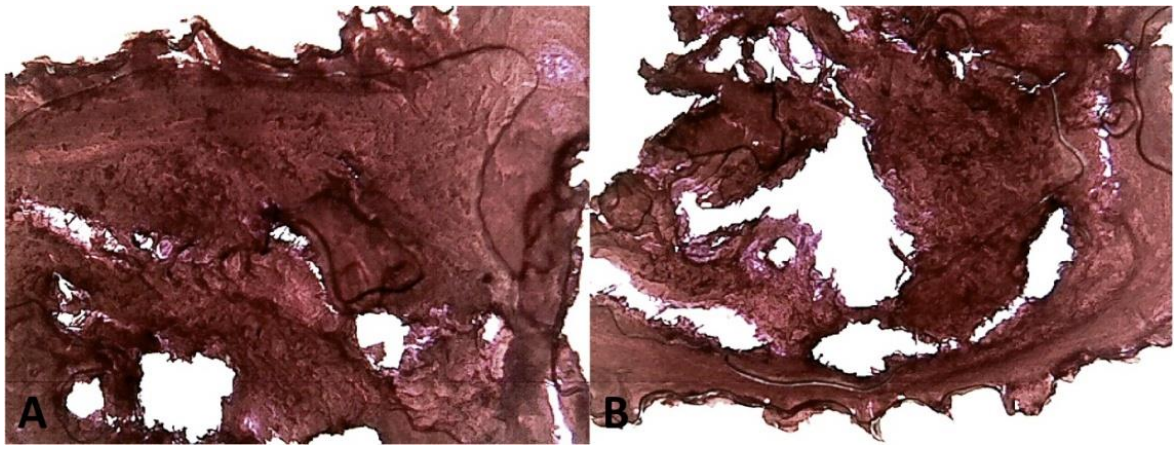

Figure 40: Autopsy 8 liver; both A and B show consistent hepatic cellular structure

Lung tissue from Autopsy 102 gave inconclusive results. Dark nontransparent granular-like material was seen throughout the section but clear fibrotic tissue could not be visualized (Figure 41).

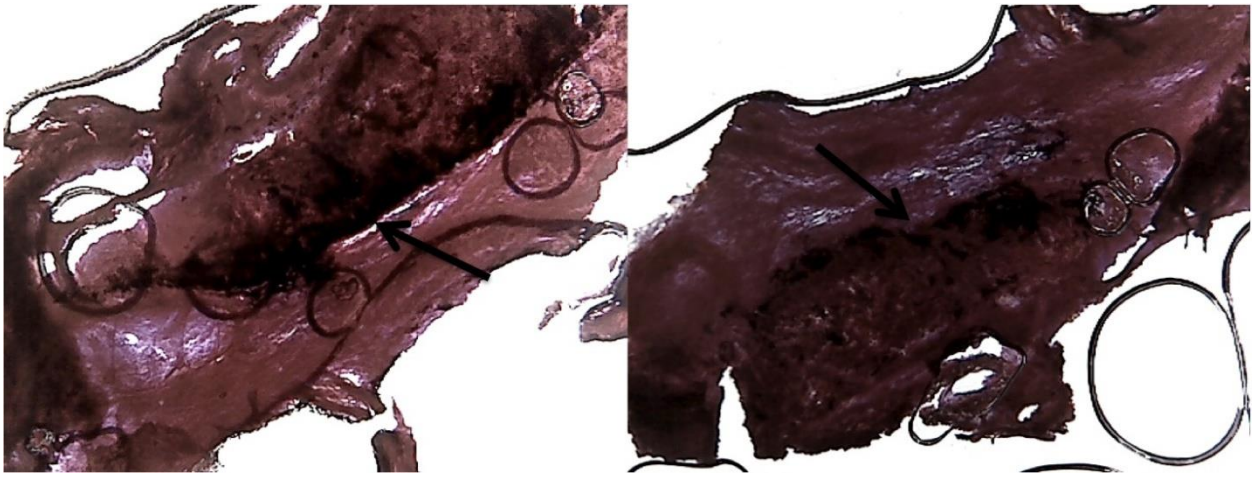

Figure 41: Autopsy 102 lung; arrows indicating dark granular material

Liver tissue from Autopsy 107 showed vague cellular structure with no sign of disease or parasite present (Figure 42).

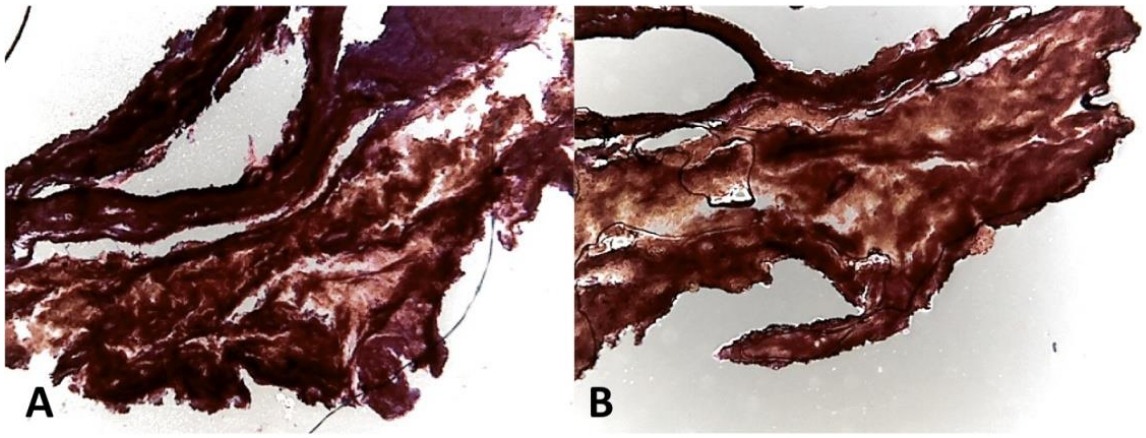

Figure 42: Autopsy 107 liver; A) Hepatic cell structure not clearly visible; B) Cellular structures slightly more visible

Liver tissue from Autopsy 108 showed course bands of fibrous tissue surrounding areas of hepatic parenchyma in a micro-macronodular organization (Figure 43) clearly revealing this young adult male suffered from cirrhosis (Junqueira and Carneiro, 2003).

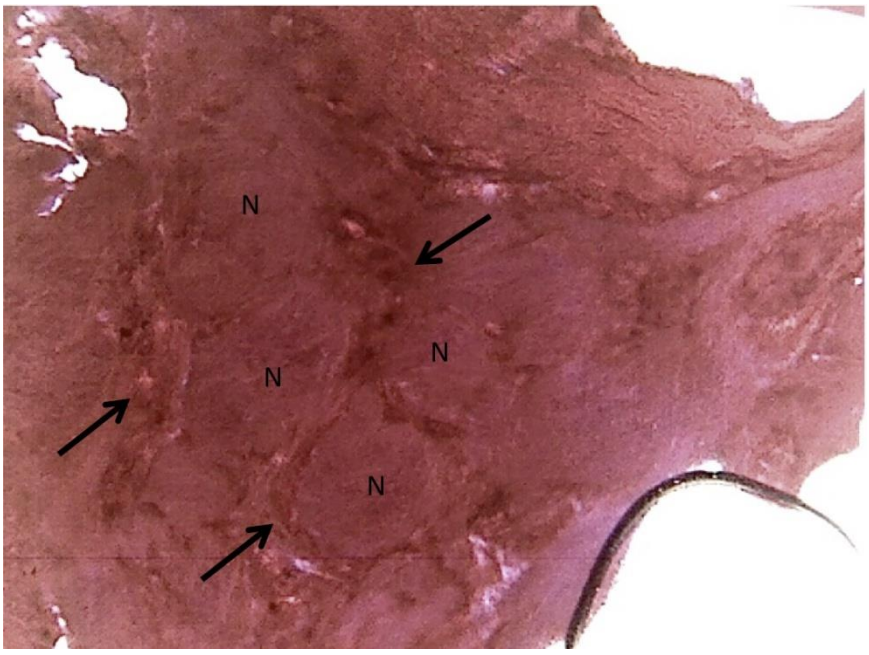

Figure 43: Autopsy 108 liver; arrows indicate fibrous tissue; N designates cirrhotic hepatic nodules

Discussion

Cook and Sheldrick (2001) note that most of the preserved lung tissue in mummies from the Kellis 1 cemetery showed the effects of living in a dry environment. Without further testing, such as mineral analysis or microscopy using electron diffraction to test for the presence of silica in lung, the contents of the dark granular material from Autopsy 102 may not be known. Even if the present material was found to be silicate crystals it is not a definitive occurrence of the disease silicosis without interstitial fibrosis (Walker et al., 1987). A possible explanation of the presence of silica without fibrosis is anthrocosis, black carbon deposits within the lung, which have been found in numerous mummified lung specimens from ancient Egypt (Walker et al., 1987). The inhalation of carbon in the form of smoke or soot would not be uncommon in ancient Egypt, even during the late Ptolemaic and early Roman period individuals working in blacksmithing, metalworking, pottery kilns and even the daily use of home hearths would regularly breath carbon in the air. Both sand pneumoconiosis and anthrocotic pigment have been previously found in individuals from Kellis (Cook, 1994; Cook and Sheldrick, 2001

Despite the typical histological presence of hepatic nodules and fibrosis in Autopsy 108's liver tissue, the etiology of this cirrhosis is difficult to determine. No parasites or parasitic ova were present in any histological sections but only a small fragment of the whole liver was tested. Schistosome parasite ova have been previously found in the rectum of an individual from Kellis 1 (Zimmerman and Aufderheide, 2010) It would not be improbable to find parasites such as schistosomes in the Oasis considering the trade routes that run through the area. Following previous analyses from Zimmerman and Aufderheide (2010) where cirrhosis was present without

parasites, it could be diagnosed as alcoholic type cirrhosis. If this is the case, than the individuals age, 21-25 years, may be a concern. Ciranni and Fornaciari (2004) describe a similar pattern of cirrhosis without parasite activity but state that their individual, 22-26 years, is too young for alcoholic type cirrhosis to have occurred.

Wine and beer were prevalent in Egypt throughout antiquity. Kharga, a large oasis east of Dakhleh connected by trade routes, was well known for its wine production through the Roman era (McGovern, 1997; Ikram, 2008). It is not unreasonable to believe that an individual could have started consuming alcohol in their early teens and after a decade of use developed alcoholic type cirrhosis.

Another important staple in the Egyptian diet was wheat. Wheat is a significant source of carbohydrates. The percentage of carbohydrates in the diet can have an influence on nonalcoholic fatty liver disease and cirrhosis (McCarthy and Rinella, 2012). A low-fat, high-carbohydrate diet increases the chance for development of fatty liver and the risk of hepatic inflammation (McCarthy and Rinella, 2012; Hasse, 2013).

Conclusion and Future Research

Quality microscope slides and digital images were obtained from both lung and liver samples indicating this point-of-care field method is a viable option for paleohistological field analyses and identification of pathological conditions in mummified human remains. A future consideration would be to process samples from these same tissues using standard laboratory histological techniques to compare image quality and tissue integrity.

The sample size used in this study was small and only accounted for roughly 16% of the available autopsy samples in the DOP Tissue Bank. For future study, it would be interesting to see how prevalent cirrhosis and especially anthrocosis is in this population. The experimental paleohistological field methodology used to process and examine these tissues has its limitations including the restriction of microscopic techniques. The potential use of a polarized light filter for the portable microscope could aid in the identification of particles in the lung. Additionally, other histochemical stains, such as the use of Masson's trichome (Walker et al., 1987) for better histologic detail of the lung or stains used in the identification of fungi or bacteria, such as tubercle bacilli possibly present in the lung. Unfortunately, not all necessary chemicals are available in the field.

CHAPTER 4: CONCLUSIONS

With continual restrictions on the export of archaeological material, it is becoming increasingly necessary to collect as much data as possible in the field. With regards to mummified remains, a more in-depth evaluation of paleopathological conditions may include histological examination of preserved mummified soft tissue. Previously, this was only possible in a laboratory setting. This study has established a paleohistological field methodology for the processing of mummified tissues in a remote area of the Dakhleh Oasis, Egypt.

Chapter two provides a step-by-step description of the field paleohistology protocol and the possible costs associated with putting together a paleohistology field kit. Although problems with field processing did occur, cellular structure was visible in all four types of tissue processed. Further study is needed to find chemical alternatives that may be more readily available in field conditions. For example, replacing the 5% aqueous DMSO rehydration solution with a fabric softener based solution would eliminate the need for hydrochloric acid. The basis of this methodology and the modified microwave washes created by Calhoun et al. (2011) was for immediate biopsy processing for medical missions. This quick turnaround is not necessary for mummified material and the microwave washes may be too harsh of a process for some mummified tissue (e.g. lung). Further study is needed to see if a modified laboratory process of dehydration, clearing and infiltration with graded alcohols is possible in the field.

Chapter three discusses lung and liver paleopathologies detectable through histological examination. With the use of a field paleohistology protocol, mummified tissues of lung and liver from the Kellis 1 Cemetery in the Dakhleh Oasis, Egypt were evaluated for the presence of any possible pathologies or parasites. Cirrhosis of the liver was clearly found in a young adult

male, possibly caused by a diet rich in carbohydrates and alcohol. Dark granules were found in lung tissue from a juvenile male but it was undetermined whether they were silica deposits from sand or carbon deposits from smoke inhalation. The presence of both lung pathologies in one individual would not be uncommon due to working conditions and the dry environment of a desert.

The overall purpose of this study was to achieve quality microscopic images from mummified tissue using an experimental field paleohistological protocol. It was found to be successful in both the lab and field setting with histological analysis resulting in identification of two different pathological conditions from two different individuals. With continued research in the field of paleopathology and paleohistology a greater understanding of diseases affecting ancient populations can be achieved.

APPENDIX A: AUTOPSY & SAMPLE TISSUE IMAGES

Autopsy 1
File Code: EG-DK2-3-A-0
Field Accession Code: 31/420-C5-1/3, Body A

Analyzed by A.C. Aufderheide
Date: 2-Dec-93

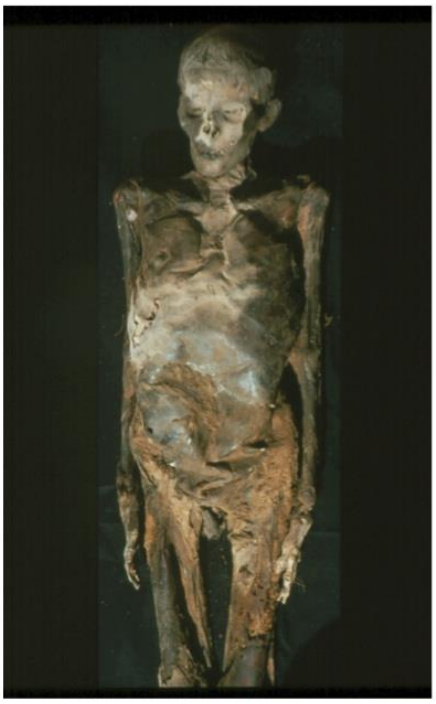

Figure 1: Autopsy 1, mummy anterior view

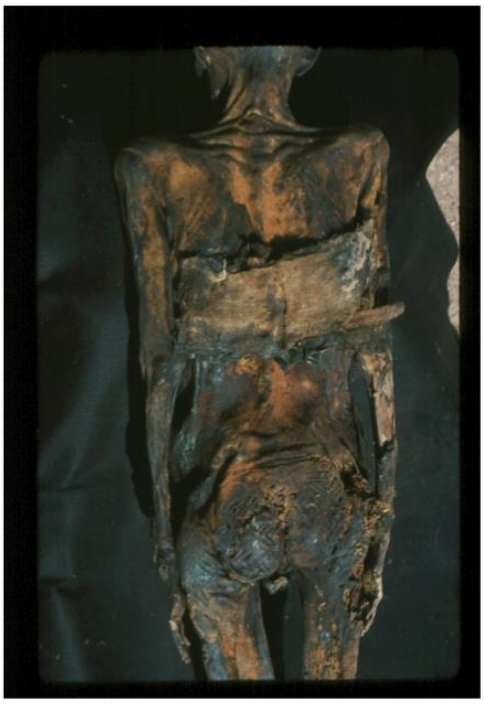

Figure 2: Autopsy 1, mummy posterior view

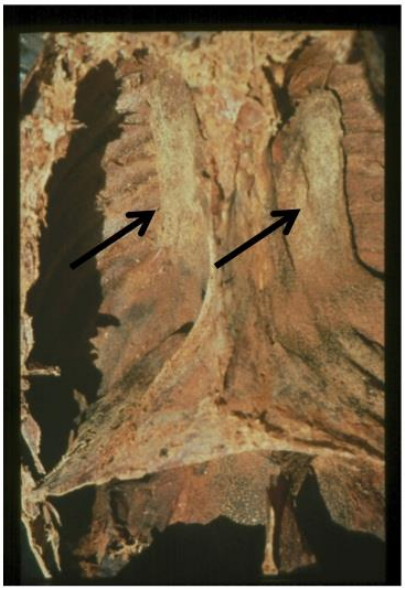

Figure 3: Autopsy 1, exposed chest cavity showing collapsed lungs (arrows)

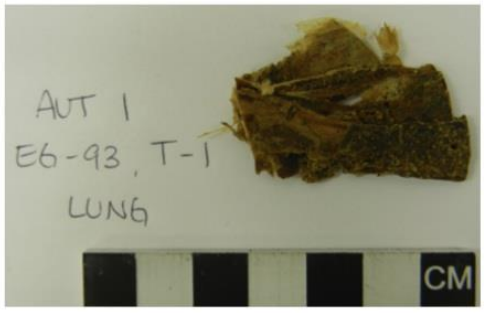

Figure 4: Autopsy 1, lung sample

Autopsy 4
File Code: EG-DK2-4-E-0
Field Accession Code: 31/420-C5-1/4, Body E

Analyzed by Larry Cartmell
Date: 5-Dec-93

Figure 1: Autopsy 4, mummy anterior view

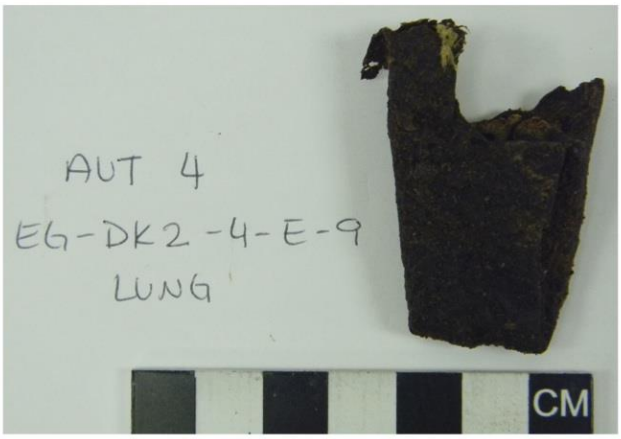

Figure 2: Autopsy 4, lung sample

Autopsy 8
File Code: EG-DK2-3-C-0
Field Accession Code: 31/420-C5-1/3, Body C

Analyzed by A.C. Aufderheide
Date: 7-Dec-93

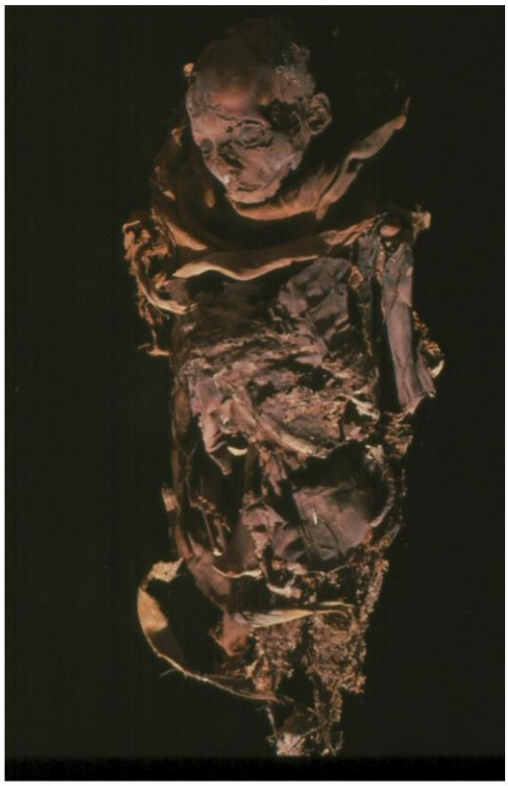

Figure 1: Autopsy 8, mummy with wrappings, anterior view

Figure 2: Autopsy 8, liver sample

Autopsy 13
File Code: EG-93-A-13
Field Accession Code:

Analyzed by Zlouis
Date: 12-Dec-93

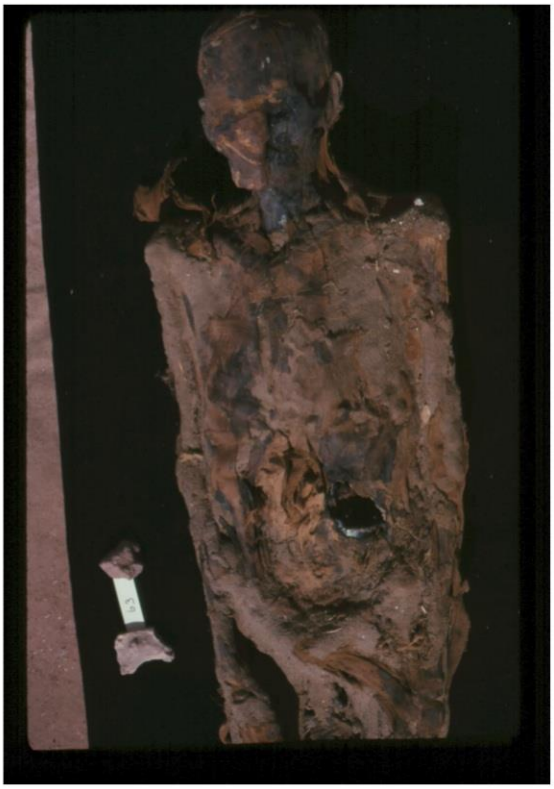

Figure 1: Autopsy 13, mummy with wrappings, anterior view

Figure 2: Autopsy 13, lung sample

Autopsy 102
File Code: EG-A-102
Field Accession Code: 31/420-C5-1/16/B

Analyzed by Larry Cartmell
Date: 3-Dec-98

Figure 1: Autopsy 102, full mummy with wrappings, anterior view

Figure 2: Autopsy 102, lung sample

Autopsy 105
File Code: EG-DK-16-A105
Field Accession Code: 31/420-C5-1/16/4

Analyzed by Larry Cartmell
Date: 2-Dec-98

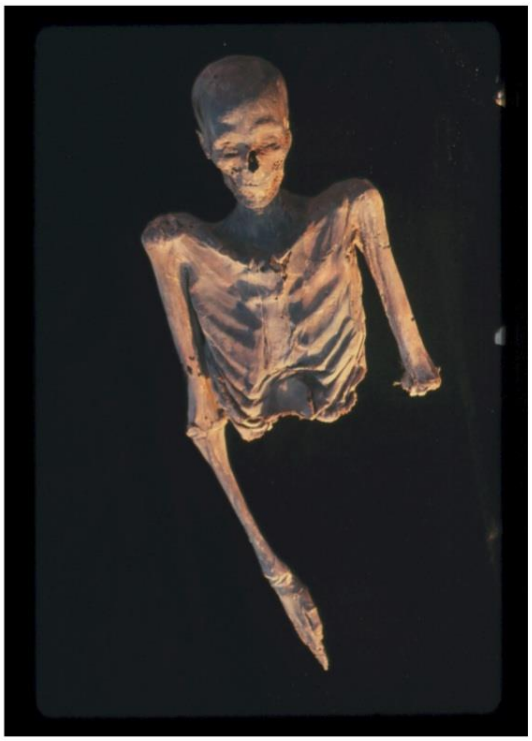

Figure 1: Autopsy 105, full mummy, anterior view

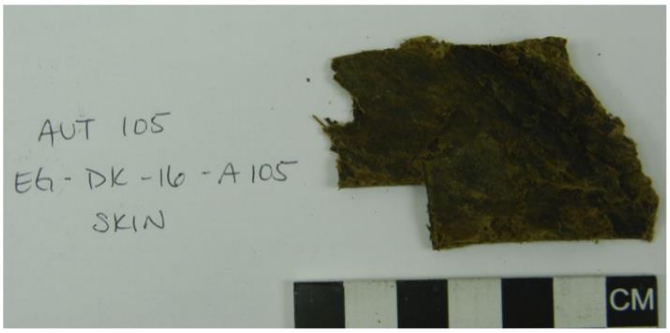

Figure 2: Autopsy 105, skin sample

Autopsy 107
File Code: EG-A-107
Field Accession Code: 31/420-C5-1/16/C

Analyzed by A.C. Aufderheide
Date: 3-Dec-98

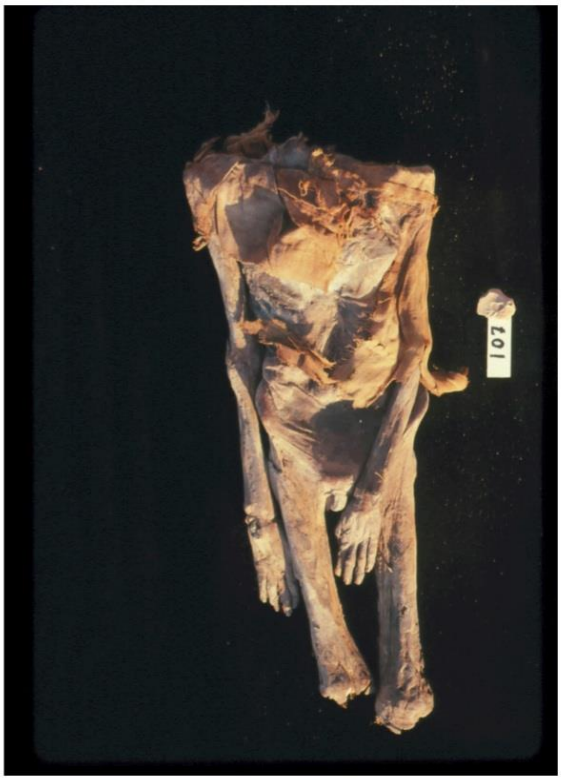

Figure 1: Autopsy 107, full mummy, anterior view

Figure 2: Autopsy 107, liver sample

Autopsy 108
File Code: EG-A-108
Field Accession Code: 31/420-C5-1/16/E

Analyzed by A.C. Aufderheide
Date: 1-Dec-98

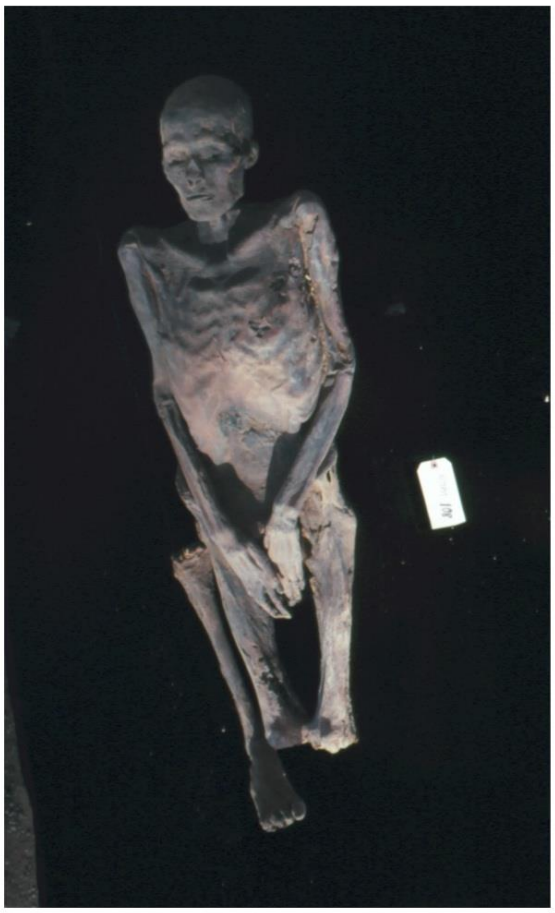

Figure 1: Autopsy 108, full mummy, anterior view

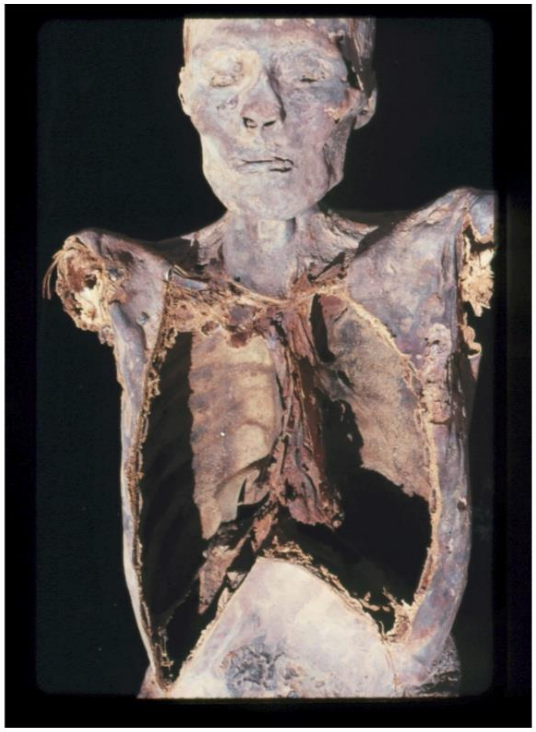

Figure 2: Autopsy 108, exposed chest cavity

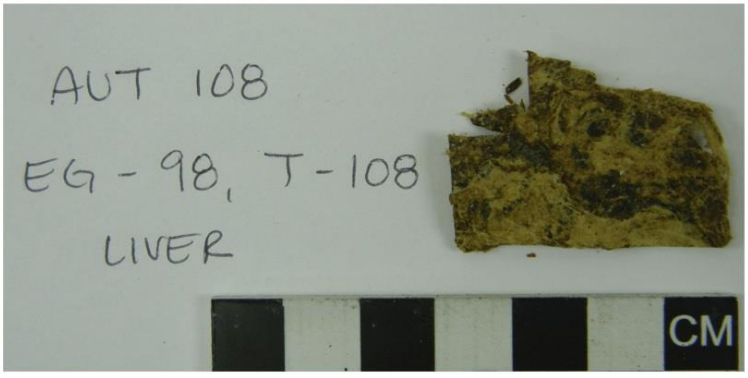

Figure 3: Autopsy 108, liver sample

Autopsy 110
File Code: EG-A-110
Field Accession Code: 31/420-C5-1/17/E

Analyzed by A.C. Aufderheide
Date: 5-Dec-98

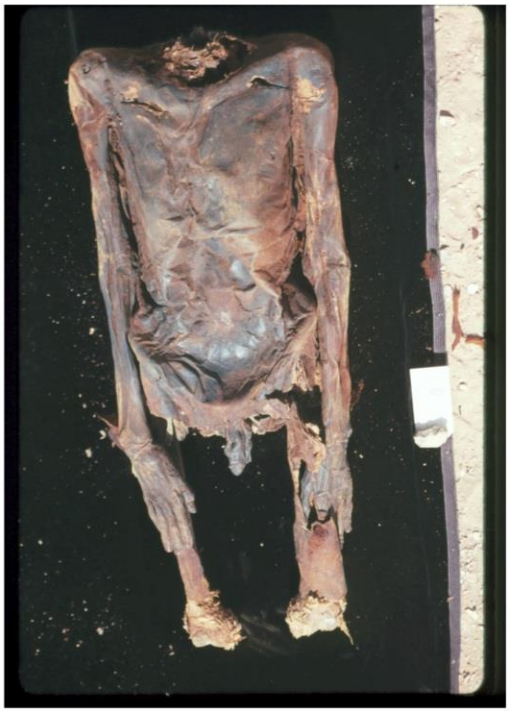

Figure 1: Autopsy 110, full mummy, anterior view

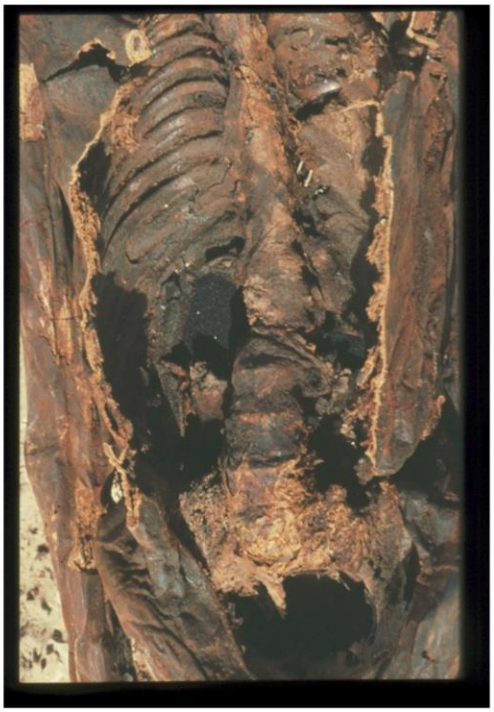

Figure 2: Autopsy 110, exposed chest and abdominal cavity

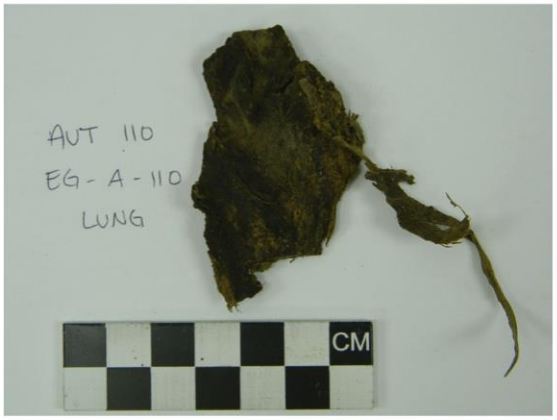

Figure 3: Autopsy 110, lung sample

Autopsy 111
File Code: EG-A-111
Field Accession Code: 31/420-C5-1/18/D

Analyzed by A.C. Aufderheide
Date: 12-Dec-98

Figure 1: Autopsy 111, full mummy in wrappings, anterior view

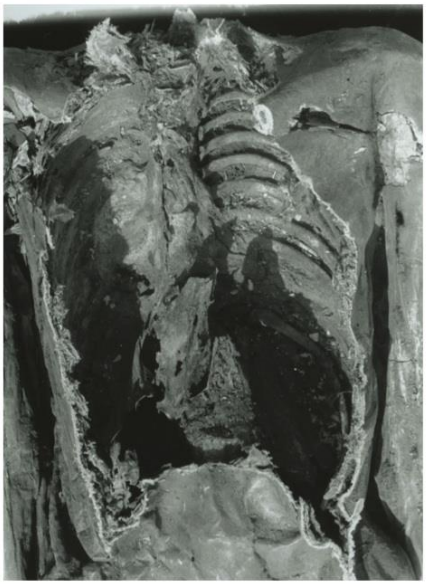

Figure 2: Autopsy 111, exposed chest cavity

Figure 3: Autopsy 111, liver sample

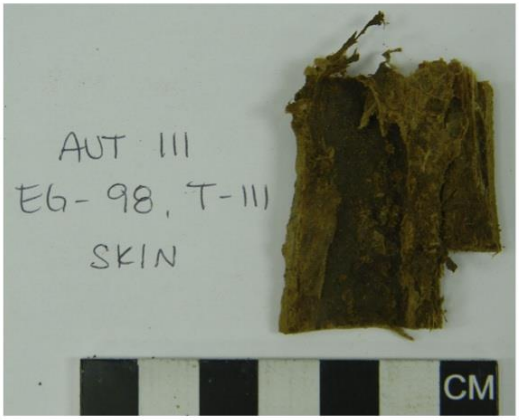

Figure 4: Autopsy 111, skin sample

Autopsy 123
File Code: EG-A-123

Field Accession Code:

Analyzed by A.C. Aufderheide
Date: 7-Dec-98

Figure 1: Autopsy 123, mummy in wrappings, anterior view

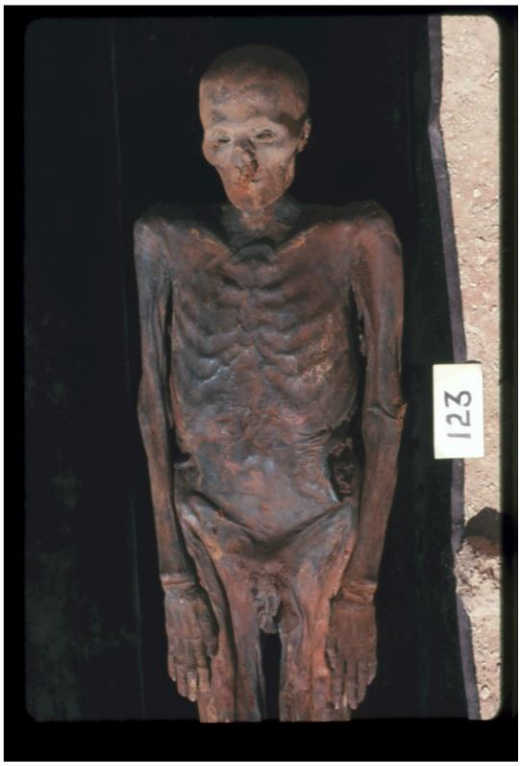

Figure 2: Autopsy 123, mummy, anterior view

Figure 3: Autopsy 123, evisceration wound

Figure 4: Autopsy 123, skin sample

Autopsy 129
File Code: EG-A-129

Field Accession Code: 31/420-C5-1/18/A

Analyzed by A.C. Aufderheide
Date: 12-Dec-98

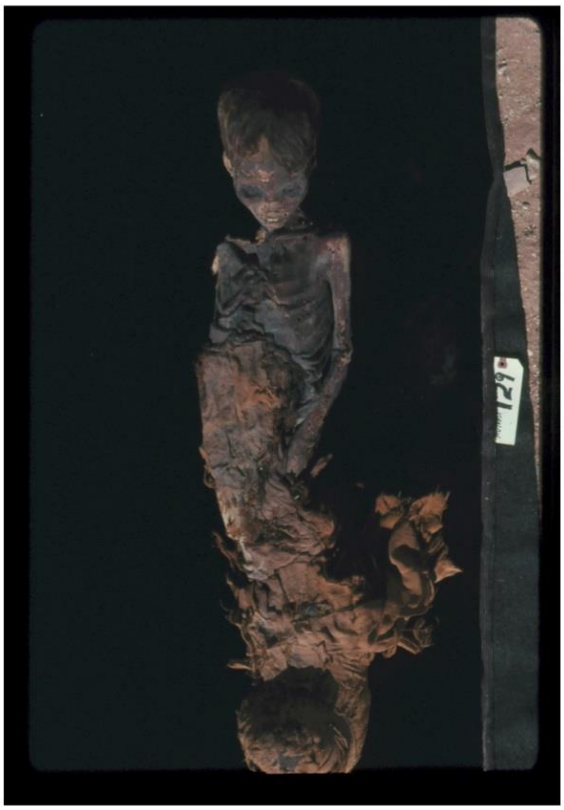

Figure 1: Autopsy 129, full mummy in wrappings, anterior view

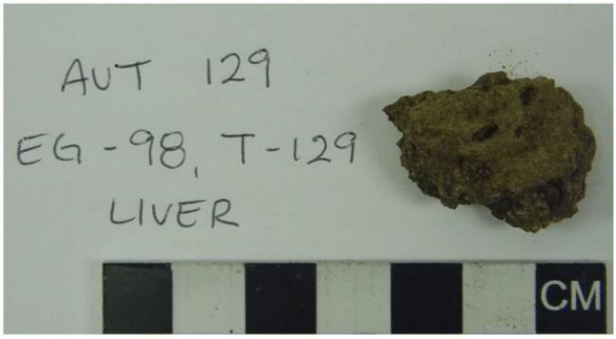

Figure 2: Autopsy 129, liver sample

Deir Abu Metta
DAM TR5 32

Analyzed by Dakhleh Oasis Project Bone Team
Date: 10-Dec-12

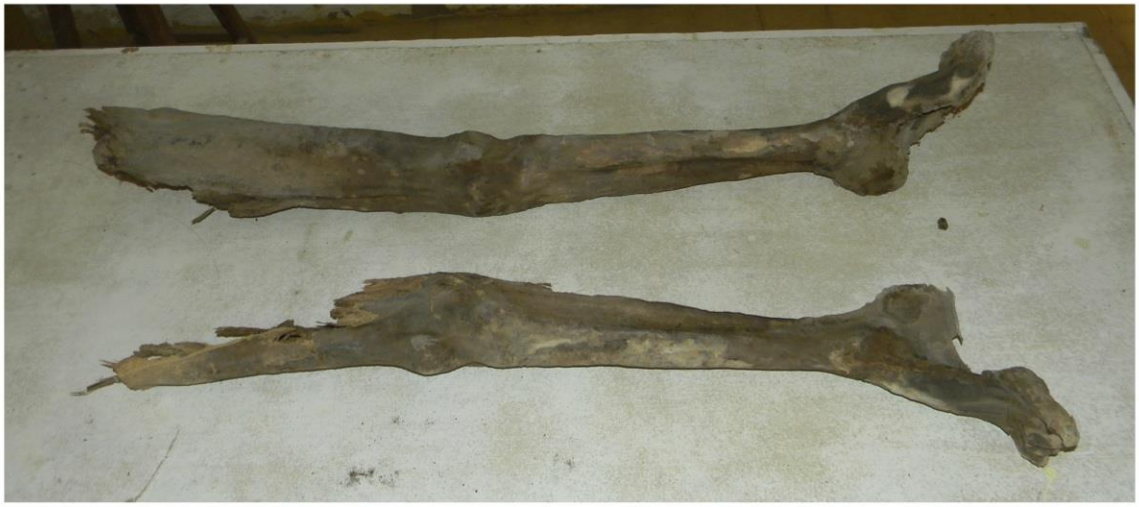

Figure 1: DAM TR5 32, mummified legs

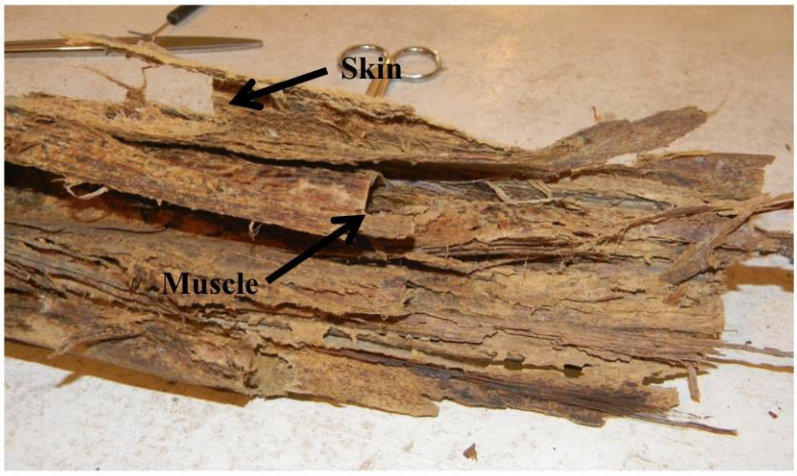

Figure 2: DAM TR5 32, section of interior leg where skin and muscle was removed

APPENDIX B: DAKHLEH OASIS PROJECT TISSUE BANK INVENTORY & SAMPLES SELECTED

Autopsy#	Sex	Age	SM/AM	Sample Selected	Tissue
1	M	12-17	SM		L R knee cartilage
					intervertebral disc
					rib cartilage
					muscle
					soft tissue
					skin/muscle
					textile
					R ear
					mass L ear canal
				X	R L lung
					diaphragm
					heart
					pericardial sac
					liver
					kidney?
					R L eye
					teeth
					hair
					scalp/hair
2	U	18-24mo	SM		external wrapping
					hair
3	M	25	AM		penis/scrotum
					scrotum
					muscle from femur
					muscle
					skin/muscle from leg
					textile
					fabric/hard material from abdominal wound
					wrappings from body
					blackened material from abdomen
					fabric adjacent to abdominal wound
					wrappings from head
					material from abdominal wound
					upper mediastinum/esophagus
					pericardial sac
					heart
					R L eye
					fingernail
					toenail
					teeth
					blackened material from chest
					hair
					material from under chin
4	F	20-22	SM		L breast

					R breast
					intervertebral disc
					rib cartilage
					muscle
					skin
					R ear
				X	R L lung
					diaphragm
					heart
					liver
					R L eye
					fingernail
					toenail
					teeth
					hair
					insect from chest
5	M	45-40	AM		penis/scrotum
					resin from cranium
					rib cartilage
					knee meniscus
					muscle
					skin/muscle
					internal portion of nasal tampon
					external portion of nasal tampon
					R L ear
					diaphragm
					pericardium
					heart
					liver
					fingernail
					toenail
					crystal from eye
					material cranial vault
					black crystalline material from chest cavity
					R retroperitoneal crystal/ blood?
6	F	25	SM		intervertebral disc
					material from pelvis prob tissue
					R breast
					muscle from leg
					R ear
					L lung
					R L eye
					fingernail
					teeth
					debris from posterior abdomen/chest
					hair
7	F	23	AM		resin
					cloth from L supra clavicular space

					nasal tampon
					bowel
					L ear
					L eye
					stick from head
					material from spinal canal
					free-lying black material from thorax
					material from pelvis
					material from L chest
					black material from surface of R mandible
					stick from vertebral column
					hair
8	U	8-11	SM		intervertebral disc
					rib cartilage
					bowel
					L ear
				X	liver
					L kidney?
					R L eye
					LUQ content
					intral cranial content
					stick in skull
					hair
9	M	5.5	AM		intervertebral disc
					black material /linen from perineum
					bowel
					R ear
					diaphragm
					trachea/mediastrinum
					liver
					R L eye
					tongue
					fingernail
					hair/scalp
10	F	45	SM		muscle from leg
					material overlying vertebral column - possible bowel
					R ear
					diaphragm
					R L eye
					fingernail
					teeth
					material from interior of chest
					hair
11	F	20-35	AM		L breast nipple
					intervertebral disc
					rib cartilage
					wrapping

					wrapping R thorax
					botanical items from chest cavity
					stick in spine
12	M	55-60	SM		skin/muscle
					muscle
					abdominal skin
					R L eye
					teeth
					hair/scalp
13	M	55	AM		penis/scrotum
					intervertebral disc
					muscle from leg
					soft tissue from pelvis
					soft tissue from over lumbar vert
					resin coated fabric
					colon
					R ear
				X	R L lung
					diaphragm
					trachea
					heart
					liver
					R L eye
					hair/scalp
					insects L eye
14	M	55-60	AM		skin
					textile
					hair
15	F	45-50	AM		skin
					eye
					finger with nail
					asceptic tampon
					hair
17					textile
					hair/scalp
19					textile from inside skull
28					textile from head
21					resin from hand
					hair/scalp
30					textile from head
33					hair
35					hair
37					hair
38					hair/scalp
40					coprolite
41					hair
56					R L eye
101	M	20-30	SM		penis/scrotum

					resin?
					intervertebral disc
					muscle
					muscle from arm
					scalp
					skin from arm
					textile
					colon
					colon contents
					small intestine
					cecum contents
					coprolite
					coprolite deep rectum
					rectal coprolites
					ear
					R L lung
					diaphragm
					pericardium
					liver
					tongue
					R L eye
					R thumb nail
					teeth
102	M	9-13	AM		penis/scrotum
					semilunar cartilage
					chordae tendinae
					skin
					bowel
					brain
				X	trachea/lungs bilateral
					liver
					R L eye
					tongue
					fingernail
					teeth
					hair
103	M	adult	AM		penis/scrotum
					meniscus
					tendon
					muscle
					skin lower leg
					coprolite
					R L eye
104	F	50+	AM		resin post thorax
					skin/resin
					wrappings
					resin cloth from abdomen
					clay from abdomen

					support palm rib cervical
105	M	?	AM		costo chondral cartilage
					intervertebral disc
					tendon hand
				X	skin
					R ear
					L lung
					larynx/trachea
					R L eye
					hair
106	F	13-17	AM		breast nipple
					labia
					intervertebral disc
					small bowel
					coprolite
					ear
					diaphragm
					pericardial sac
					tongue
					teeth
					hair
107	M	7.5-8.5	AM		intervertebral disc
					skin/muscle
					textile from chest cavity
					external textile
					bowel
				X	liver
108	M	21-25	AM		penis/scrotum
					resin skin of back
					rib cartilage
					skin from arm
					textile
					colon
					ear
					lung
					ascending aorta
					heart
					pericardium
				X	liver
					R L eye
					tongue
					fingers with nail
					teeth
					anterior neck structure
					hair
109	U	8-12	AM		hair/scalp
110	M	30-40	AM		penis/scrotum
					resin pelvis

					muscle
					skin from thigh
					textile
					bowel
				X	L lung
					trachea bronchi R lung
					pericardium
					fingernail
111	M	20-26	AM		penis/scrotum
					skin
					ear
					lung
					diaphragm
				X	liver
					R L eye
					fingers with nail
					toes with nail
					hair/scalp
112	U	7-8	AM		intervertebral disc
					muscle from thigh
					skin/muscle
					skin
					rectum
114	M	6-8	AM		penis
					resin present in embalming incision
					intervertebral disc
					muscle
					skin
					urinary bladder
					fingernail
					toenail
115	F	28-32	AM		R breast
					resin abdominal cavity
					resin from exterior of mummy wrappings
					achilles bilateral tendon
					umbilicus
					resinous material from L lower leg wrapping
					resin thoracic cloth
					external wrapping
					ear
					R L eye
					toenail
					hair
117	M?	adult	AM		skin
					linen
					brain
					R L eye
					hair/textiles

					hair/scalp
118	U	6-8	SM		skin
					eye
					hair/scalp
119	M	40-50	AM		resin from cranium
					skin
					external wrapping
					nasal tampon
					L ear
					teeth
					hair
120	U	9-11	AM		nasal tampon
					ear
					R L eye
					teeth
					hair/scalp
121	U	6-8	SM		skin
					ear
					L eye
					hair/scalp
122	M	35-39	AM		pubic hair
					resin from skull
					resin R chest
					external genitalia
					semilunar cartilage
					muscle
					skin/muscle
					skin
					nasal tampon
					external wrapping
					ear
					diaphragm
					L kidney crystals
					resin coated liver?
					R eye
					toenail
					teeth
					hair/scalp
123	M	50+	AM		penis/scrotum
					resin
					muscle
				X	skin
					R L eye
					fingers with nail
					toes with nail
					teeth
					hair
					hair/scalp

124	M	50+	AM		rib cartilage
					muscle
					skin from chest
					skin from face
					R L eye
					hair/scalp
125	F	47-43	AM		resin
					muscle
					skin
					ear
					pericardium
					liver
					R L eye
					teeth
					hair/scalp
					insects cranial cavity
					insects thigh
					insects feet
					LLQ evisceration wound
126	M	30-40	AM		penis/scrotum
					resin chest cavity
					rib cartilage
					muscle
					skin
					rectal contents
					R L Lung
					diaphragm
					trachea/aspirated tooth
					trachea
					ascending aorta
					teeth
					insects
127	M	adult	AM		muscle
					nails
					skin
128	F?	6-8	AM		resin
					skin
129	M	5.5-6.5	AM		penis/scrotum
					muscle
					skin from leg
					bowel content
					ear
				X	liver
					R L eye
					fingers with nail
					toes with nail
					hair/scalp
130	M	6-8	AM		penis/scrotum

				resin thorax
				skin/muscle
				textile
				fingers with nail
				hair
131	M	7.5-8.5	AM	muscle
				skin
				ear
				R L eye
				fingers with nail
				teeth
				hair/scalp
132	U	3-5	AM	resin
				skin/muscle
				cord
				hair
134	F	50+	AM	resin
				fingers with nail

APPENDIX C: FIELD PALEOHISTOLOGY PROTOCOL

Field Paleohistology Protocol

- Rehydration:
    - Prepare rehydration fluid*
        - 1L of 5% aqueous dimethyl sulfoxide (DMSO) in Tris buffer:
          -6.05g TRIS buffer
          -900mL distilled water
          -38.5mL HCl

* if DMSO is not available, see Mekota and Vermehren (2005) for other rehydration options

        - Dissolve TRIS buffer in distilled water
        - Add HCl to water/buffer solution

    - Submerge selected tissue sample in rehydration fluid for 24-48 hours or until optimal rehydration is reached
        - Continually check tissue every 24 hours

- Fixation:
    - Rinse sample with distilled water, about 3 swirls

    - Submerge sample in 10% buffered formalin* for at least 24 hours
        - At this point, sample can stay in formalin for several days if microwave washes and paraffin embedding cannot be carried our immediately

* if formalin is not available, samples may be fixed using a 70/30 alcohol/xylene solution or 70% alcohol solution

- Microwave Washes:
    - First wash*:
        - Fill hot pot to max level with tap water
        - Place thermometer in hot pot and increase temperature to between 95°C and 99°C
            - While water is heating:
                - place samples in 8 dram glass tubes and fill with 30mL of ethyl alcohol
            - Prepare tubes to be suspended on side of hot pot with rubber band and paperclip
        - Once water has reached desired temperature, suspend tube with sample on the side of the hot pot
        - Keep at consistent temperature, between 95°C and 99°C, for 15 minutes
        - Remove from hot water bath

* if only one alcohol type is available, one wash may be sufficient to dehydrate the sample or two washes in the same alcohol

****CAUTION: GLASS AND CONTENTS ARE HOT****

- Second wash:
  - Fill hot pot to max level with tap water
  - Place thermometer in hot pot and increase temperature to between 95°C and 99°C
    - While water is heating:
      - discard ethyl alcohol waste, keep sample in tube
      - fill tubes with 30mL of isopropyl alcohol
  - Once water has reached desired temperature, suspend tube with sample on the side of the hot pot
  - Keep at consistent temperature, between 95°C and 99°C, for 15 minutes
  - Remove from hot water bath

****CAUTION: GLASS AND CONTENTS ARE HOT****

- Third wash:
  - Fill hot pot to max level with tap water
  - Place thermometer in hot pot and increase temperature to between 95°C and 99°C
    - While water is heating:
      - discard isopropyl alcohol waste, keep sample in tube
      - fill tubes with 30mL of xylene* ** ***

* this must be done in a well-ventilated area
** xylene must only be used in glass containers
*** if xylene is not available, acetone may be used as a replacement in this step only

  - Once water has reached desired temperature, suspend tube with sample on the side of the hot pot
  - Keep at consistent temperature, between 95°C and 99°C, for 15 minutes
  - Remove from hot water bath*

*DO NOT LET SAMPLE COME TO ROOM TEMPERATURE AFTER THIS WASH

****CAUTION: GLASS AND CONTENTS ARE HOT****

- Wax Embedding:
  - Prepare PVC molds:

THIS SECTION SHOULD BE DONE BEFORE LEAVING FOR THE FIELD

- Cut 1-inch diameter PVC pipe into 2-inch segments
- Cut rectangle from plastic Solo® plate to the needed length and width of each PVC mold (this may be done in the field)
- Cut 3x3-inch plastic sheet from Glad® Press'n Seal® wrap, one for each PVC mold (this may be done in the field)

THIS SECTION TO BE DONE AFTER THIRD MICROWAVE WASH

- Melt paraffin:

    - Fill hot pot to max level with tap water
    - Reduce hot water bath temperature to around 40˚C
    - Fill glass carafe with paraffin chips*

* if using glass carafe from cappuccino maker, filling to about the 2 cup mark is enough to embed 3 samples

    - Place glass carafe in hot water bath
        - Keep tubes with samples still in xylene warm in the water bath while wax is melting
    - Spray gloved fingers with canola oil to lubricate the inside of the PVC pipe and plastic divider

- Assemble PVC molds:

    - Place plastic divider in PVC pipe to form a crescent shape
    - Place 1 3x3-inch Glad® Press'n Seal® wrap sheet at the bottom of the pipe and secure with a rubber band*

* be sure to gently press the PVC mold into the bottom plastic wrap sheet to form a seal around the bottom edge (it is Press'n Seal® wrap after all!)

- Once wax has melted and molds are ready:

    - Discard xylene waste

****CAUTION: GLASS AND CONTENTS MAY BE HOT****

    - Gently pour a few centimeters of wax into the mold
    - Remove warm sample from tube and place in warm wax*
    - Fill entire mold with wax**
    - Allow to cool and harden overnight*** ****
    - Remove wax block from mold and trim to fit the opening of the microtome

* the goal here is to suspend the sample a few centimeters from the bottom of the mold, place the sample too high and it may become exposed if the wax caves-in while cooling
** this creates the 'tail' that the microtome will hold on to when sectioning
*** if air bubbles form, fill in with melted wax
**** an alternative to cooling overnight is to harden on ice for 10 minutes

- Sectioning
    - Mount the trimmed wax block in the sliding microtome
    - Face block to achieve uniform thickness between 10 and 20 µm
    - As cut sections are formed, place them in a warm water bath (35°C to 40°C)
    - Troubleshooting:
        - Blade may need to be reinforced with additional washers for stability
        - If blade becomes sticky or coated in residual paraffin, it can be cleaned with a few drops of xylene on a Kimwipe®; wipe upwards, towards the cutting edge of the knife
        - Once block housing has reached its full extended position on the microtome the block must be repositioned and re-faced (that's why it's important to keep the sample towards the bottom of the block)
        - If block is too cold, use an external light source to heat the wax for smooth sectioning
            - This may also be achieved by breathing on the block and blade
        - If sections curl while being cut, use an angled probe from a dissection kit to coax the section into a flatter plane when coming off the blade
        - If sections are wrinkled or compressed, the paraffin may be too soft (warm), allow to cool.
        - If block is too large to be cut by the blade, a lip of wax may build-up on the upper portion of the block; this will need to be periodically removed with a scalpel
    - Sections can be periodically scooped out of the warm water bath, as they flatten and smooth, onto glass slides
    - Once all cut sections are on slides, allow them to dry for at least 10 minutes or overnight
- Staining:
    - See Hematoxylin & Eosin (H&E) staining procedure

References Cited:

Mekota AM, Vermehren M. 2005. Determination of optimal rehydration, fixation and staining methods for histological and immunohistochemical analysis of mummified soft tissues. Biotech Histochem 80(1):7-13.

APPENDIX D: HEMATOXYLIN & EOSIN STAINING PROTOCOL

Hematoxylin and Eosin (H&E) Staining Procedure

- Place slides containing paraffin sections in a coplin jar

- Deparaffinize and rehydrate sections:

3 x 3 minutes	Xylene* *(blot excess xylene before going into ethanol)*
3 x 3	100% ethanol
1 x 3	95% ethanol
1 x 3	80% ethanol
1 x 3	distilled water** ***

* if xylene is not available, a mixture of isopropanol and mineral oil or dishwasher soap (Buesa and Peshkov, 2009) could be used
** at this stage, sections may lift off of slides, be sure only one tissue sample is being processed in one coplin jar or separate them into individual petri dishes; use forceps to place sections back slides for staining
*** be careful when decanting liquid from coplin jar, keep lifted sections in the jar

- Remove slides from coplin jar and place on flat surface. Blot excess water from slides before covering with hematoxylin. Using a pipette, drop enough on each slide to cover tissue, keeping stain confined to slide. Rinses can be done in coplin jar or glass beakers.

- Hematoxylin staining:

1 x 3	Hematoxylin
Rinse	distilled water
1 x 5	tap water *(to allow stain to develop)*
Rinse	1 x 2 distilled water

- Remove slides from coplin jar or beaker and place on flat surface. Blot excess water from slide before covering with eosin.

- Eosin staining and dehydration:

1 x 30 seconds	Eosin *(up to 45 seconds for older batch of eosin)*
3 x 5 minutes	95% ethanol
3 x 5	10% ethanol *(blot excess ethanol before going into xylene)*
3 x 15	Xylene*

- You can leave slides in xylene overnight to get good clearing of any water**

* see note above about xylene replacement
** before cover slipping, position sections on slides trying as hard as possible to prevent tissue folding; it may be easier to scoop sections back onto slide (much like you did out of the warm water bath after sectioning) using a large beaker of xylene

- Coverslip slides using Per/Mount®

- Place an adequate amount of Per/Mount® on the slide using a glass rod, taking care to leave no bubbles

- Angle the coverslip and let fall gently onto the slide. Allow the Per/Mount® to spread beneath the coverslip, covering all the tissue.

- Dry overnight in the hood or well ventilated area.

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