

INFRARED CHEMICAL IMAGING FOR PATHOLOGY AND FORENSIC BIOLOGY

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CERTIFICATE OF AUTHORSHIP / ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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PREFACE

Tissue pathology assessment is primarily performed using simple human visual examination and interpretation. While various physico-chemical or biochemical treatments may be used to enhance or highlight components within the sample, the ultimate conclusion regarding the 'identity' of the sample lies with the very human examiner making comparisons with samples of known origin. Although this process sounds straightforward, more often than not the samples are less than ideal, and will be subject to natural variations which can introduce a whole gamete of artefacts which the examiner must integrate into their interpretation process. Suddenly, the comparison process goes from distinct, 'black and white' decisions, to an entire greyscale in-between.

Pathologists garner their knowledge not just from theory, but by training themselves over many years to recognise the normal from the abnormal. The pathologist must be able to deliver, with confidence, a diagnosis or ruling to a patient, treating medical professional, or (in some cases) a court. Their findings are based on a comparison of the subject tissue with many tissue samples of known prognosis, and the recognition of cell and tissue structures that 'do not belong'. Hence, it is years of exposure to endless numbers of cases which proves to be critical in achieving consistent and correct diagnoses.

Any tool that can improve the visual appearance of a sample in such comparison work is always of interest to the practitioner. Reducing the subjective nature of any scientific examination process, and more importantly reducing the potential for human error, is something all researchers strive for. The ability to confirm a pathological finding with an objective, computerised result, may also offer greater confidence to the information receiver, be they a patient or jury. It is the development of a more objective approach to tissue analysis that forms the fundamental premise of this work.

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ABBREVIATIONS

%CC	Percentage of Correctly Classified
AAS	Atomic Absorption Spectroscopy
AIHW	Australian Institute of Health and Welfare
AJCC	American Joint Committee on Cancer
ANN	Artificial Neural Networks
ALM	Acral Lentiginous Melanoma
ANOVA	Analysis of Variance
ASCII	American Standard Code for Information Interchange
ASD	Average-Standard Deviation
AUC	Area Under the Curve
Avg	Average
BA	Boric Acid
BCC	Basal Cell Carcinoma
BMU	Best-Matching Unit
BTEM	Band-Target Entropy Minimisation
CC%	Correct Classification Percentage
CCD	Charge Coupled Device
CDK	Cyclin/cyclin-dependent kinase
CI	Chemical Imaging
CLND	Completion Regional Lymph Node Dissection
CT	Computed Tomography
DNA	Deoxyribonucleic acid
ED	Euclidean Distance
EFOV	Expanded Field Of View
EGF	Epidermal Growth Factor

ELISA	Enzyme-linked Immunosorbent Assay
ELND	Elective Lymph Node Dissection
EMSC	Extended Multiplicative Signal Correction
FGF	Fibroblast Growth Factor
FIR	Far-infrared
FISH	Fluorescence <i>in situ</i> Hybridisation
FNB	Fine Needle aspiration Biopsy
FPA	Focal Plane Array
FRCPA	Fellow of the Royal College of Pathologists of Australasia
FTIR	Fourier Transform Infrared
FTIR CI	Fourier Transform Infrared Chemical Imaging
G0	Gap 0
G1	Gap 1
G2	Gap 2
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
H&E	Haematoxylin and Eosin
HCA	Hierarchical Cluster Analysis
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
IFN	Interferon
IGF	Insulin-like Growth Factor
IL	Interleukin
IP	Intraperitoneal
ISEL	<i>In Situ</i> End labelling
LA-ICP-MS	Laser Ablation Inductively Coupled Plasma Mass Spectroscopy
LDA	Linear Discriminant Analysis

LDH	Lactate Dehydrogenase
LN	Lymph Node
LOO	Leave One Out
LT	Leukotriene
LX	Lipoxin
MALDI-IMS	Matrix-assisted Laser Desorption Ionisation Imaging Mass Spectrometry
MCT	Mercury Cadmium Telluride
Med	Median
mf	Myofibroblasts
MF	Matched Filtering
Min/Max	Minimum / Maximum
MIQR	Median-Interquartile Range
MIR	Mid-infrared
MMP	Matrix Metalloproteinase
MNF	Minimum Noise Fraction
MQE	Mean Quantisation Error
MRDF	Maximal Multinomial Logistic Regression
mRNA	Messenger Ribonucleic Acid
MTMF	Mixture-Tuned Matched Filtering
MVN	Multivariate Normal
m/z	Mass to Charge Ratio
NADH	Nicotinamide Adenine Dinucleotide
NIR	Near-infrared
NMSC	Non-melanoma Skin Cancer
NN	Nearest Neighbour
NOR	Nucleolar Organiser Region
NR-Laser-SNMS	Non-resonant Laser Secondary Neutral Mass Spectrometry

NSW	New South Wales
OPD	Optical Path Difference
PAP	Papanicolaou
PAS	Periodic Acid-Schiff
PBS	Phosphate-buffered Saline
PC	Principal Component
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PG	Prostaglandin
PPI	Pixel Purity Index
pTNM	Primary Tumour Node Metastasis
QDA	Quadratic Discriminant Analysis
RCPA	Royal College of Pathologists of Australasia
RGP	Radial Growth Phase
RNA	Ribonucleic Acid
ROI	Region Of Interest
RPAH	Royal Prince Alfred Hospital
RT-PCR	Reverse Transcriptase-polymerase Chain Reaction
SAM	Spectral Angle Measure
SCC	Squamous Cell Carcinoma
SCS	Spectral Correlation Similarity
SIMCA	Soft Independent Modelling of Class Analogy
SLNB	Sentinel Lymph Node Biopsy
SMLR	Sparse Multinomial Logistic Regression
SN	Sentinel Node (<i>aka</i> Sentinel Lymph Node)
SOM	Self Organising Map

SPF	Specific Pathogen Free
SSV	Spectral Similarity Value
SVM	Support Vector Machine
SVP	Spectral Vector Properties
TE	Topographic Error
TGF	Transforming Growth Factor
THSIS	Thermal and Hyperspectral Imaging System
TIL	Tumour Infiltrating Lymphocyte
TNF	Tumour Necrosis Factor
TNM	Tumour (T) / Lymph Nodes (N) / Metastasis (M)
TOF-SIMS	Time-of-flight Secondary Ion Mass Spectrometry
UTS	University of Technology, Sydney
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
VCA	Vertex Component Analysis
VGP	Vertical-Growth Phase
WLE	Wide Local Excision

ABSTRACT

The objective of this research was to explore the capability of Fourier Transform Infrared Chemical Imaging (FTIR CI) for two specific pathology applications: 1) the analysis of human tissues for the diagnosis of melanoma, and 2) incised skin wound age determination for the purpose of forensic investigation.

For the melanoma study, thin serial sections were obtained from an archival tissue bank that consisted of pathologist pre-diagnosed ("gold-standard"), paraffin-embedded, human skin and lymph node tissues. Thin sections from each block were mounted on infrared reflective microscope slides and imaged, and a selection of the total images nominated as either training or test samples. Each training sample image was then compared to its corresponding haematoxylin and eosin (H&E)-stained section and reference library spectra extracted. Vertex component analysis (VCA) as a spectral feature extraction method was also explored. Classification of the test sample images was then performed using the spectral angle mapper (SAM) algorithm and the accuracy assessed by comparing the resulting classification images to the H&E-stained tissue sections. The tissue classification model developed produced a range in result quality, and highlighted various critical aspects in the construction of such methodologies. The taking of spectral derivatives improved image classification, as did the removal of paraffin from the tissue (although no data treatment targeting the paraffin was conducted on the non-deparaffinised tissues). Although the accuracy achieved in this study fell short of that required for clinical practice, the results obtained demonstrate that further investigation into the SAM algorithm as a tissue classifying tool is certainly warranted.

The second pathology application explored the ageing of wounds, a determination that may be critical in criminal investigations, particularly in homicide investigations, in which the timing of wound infliction may be crucial evidence. For this study, incised wounds were

inflicted on rats in a controlled manner and the tissue excised following a known amount of healing time ranging from 5 minutes to 288 hours (12 days). Thin sections of the wounds were mounted on infrared reflective slides, deparaffinised and then imaged using FTIR CI. Although four classification models were attempted, none were capable of producing highly accurate wound age determination. Spectral variation was observed between earlier and later wound ages using some of the classification methods, but the ability to correctly group the test samples into their respective age groups was not achieved. Based on the number of variables which must be taken into consideration when performing such a study, and the number of areas identified as needing further improvement (e.g. spectral data quality), the fact that even a limited form of discrimination was achieved using FTIR CI was encouraging.

INTRODUCTION

INTRODUCTION

Pathology, the examination of biological tissues for disease and abnormality, is an important means by which a trained medical professional may obtain diagnostic information on a patient's state of health. This approach is based on the hypothesis that a gross change in an individual's normal state of health will be accompanied by changes at the cellular, and hence biochemical, level. Hence, by examining the tissue of an individual at the microscopic level using a standard biological visible light microscope, and perhaps additionally employing various chemical testing techniques, the responsible scientist may potentially determine what changes are occurring, or have occurred, in the patient, and what these changes are symptomatic for.

The effectiveness of any medical treatment relies on accurate diagnosis of the causative agent. Prescription of the wrong medication, or the failure to detect the need for specific medical treatment, can result in increased recovery time for the patient or potentially death in the case of gross misdiagnosis. An aside to these potential problems is the role that public litigation plays in modern medical practice, particularly malpractice suits for mistaken diagnosis and the forwarding of misleading prognostic information to the patient.

Inaccuracy in post-mortem examination of biological tissue can also lead to erroneous conclusions being drawn about the factors involved in an individual's death. An incorrect or incomplete post-mortem finding may mislead a criminal investigation and in turn produce a gross miscarriage of justice. Similarly, failure to detect the correct cause or manner of death may affect the findings produced during coronial inquests. This in turn may prevent potentially dangerous products or procedures from being identified and the necessary life-saving changes being made before further loss of life.

With these motivations it is no wonder that the medical and forensic science industries constantly strive for greater accuracy in diagnosis, and it is this need which drives and

justifies ongoing research into pathological techniques. The need for accuracy has always been of great concern in medical research, as in all scientific fields of endeavour, but in today's litigious society never before has an incorrect diagnosis been a more emotionally and economically costly issue. Although there is uncertainty in every measurement, a strong movement away from subjective analysis methods has been witnessed across all scientific disciplines, particularly with the introduction of computerised and automatable analysis techniques. However, processes such as pathological tissue analysis that involve the visual examination and comparison of objects, still rely significantly on the ability of the human eye and brain.

In many cases, there is often no one distinct, reproducible physical feature in the object being examined that can be used to describe a particular feature across a group of distinctly separate images. What is required is further depth to the information obtained from the sample. To obtain this extra dimension of data, there are a number of means at the examiner's disposal. While descriptive variables may be collected for each sample to assist in some studies, such as patient age, gender, weight, etc, such data may not offer much in the way of assistance in cases where a high degree of intra-sample variability is observed. An alternative is the collection of hyperspectral data for each image, where the spectral dimension is equivalent to that obtained during infrared or fluorescence measurements. Using this approach, the additional dimension to the data may be gathered on a point by point (pixel by pixel) basis, so that areas of particular interest identified from their morphology may then be further examined from a biochemical perspective.

▪ INFRARED SPECTROSCOPY AND BIOLOGICAL RESEARCH

Infrared spectroscopy is an extremely powerful analytical tool for the biologist in that, with the exception of a few homonuclear molecules, all molecular species absorb infrared radiation, and hence it is possible to identify the various organic compounds of which many biological samples are comprised. Developments in infrared technology

over recent decades have seen a shift from the analysis of separated tissue components to direct sampling of tissue specimens, particularly in diagnostic pathology. The motivation behind such research is the concept that all changes in tissue composition which are a result of disease are accompanied or preceded by biochemical changes, and that the chemical species involved in these biochemical changes may potentially be detected and identified using infrared analysis methods. Hence, the innumerable biochemical components which comprise a biological tissue sample may be employed as a means by which different cell types are able to be identified and classified using Fourier Transform Infrared Chemical Imaging (FTIR CI) data. The information thus obtained may then be used to assist the pathologist in reaching his or her diagnosis, and subsequently provide the patient with more a more accurate prognosis. Hence, FTIR spectroscopic analysis of biological tissues offers the potential to be a useful additional technique in pathological evaluations.

▪ **RESEARCH AIMS**

It is apparent from the information provided above that FTIR CI offers great potential in the field of anatomical pathology. From a purely fiscal standpoint, the opportunity to even partially automate a highly labour-intensive activity is instantly attractive. However, the financial viability of such technology is itself limited by instrumentation purchase price, maintenance and running costs, as well as the expected life-span of the mechanical componentry and associated consumables. The need for staff training, instrumentation qualification and method quality assurance investigation costs must also be factored into any considerations of employing such new technology into routine pathology workflows. It can be difficult to accurately quantify the monetary benefit in obtaining potentially lower error rates (which in turn result in lower litigation risks) and improved diagnostic information (such as for criminal investigations). Associated potential benefits from implementing new technology, such as a reduction in mortality (due to

faster and more accurate diagnoses) and wrongful incarceration time (as a result of more accurate evidence examination), would be beyond value to the individuals and families they concern. Hence, any investigation into the feasibility of employing FTIR CI may be deemed at the very least worthwhile.

The two pathology applications explored in this research were selected based on current issues with their routine diagnostic techniques. In both cases, respected and well-established analysis methods are available to the pathologist, but these rely heavily on the skill and experience of the pathologist, and are fundamentally subjective in nature. It is not in the scope of this research to seek to eliminate the involvement of the diagnosing pathologist, rather, the primary goal is to further the development of instrumental techniques which may assist or independently confirm the assessment made by the investigating pathologist.

Hence, it is the aim of this research to explore the possibility of developing a FTIR CI method which may reliably and objectively assess:

1. skin and lymph node tissue obtained from patients for the presence of primary melanoma and metastases, and hence to develop FTIR chemical imaging as a cancer diagnostic tool (to distinguish between normal and cancerous tissues);
2. skin wound variation, in an effort to determine if cellular changes in mechanically damaged tissues can be detected and aged spectroscopically. This information would assist the consulting pathologist in suspicious death enquiries where wound age is at issue and ultimately assist the court/criminal justice system/coroner.

- **THESIS STRUCTURE**

To assist the reader, general background information on pathology and infrared chemical imaging is provided in Chapter 1, followed by the application-specific chapters. A brief outline of the chapters and their contents follows below:

- **Chapter 1: Pathology and Infrared Chemical Imaging Theory**

Besides outlining the aims of the research undertaken, Chapter 1 provides a general introduction to the scientific principles behind the studies of pathology and infrared spectroscopy. It includes background information on tissue pathology sample preparation and examination, and examples of how diagnostic pathology is used in modern medical practices. The second component of this chapter looks at infrared chemical imaging, starting with the fundamentals of infrared radiation theory and basic spectroscopic instrumentation, and then progresses onto the development of focal plane arrays, the treatment of infrared chemical imaging data, and examples of recent research applications.

- **Chapter 2: Melanoma Study Part 1 (*Background and Method*)**

The first section of Chapter 2 focuses on the background literature surrounding the diagnosis and treatment of melanoma, as well as current research into alternative diagnostic tools. Covered in this chapter is a discussion on previous cancer research conducted using infrared chemical imaging, as well as an overview of the various data analysis tools commonly employed in these investigations. A detailed description of the samples used in this study, as well as the sample analysis method(s) employed, has also been provided.

- **Chapter 3: Melanoma Study Part 2 (*Results and Discussion*)**

Chapter 3 reports the results of the infrared chemical imaging analysis of the lymph node samples as well as detailing the outcomes of the “automated” image classifications. A discussion of the results obtained then follows, examining the success of the method chosen and any problematic issues.

- **Chapter 4: Wound Study Part 1 (*Background and Method*)**

Chapter 4 provides the literary background to the examination of wound age for forensic purposes, and discusses the biological nature of wound healing. An overview of the wound ageing methods examined to date is provided, along with a description of the study’s infrared imaging approach.

- **Chapter 5: Wound Study Part 2 (*Results and Discussion*)**

The results of the wound ageing study are presented in Chapter 5, along with a comparison of the computerised classification approaches and their outcomes. A discussion as to the ability of the chosen sample and data analysis methods to effectively discriminate between wound ages is then presented.

- **Chapter 6: Conclusions & Future Directions**

This chapter provides an overall critique of infrared chemical imaging as a tool in pathology. The results from the two studies are summarised, and the pros and cons of each proposed chemical imaging application discussed in both research and clinical settings. Also discussed are potential areas for further work to be conducted for melanoma and wound ageing, as well as pathology diagnostics as a whole.

CHAPTER 1:

PATHOLOGY AND INFRARED CHEMICAL IMAGING THEORY

1.1 PATHOLOGY

The science of pathology is often defined as the study of an organism (or its component cells, tissues and organs) for the origin, nature and course of a disease, with the term itself deriving from the Greek words of *pathos* (meaning suffering or harm) and *logos* (reason). Pathology, in its simplest definition, is the study of how the organs and tissues in a healthy body differ to those observed in a person exhibiting illness or death. The principles of pathology have been practised for centuries in the form of autopsies and physiological observations, with records of human dissections being performed in Alexandria to study the changes brought about by disease as early as the third century BC.^[1] The development of the light microscope in the seventeenth century meant that scientists could begin to examine disease processes at a cellular level. More recent advancements in scientific instrumentation (such as the scanning electron microscope and mass spectrometry) have led to the development of techniques which can analyse cells at the molecular and even supramolecular level.

In modern medical practice, pathology is recognised more as a specialised discipline of medicine, and in hospitals it is often divided into two main strands: clinical and anatomical pathology. Clinical pathology concerns itself with the biochemical and/or biological examination of substances excreted by the body, such as urine and cerebrospinal fluid, as well as blood and tissue fluids. Anatomical pathology, on the other hand, employs gross, microscopic and molecular examination techniques on whole bodies, organs and tissues, to study structural abnormalities. More specialised types of pathology have also arisen to address specific needs in various other arenas, such as forensic pathology (the combination of medical pathology knowledge in relation to legal issues, such as time or cause of death coronial investigations), veterinary pathology (the study of disease in animal tissue and bodily fluids) and even plant pathology (the examination of diseases in plants brought about by pathogens and environmental conditions).

1.1.1 The Pathologist

Pathology is often referred to as the science that underpins modern medical practice as it plays such a vital role across all aspects of medical science. Doctors will make informed decisions about disease diagnosis and treatment often based on the information provided to them by a trained pathologist. Consequently, the result delivered by the pathologist is dependent on their knowledge and diagnostic skill base, and is the product of years of training and practice. Pathologists most often commence their careers as general medical practitioners, and then go on to pursue a specific career in pathology. For example, the Royal College of Pathologists of Australasia (RCPA), which accredits laboratories for pathology training as well as being responsible for the training and professional development of pathologists, requires registered medical practitioners to have at least one year of postgraduate experience prior to initiating their specialist pathology training and to be employed in an accredited pathology laboratory.^[2] Successful applicants must then undertake a minimum of five years pathology laboratory training in general or clinical pathology or a nominated specific discipline and sit three examinations comprised of a basic pathological sciences component as well as two discipline-specific tests, before they may be certified as a qualified pathologist and Fellow of the College (FRCPA).

1.1.2 Current Issues in the Pathology Discipline

Most diseases at present are diagnosed by pathology, and the effectiveness of many treatments is also monitored by trained pathologists. According to the RCPA, “pathologists diagnose every detected cancer in the world”,^[3] and therefore it is vital that the pathologist has extensive knowledge and experience in the field of pathology, and is able to utilise the most accurate and efficient techniques to assist them in reaching their conclusions. However, the lengthy training process and a general lack of training support from governments and medical service providers has led to a national and international shortage

of specialist pathologists.^[4] Additional professional burdens are also being placed on practising pathologists in the form of strict, but very necessary, quality control measures, to minimise the potential for misdiagnosis. Ever-increasing workloads brought about by an ageing population, ethnic diversity (giving rise to differing cultural needs and a large diversity in genetic disorders), as well as the need for ongoing monitoring of patients with prolonged illness, have borne witness to an over-stretched pathology workforce in Australia, particularly noticeable in 2006 when the ratio of pathologists to the Australian population was 1:15500.^[4]

There are a number of possible solutions to assist pathology services in meeting the demands of the health and legal systems. Simple, but fiscally contentious solutions could include obtaining more government funding, providing more opportunities for the training of pathologists, as well as examining the current work conditions of pathology practitioners with a thought to attraction and retention for the future. Another critical area is the development of new technology to assist the pathologist in obtaining a rapid and ever earlier disease diagnosis. Technological advances in the fields of pharmacogenetics and proteogenomics have sought to examine the individual's genetic history and make-up, promoting a shift towards more 'personalised' health care with an eye to prevention or individualised therapeutic treatment regimes. While prevention and risk factor analysis are essential in modern medicine, genetic analysis of human tissue is only one aspect of pathology work. Hence, research into new techniques which can assist the pathologist in any facet of their role must be pursued with vigour.

1.1.3 Histology

Pathologists employ a tool known as *histology* to study tissue structure at the microscopic level. In order for cellular architecture to be visualised, the tissue to be examined is excised from the patient, thinly cut (often in the order of several microns thick), mounted

on a glass microscope slide, stained to permit easier visualisation, and then examined in transmission mode using visible light under a biological compound microscope. Depending on the level of structure to be examined, and therefore the resolution required (resolution of structures by light microscopy is of the order of 0.2 - 0.6 μm),^[5] objective lenses of varying numerical aperture and magnification are employed. Additional techniques to assist in illumination and contrast within the specimen may be used, such as tissue component-specific staining techniques that can target molecules such as lipids and proteins within cells and tissues.

A brief overview of tissue composition, preparation and commonly encountered pathology techniques are given below.

1.1.3.1 Mammalian Tissue (a general overview)

The cell is the basic unit of the mammalian body, and represents the first level of organisation in living organisms. Over two hundred different cell types can be found in the human body, and they form the building blocks of a variety of tissue types such as epithelial, connective, muscular and nervous tissue.^[6] The highly specialised organs found in the mammalian body are all formed from at least two of the aforementioned tissue types. Cells, tissues and the fluids found in the mammalian body are composed primarily of water, proteins, nucleic acids, lipids, and carbohydrates.

1.1.3.2 Tissue Preparation

The main steps required in preparing tissue for light microscopy examination have been listed below. Please note however that not all of these steps listed below will be required for every tissue preparation method chosen.

Step 1: Fixation

In order to ensure that microbiological degradation of the excised tissue sample does not

occur before a thorough pathological examination may be conducted, the tissue needs to be preserved or *fixed*. Fixation commonly involves cross-linking the amino groups in proteins with nearby protein nitrogen groups via a methylene (CH_2) linkage to “fix” or precipitate the proteins out of the sample. It thus prevents any further tissue degradation by stopping enzyme activity and cell autolysis.^[5] A perfect fixative should also preserve the tissue’s normal architecture as much as possible with even penetration of the tissue and prevention of desiccation. It should also be affordable, safe, possess good stability, and not interfere with later treatments of the tissue.^[7] Neutral-buffered formalin (formalin is a 37-40% solution of formaldehyde in water) and Bouin’s fluid (a compound fixative consisting of picric acid, formalin and acetic acid) are two common fixative agents.

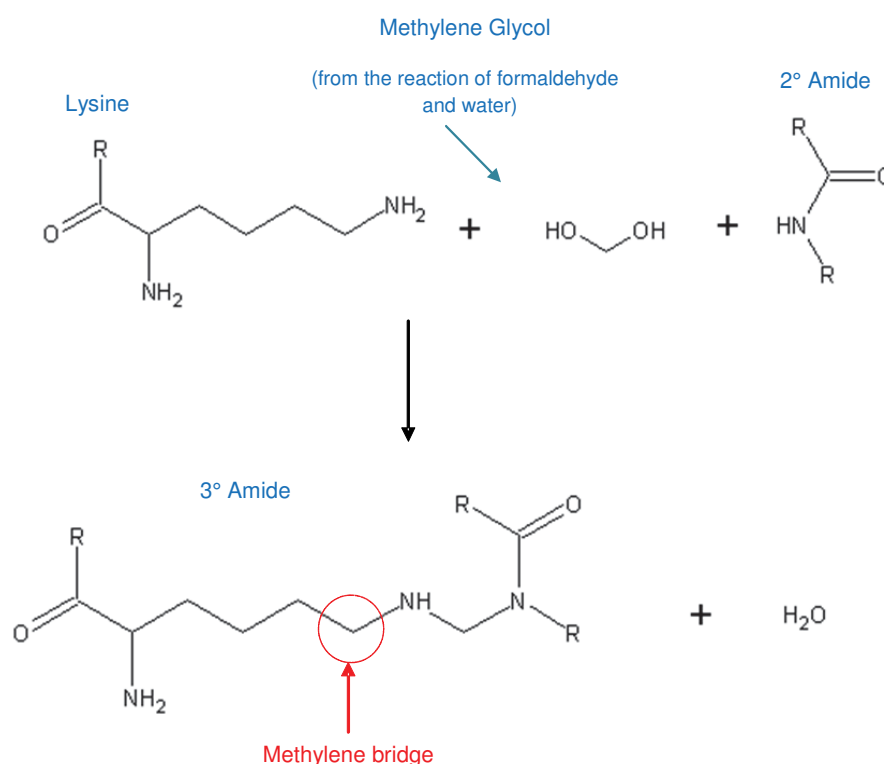


Figure 1-1: Formalin fixation results in the formation of a stabilising meshwork caused by the cross-linking of proteins. The reaction above depicts the formation of a methylene bridge during the fixation of a tissue with formalin.^[8]

Step 2: Dehydration and clearing

This step involves the removal of water (dehydration) from the cells in the tissue so that it may be replaced with a firmer material. The dehydrating agent is then usually replaced in the tissue with another reagent (clearing) which is miscible with the chemical being used for embedding. The most common method of dehydration is subjecting the tissue to a series of graded alcohol baths (usually ranging from 50 - 100% alcohol concentration), which gradually encourages the water in the tissue to diffuse out and the alcohol solution to diffuse in. A balance must be reached between improving efficiency in regards to the number of varying concentration solvent baths, the time submerged, and the prevention of damage to the tissue caused by too rapid removal of the water or an overly harsh a solvent.

There are numerous organic solvents that may be employed as clearing agents, but prior to selection the pathologist must give consideration to the speed, harshness of action, flammability, toxicity and cost of the solvent chosen.^[7] Xylene is a commonly employed clearing agent, but it is beginning to fall out of favour due to the appearance of less flammable and toxic products on the market.

Step 3: Embedding

Embedding is the technique by which tissues are converted into a block of material of suitably firm consistency for the thin sectioning, or cutting, of the tissue by a microtome. In wax embedding (with paraffin wax being the most common compound used) the clearing agent is first removed by a series of hot wax baths, via a combination of washing and evaporation. The tissue is then immersed in a fresh wax bath until it has been completely infiltrated and then subsequently placed into a small container or mould (see Figure 1-2), covered with melted wax, and allowed to harden into a wax block which can later be mounted on the microtome and easily cut.



Figure 1-2: (a) Example of a plastic histology tissue cassette;^[9] (b) a paraffin-embedded tissue block (front and back).

The largest proportion of medically examined tissues are preserved using formalin fixing and paraffin embedding.^[10] Alternative embedding materials include celloidin and synthetic resins. However, whatever the choice of embedding material, the embedding of the specimen needs to produce a block of material of uniform consistency which is easiest to cut to the desired thickness with minimal distortion. Embedding materials need to be removed prior to the next stage in the examination process, which is most commonly differential staining using dyes or histochemical procedures (refer to Figure 1-3).

It is important to note that certain tissue components such as enzymes, lipids, and cell antigens cannot survive this fixing and embedding process, and accordingly alternative tissue preparation methods may be required for the demonstration of particular antigens.^[5] One such alternative method is freezing. In this process, the tissue is rapidly frozen immediately following excision, causing the water in the tissue to freeze and the sample to harden. The rate at which the tissue is frozen will control the size of ice crystals formed within the tissue itself, as mechanical damage due to large ice crystal formation can significantly alter the tissue structure. By employing extremely low temperatures, such as those found in liquid nitrogen (-196°C), the ice crystal size can be kept quite small.

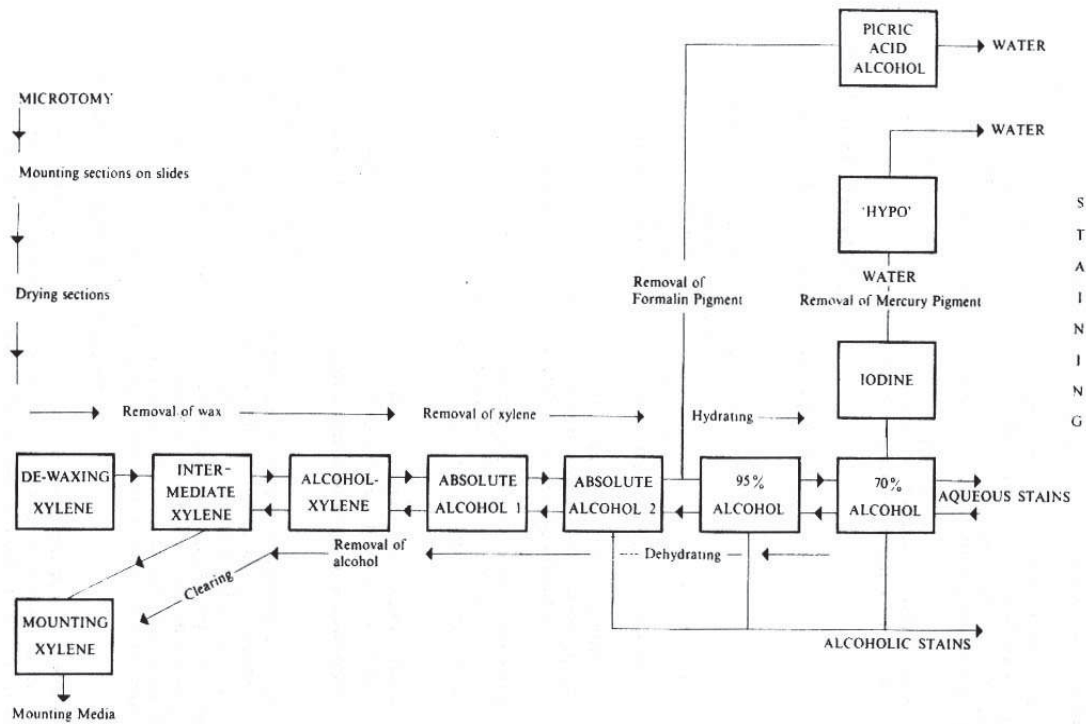


Figure 1-3: Schemes of progression of paraffin sections from de-waxing xylene to staining and dehydration and clearing to mounting.^[11]

In cases where samples possess a considerable proportion of non-water filled void space (e.g. lung tissue), the use of an embedding medium such as a water-based glycol or resin may be employed. Freeze drying is another freezing technique available to the pathologist and removes only water (by a process of sublimation). This then permits direct wax impregnation and preserves the water-soluble materials present in the sample.

Although frozen sectioning can be a very fast (taking only minutes) process and preserves the enzyme chemistry compared to tissues prepared using standard formalin fixation and paraffin embedding, there are a number of disadvantages. Crotechnique-prepared tissues can possess poor tissue morphology, the specimens pose a greater biohazardous risk at the early stages of tissue preparation, very thin or serial sections may be difficult to cut and there is limited capability to perform retrospective studies (not ideal for archive construction).

1.1.3.3 *Preparation Artefacts*

Every technique employed in histological preparation of tissues possesses its own inherent problems, particularly in the way each causes changes, known as artefacts, in the tissue being studied. One of the most common artefacts of tissue processing is loss of material. For example, the paraffin wax tissue processing regime results in the loss of small water-soluble molecules during the fixation stage (e.g. sugars, ions, vitamins and cofactors) as well as the lipids and lipid-soluble materials when the tissue is exposed to the alcohol, clearing agents and hot wax stages.^[7]

The choice of preservation method used may be critical when the pathologist is searching for specific tissue changes, as prolonged or inappropriate preservation methods can potentially remove various biochemical components indicative of particular disease states or change the architectural structure of the tissue itself. Therefore, the choice of preservation or 'fixation' method must be made with the tissue components of interest in mind, and should ensure that the cell and tissue components are preserved in a condition identical to that which existed during life.^[11]

In addition to material loss, another artefact may be that of chemical additions to the tissue. These may be in the form of chemical species introduced by the tissue processing chemicals (e.g. aldehyde-containing preservative), or by unintentional forms of contamination during handling in the laboratory. Other common artefacts include the alteration of structures caused by tissue shrinkage (which can be quite problematic if the tissue is not affected evenly), and ice crystal damage, which occurs during frozen section processing. Hence, the selection of tissue processing technique will be predetermined by the choice of components and structural features to be observed.

1.1.3.4 Tissue Cutting

Cutting, also referred to as sectioning, for the purposes of microscopic examination, involves the tissue block being finely sliced using a specialised cutting machine known as a microtome. In the case of paraffin-embedded tissue blocks, the microtome chosen may be used to cut the tissue in fine, even increments (usually 0.5 - 10 μm), producing exactly adjacent sections in the form of a ribbon. The wax slices are then floated onto a bath of warm water, which allows the tissue to recover from any wrinkles or folds created during the cutting process. The tissue section is then deftly lifted onto a clean glass microscope slide and allowed to dry.

To cut frozen tissue, a method referred to as frozen sectioning or cryotomy is employed. Frozen sectioning is similar to that of the paraffin-embedded tissue, but requires the use of a freezing microtome (only the specimen is kept cold) or more commonly a cryostat (a microtome housed in a special deep-freeze cabinet) to keep the tissue block in its frozen state. The cut tissue section can then be mounted directly onto a glass microscope slide or coverslip.

Sectioning by vibratome (an instrument similar to a microtome but employing a vibrating razor blade assembly) offers some advantages over routine fixation and paraffin embedding, as well as frozen sectioning, in that the tissue is not subjected to organic solvents or high heat (i.e. the tissue does not necessarily have to be embedded or frozen prior to sectioning), and the morphology of the tissue remains intact as no freezing or thawing of tissue is required. However, this technique is not commonly employed as it is a slow sectioning process, it is difficult to perform with soft or poorly fixed tissues,^[10] and the sections are often distorted by chatter marks or vibratome lines.

1.1.3.5 *Tissue Staining*

Staining of tissues is performed to produce contrast and colour for ease of microscopic examination, allowing tissue structure to be clearly demonstrated. Many commonly employed stains are water-soluble, requiring the removal of the paraffin (referred to as 'dewaxing') from the tissue section, and the replacement of water in the tissue prior to staining, if wax was used for embedding (frozen sections are exempt from this step). In a sense, the dewaxing and rehydration process is almost the reverse of the tissue processing method (although much faster as one is only dealing with a thin section), first involving removal of the wax with xylene (or any other suitable solvent), followed by exposure of the tissue slide to a series of graded alcohol solutions (100 - 70% alcohol concentration) and then finally to distilled water.

Stains utilise differences in the chemistry of the cell or tissue components to highlight various features within the tissue. There are three classes of tissue stains:

- 1) those that can distinguish between acidic and basic cell components;
- 2) specialised stains to highlight components in the extracellular matrix such as connective tissue; and
- 3) metallic salt solutions which make use of ion exchange reactions to form coloured metal precipitates on the tissue structures.^[12]

Two common employed tissue stains include Haematoxylin and Eosin (H&E) and toluidine blue. H&E stains the acidic (or basophilic) components of the cell (such as the deoxyribonucleic acid (DNA)- and ribonucleic acid (RNA)-rich nuclei) purple/black and the basic components of the cell cytoplasm pink/red (an example is given in Figure 1-4). Toluidine blue stains tissues blue except for those rich in polyanions (and consequently causes the molecules of the stain to polymerise with each other), which are stained purple.^[12]

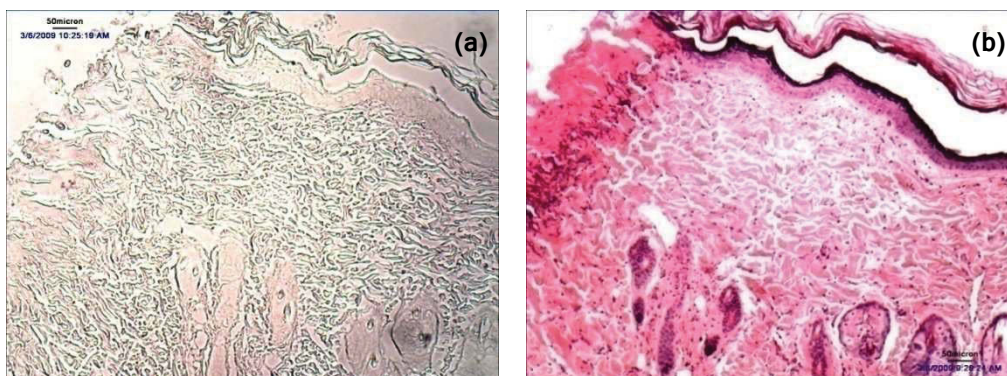


Figure 1-4: Examples of unstained and H&E stained tissue. Image (a) demonstrates a lack of contrast which makes cell and tissue type identification and observation difficult. Image (b) shows the improvement gained from employing a simple tissue stain such as H&E.

Differential staining using dyes such as H&E is not very chemically specific. For improved chemical specificity on a more precise physicochemical basis, the pathologist must turn to alternative techniques such as histochemistry and immunology.

1.1.4 Histochemistry

Unlike the tissue staining described above, histochemistry utilises specific chemical reactions (such as enzyme activity or chemical reactivity) to provide information on the presence and location of particular intracellular and extracellular macromolecules or chemical groups. Histochemical stains react with the chemical species of interest to form insoluble precipitates which possess specific, visible colours or appear as a metallic deposit. For example, the Periodic Acid-Schiff (PAS) staining technique, which is used to identify protein-bound carbohydrates, forms a magenta precipitate (purple fuchsin) with glycogen and carbohydrate-rich molecules.

The application of histochemical stains can require a reasonable level of care in application, and consideration must be given to the employment of positive and negative controls. For example, the PAS stain is not specific for glycogen, and the use of a negative control (in the form of a consecutive section which has undergone enzyme digestion with amylase) will enable the differentiation of glycogen from other PAS-positive materials such as mucins, glycoproteins and reticulin.^[7]

Histochemistry may also be used to study the tissue distribution of specific enzymes. Enzyme histochemistry is usually performed on unfixed frozen tissue sections due to the highly reactive nature of biological enzymatic systems with the tissue fixation and processing reagents. The tissue sections are placed in an incubating solution containing the specific enzyme substrate, along with any inhibitors or cofactors necessary for the reaction. The enzymes of interest in the tissue then bind to the substrate, which may then be observed by the subsequent addition of a visualizing agent (the visualizing agent can alternatively be included in the incubating solution).

1.1.5 Immunohistochemistry

While histochemical stains enable the visualisation of the products of enzymatic reactions, more precise localisation of macromolecules can be achieved using immunohistochemistry. For example, when a cell is exposed to a change in environment, such as that brought on by a harmful stimulus such as a tumour-associated antigen, the cell responds by turning off most genes and activating a set of genes which code for what are known as cell stress proteins. These proteins have a cytoprotective effect (they can protect the cell from noxious chemicals or other harmful stimuli),^[13] but their activation will not necessarily produce any routine morphological, and hence visibly detectable, changes in the cell. Immunohistochemistry employs labelled antibody reagents which interact (antigen-antibody) with antigens in the tissue sections to detect the presence of specific substances (usually a

protein or other macromolecules). The areas of tissue affected are visualised with a marker, such as a radioactive element, fluorescently labelled enzymes, or a chromogenic substrate which may then be seen through a standard light or fluorescence microscope.

The first step in immunohistochemistry is to identify the macromolecule, or antigen, of interest in the tissue sample. The tissue section is then exposed to a solution of labelled antibodies (e.g. horseradish peroxidase or alkaline phosphatase) specific to the antigen selected. In the case of the enzymatically labelled antibodies, the tissue section is then treated with a solution containing the enzyme label substrate, which then reacts with the enzyme label to produce a coloured product, providing the specific location of the antigen in the tissue section (*direct method*). Alternatively, the tissue can be initially treated with an unlabelled primary antibody, rinsed of any remaining unbound primary antibody, and then subsequently treated with a labelled secondary antibody (*indirect method*). One advantage of the indirect method is the increased number of different antigenic binding sites available on the primary antibody, which in turn increases the method's sensitivity. Another advantage is the reduced need for the primary antibody (which may be difficult to obtain in large quantities).^[12]

Employment of immunohistochemical techniques also means that many antigens can be successfully visualised in formalin-fixed, paraffin-embedded tissue sections when combined with antigen retrieval techniques.^[10] In the cases where frozen sections are required, the tissue should be fixed with acetone or alcohol prior to implementing any immunohistochemical staining methods.

1.1.6 The “Gold Standard” of Pathology

So what comprises a standard in anatomical pathology? This is a critical question in the pathology arena, as individuals suffering from the same disease can display what may appear to the untrained pathologist to be differing cellular morphology. Knowledge of what

constitutes 'normal' and 'healthy' cells or tissue in individuals can only be gathered from numerous observations of the same cell or tissue type from the one individual and then comparison with the corresponding tissue collected from innumerable individuals who do not display any associated symptoms of a particular disease. However, the question arises as to what may be defined as the "standard" for comparison. While diseased cells grown in cultures may be considered 'purified' representations of the disease, often there are subtle changes in the like cells found in a living organism that the pathologist must learn to detect and then determine as 'self' or 'non-self'.

For example, the cells present in the human female cervix have often been the source of much ire to pathologists in the range of cellular groups and changes which may be observed in the one sample. Papanicolaou (PAP) smear diagnostic error or 'false-negative' rates of between 1% to 93% have been reported in the literature.^[14] For medical service providers the appearance of articles, such as that depicted in Figure 1-5, is highly undesirable.



Figure 1-5: Example of the bad publicity and treatment consequences from erroneous results.^[15]

1.1.7 Histology - Where do we go from here?

The many component techniques of histology provide the pathologist with a large arsenal of diagnostic tools. Simple tissue staining techniques such as that of H&E have become the

mainstay of many pathological examinations. When the pathologist wishes to search for the signs of tissue damage or disease that may not be structurally visible using simple staining and a standard biological microscope, more sensitive and specific histological techniques are also available, such as immunofluorescence and scanning electron microscopy. The cost, time-requirements and technical skill required for some histological tests may make them unwieldy for routine, high-throughput hospital environments. Multiple tissue sections may be required per case to enable a variety of histological tests to be performed to achieve accurate diagnosis also. While automation of the tissue processing has reduced some of the burden on histology laboratories, a considerable amount of labour is still expended in the treated tissue examination stage, particularly where automation of slide examination is not available or feasible.

Although standard pathological examination of tissue sections using the light microscope is excellent for relatively rapid, routine analysis of specimens, there is considerable concern over the potential for misdiagnosis, particularly as the testing performed ultimately relies on a human pathologist to visually examine the tissue. The ability to offer additional techniques to assist the pathologist in reaching a diagnosis is of considerable scientific interest, particularly in pathologically ambiguous cases where the gross physical changes in tissue composition have yet to appear. It is to the biochemical changes which accompany or precede the physical changes in tissue composition that the researchers are now turning, as evidenced in the large volume of immunohistochemistry techniques already developed. Recent technological developments mean that there are now even more techniques available to the pathologist, and one of particular interest is the use of infrared spectroscopy to obtain chemical information for biological samples.

1.2 INFRARED SPECTROSCOPY AND CHEMICAL IMAGING

1.2.1 Electromagnetic Radiation

Fundamental to the existence of life on our planet is the light produced by the sun. Light is a form of energy referred to as electromagnetic radiation, and is considered to be dualistic in nature as it possesses both wave and particle properties. Electromagnetic waves exist and travel through space in the form of alternate and perpendicularly positioned electric and magnetic fields (see Figure 1-6). Electromagnetic radiation can possess a broad spectrum of frequency (represented by the symbol f) and wavelength values (represented by the symbol λ), with the particular feature of the wave (e.g. colour) defined at its source and remaining unchanging, even as it moves through different media.

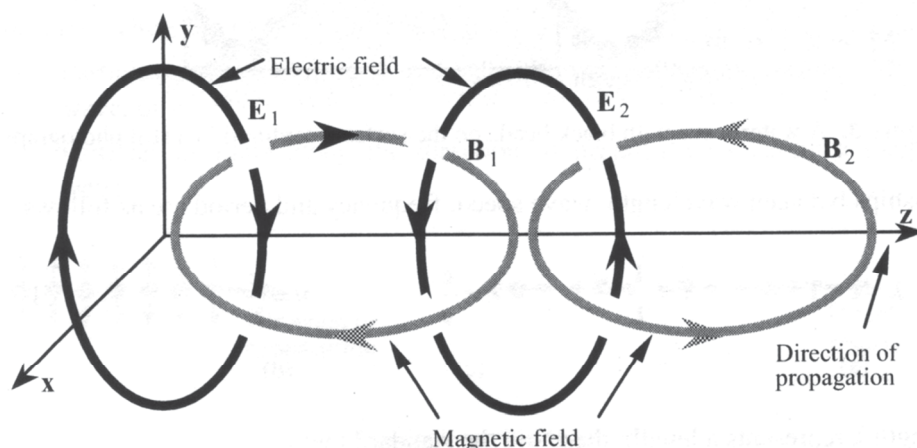


Figure 1-6: Diagrammatic representation of the dual nature of the electromagnetic wave. Note that the electric and magnetic fields are perpendicular to each other as well as both being transverse to the direction of the wave propagation.^[16]

The equation of wave propagation, and the relationship between frequency and wavelength, can be described using the following equation:

$$f = \frac{c}{\lambda} \quad (1)$$

where c is defined as the propagation speed of the (light) wave in a vacuum (299,792,458 m/s).

The electromagnetic spectrum is a model used to describe a range of electromagnetic waves, ranging from the higher frequency gamma rays to the lower radio and television wave frequencies (a simple diagrammatic representation of the electromagnetic spectrum is depicted in Figure 1-7).

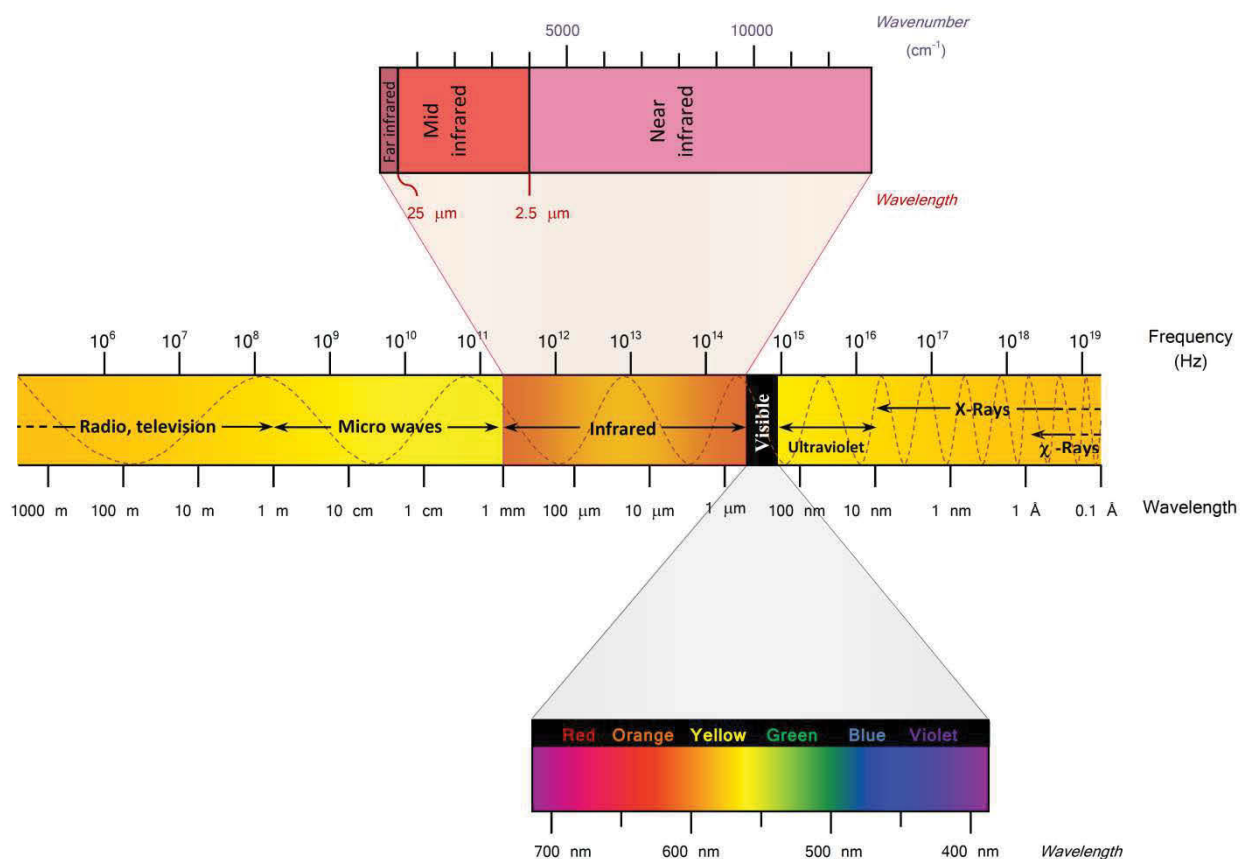


Figure 1-7: The electromagnetic spectrum. The boundaries between the bands depicted are arbitrary and do not delineate strict borders.

While the movement of light through space (such as refraction and reflection) is easily described using the wave theory, this model suffers as one begins to consider the interaction of light energy with matter, such as its variable absorption by materials at the microscopic scale (e.g. molecules and atoms). To effectively describe the manner of electromagnetic energy interaction with materials, the particle theory of light was developed. Particle theory states that light energy may also be physically defined as a stream of discrete energy particles known as photons. Each of these photons possesses a finite amount of energy (E), which is in turn related to the frequency of the light, as can be demonstrated in equation 2 below:

$$E = hf \tag{2}$$

Where h is Planck's constant (6.63×10^{-34} J s).

When a photon strikes an object it may be absorbed by the atoms or molecules comprising the item, elastically scatter ("bounce off"), or it may inelastically collide and exchange some of its energy with an electron in the object. The selective interaction of materials with different wavelengths of light is dependent on the energetic properties of that specific material, which in turn will depend on the molecular and in some cases atomic structures from which the item is comprised.

1.2.2 Visible light

The human brain gathers image information using signals detected by the eye. These signals are generated based on an object's ability to absorb or reflect visible light. While the average human can detect a myriad of different colours, our optical systems are restricted to the region of the electromagnetic spectrum often referred to as visible light. Visible light is actually a form of electromagnetic radiation that possesses a wavelength in the range of approximately 400 to 700 nanometers (nm).

A standard compound light microscope is the primary tool for an anatomical pathologist, and while it assists the human operator to obtain information at the microscopic level, only with the aid of tissue stains or specialised filters can it provide some further, if very limited, information on the chemical make-up of the material being examined. While the morphology of a sample can be highly indicative of abnormality in the tissue being examined, simultaneous collection of biochemical data from the same sample offers a more complete overview of the nature of the changes occurring therein. As can be seen in Figure 1-7 the human eye can only detect a small band of the electromagnetic spectrum, but the material being examined could also be capable of absorbing 'invisible' radiations. The selective absorption, reflection or transmittance of a material of various electromagnetic radiations can, like colours, be used to obtain data to describe a material. One source of useful invisible radiation in the biological realm is the infrared region of the electromagnetic spectrum.

1.2.3 Infrared Light

The infrared region of the electromagnetic spectrum spans the frequency range of approximately 3×10^{11} to 4×10^{14} Hz and a wavelength range of approximately 1 mm to 750 nm. For convenience, wavenumbers (assigned the unit cm^{-1}), the inverse of the corresponding wavelength value, are used when describing infrared measurement parameters. The infrared portion of the electromagnetic spectrum is often divided into three regions: the far-infrared (FIR), mid-infrared (MIR) and near-infrared (NIR).

As may be observed in Figure 1-8, the far-infrared region of the electromagnetic spectrum possesses waves with very low energy. However, this region may be used in rotational spectroscopy studies. In contrast, the near-infrared region, due to its high energy waves, can excite overtone or harmonic vibrations in molecules, and is a wavelength range often employed in Raman spectroscopy lasers. Of particular interest to analytical chemists is the

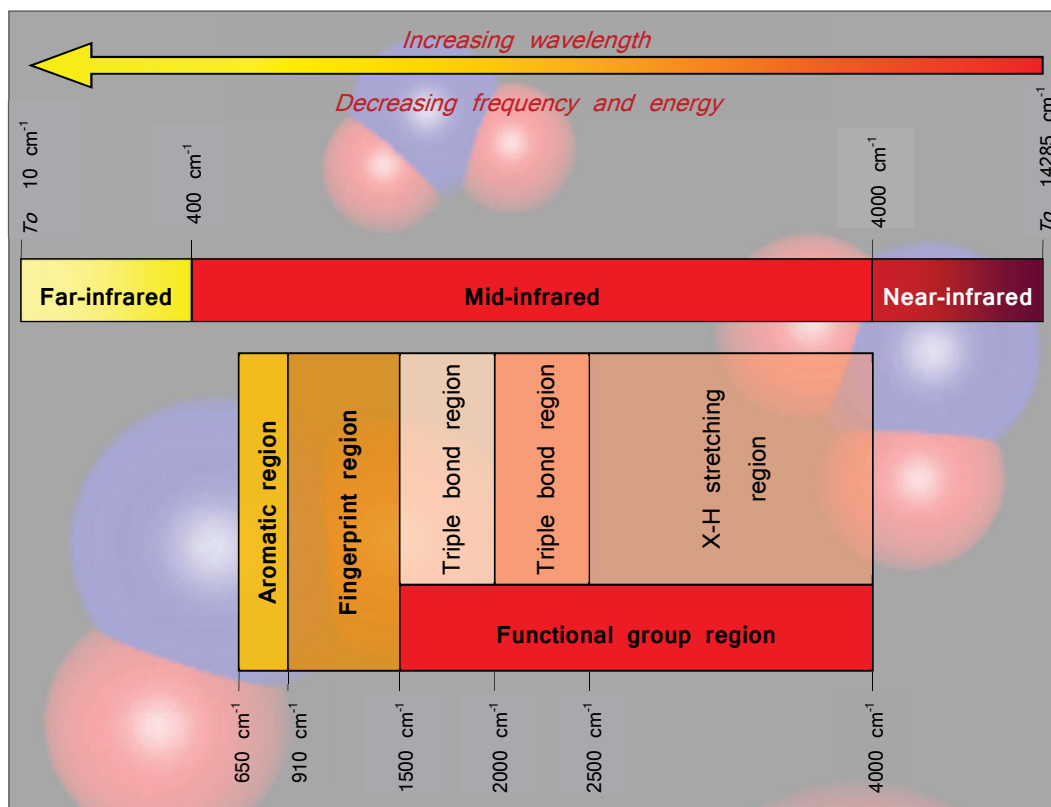


Figure 1-8: The three main regions of the infrared portion of the electromagnetic spectrum and their approximate corresponding wavenumber ranges

mid-infrared region, which is itself often divided into three sub-regions referred to as the functional, fingerprint and aromatic regions. The functional group region is concerned with the absorption bands arising from the various functional groups comprising a molecule, and can provide general information on the structure of the compound being analysed. In contrast, the fingerprint region is concerned with the motion of the bonds which are responsible for the linking of a substituent group to the remainder of the molecule, such as bond stretching and bending vibration frequencies, and may be used to obtain highly specific and individualizing information about a molecule.

The mid-infrared region is commonly used to elucidate molecular structural information from induced changes in the vibrational and rotational state of molecular bonds. The absorption

of infrared radiation by a material will be dependent on the rotational and vibrational motions of the molecules comprising that sample. When infrared radiation impinges on a sample, the photons may interact (or 'couple') with the molecular bonds, producing an oscillating dipole moment which, in simple terms, results in a change in the vibrational and rotational state of the molecular bond.^[17]

Molecules possessing zero dipole moment for any bond length, such as in the cases of homonuclear diatomic molecules (e.g. O₂, N₂), will not interact with the photons of infrared light. Conversely, the absorption of infrared radiation by highly complex molecules will be affected not only by the vibrational and rotational state of the bond, but also subtly by the interaction of that bond with its neighbouring atoms or molecular groups.

The frequency of infrared radiation at which the material under investigation will absorb will be dependent on three factors:^[18]

- 1) the identity of the atoms comprising the molecule;
- 2) the nature of the bonds between the component atoms (e.g. single as opposed to double or triple bonds);
- 3) the molecular structure (e.g. even isomers, with the exception of enantiomers, will have differing absorption properties).

Most materials, particularly those of a biological origin, are composed from various compounds possessing any number of molecular conformations which will in turn absorb different frequencies when exposed to infrared radiation. This in turn can produce highly complex absorption profiles that are uniquely characteristic of the component functional groups of the molecule as well as the overall molecule configuration. Therefore, by measuring and plotting the amount of energy absorbed (or transmitted) by a material against the frequency of the impinging infrared radiation, one can obtain a 'profile' or 'fingerprint' of

the material being examined, which may then be compared to a library of reference spectra for identification purposes.

1.2.4 Infrared Instrumentation and Measurement Theory

A basic infrared spectrometer is comprised of an infrared energy source, the energy of which is collimated and modulated onto a sample. Depending on the sampling method selected, the infrared light may be collected once it has reflected off the sample (reflectance mode), or by measuring the amount of light transmitted through the sample (transmission mode). The reflected or transmitted infrared energy from the sample is then detected, the signal amplified where required, and the signal converted into an easily interpreted form and displayed using a computer or, in the cases of older instrumentation, a line plotting device.

The most common commercial sources of infrared light are the Globar (a silicon carbide rod) or Nernst filament (zirconium, yttrium and erbium oxide mixture). These sources produce an unmodulated beam of infrared energy. While it is possible to employ a dispersive grating system to vary the wavelength of the infrared light one frequency at a time, modern instrumentation manufacturers prefer to make use of the interferometric modulation of radiation, permitting simultaneous measurement of numerous wavelengths. To perform this, a Michelson interferometer (Figure 1-9) is commonly employed.

The Michelson interferometer is a device consisting of perpendicularly positioned plane mirrors, one moving, one fixed, and a semi-reflecting film placed between, known as the beamsplitter, which can produce an optical path difference that can then be used to modulate the beam of infrared light produced by the light source. The light from the interferometer strikes the sample, which is collected at the detector in the form of an interferogram, a data signal which can then be recalculated into a standard absorbance (the

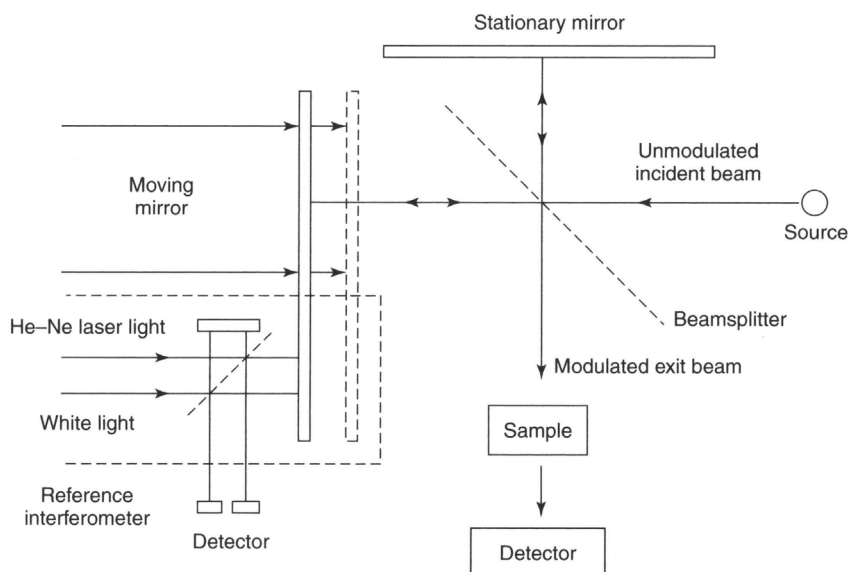


Figure 1-9: Michelson interferometer.^[19]

'units' of which are sometimes assigned the value a.u. (arbitrary units) in texts) versus wavenumber plot using fast Fourier transform algorithms. If a step-scan interferometer is employed, the rate of data flow from the sample is determined by the rate of the stepping of the moving mirror. As soon as the mirror moves, the detector initiates its data gathering and transmission again. The data collected by the detector is a function of the interferometer optical path difference (OPD), but cannot be represented in the form of spectral frequency and intensity until Fourier transformation has taken place.^[20] By employing an interferometer in the place of an IR tunable filter, different component frequencies in the infrared light beam can be measured simultaneously, and one obtains both a multichannel and multiplex advantage, improving measurement efficiency.^[20]

Once the infrared light is focused onto the sample, the photons of varying wavelengths interact with the molecules in the sample, and infrared radiation is transmitted or reflected (depending on the mode of instrument operation) by the sample. These wavelengths are then focused using a variety of lenses and mirrors onto the detector. Modern-day spectrometer manufacturers tend to offer a variety of infrared sensitive materials for

detectors, such as mercury cadmium telluride (MCT) and indium antimonide (InSb). However, detectors made of such materials are by their nature unstable and require cryogenic cooling to operate (~70 to 80 K).

Infrared spectrometers may be used to examine gaseous, liquid and solid samples. The sample holders are composed of infrared-transparent materials such as calcium fluoride (CaF₂), sodium chloride (NaCl) or potassium bromide (KBr), which are in turn non-reactive with the sample being examined.

1.2.5 FTIR Microspectroscopy

Compared to visible light, infrared waves possess relatively long wavelengths and low photonic energy, which means that they can be useful for the non-destructive analysis of biological materials. Fourier Transform Infrared (FTIR) microspectroscopy, or simply infrared microscopy, is basically optical microscopy in the infrared region of the electromagnetic spectrum. Infrared microscopy is highly useful analytical technique for the study of materials that have significant absorption or transmission bands in the infrared wavelength region, but may not possess any particular features of note when examined in the visible spectrum.

The infrared microscope itself is comprised of specially designed infinity corrected objective lenses (often referred to as Schwarzschild or Cassegrain) with reduced numerical apertures (the numerical aperture is a measure of the microscope objective's ability to gather light at a fixed object distance) which increase the depth of focus in the specimens. These objective systems are composed to two mirrors, one concave and one convex, with a hole in one mirror for the light to pass through,^[21] and are required in the place of standard microscope objectives due to the extremely poor infrared transmission properties of glass. Most infrared microscopes are also designed to permit normal sample viewing using

interchangeable standard visible light microscope objectives mounted on the same turret as the infrared objective lens.

A diagrammatic representation of the construction of a standard infrared microscope has been given in Figure 1-10.

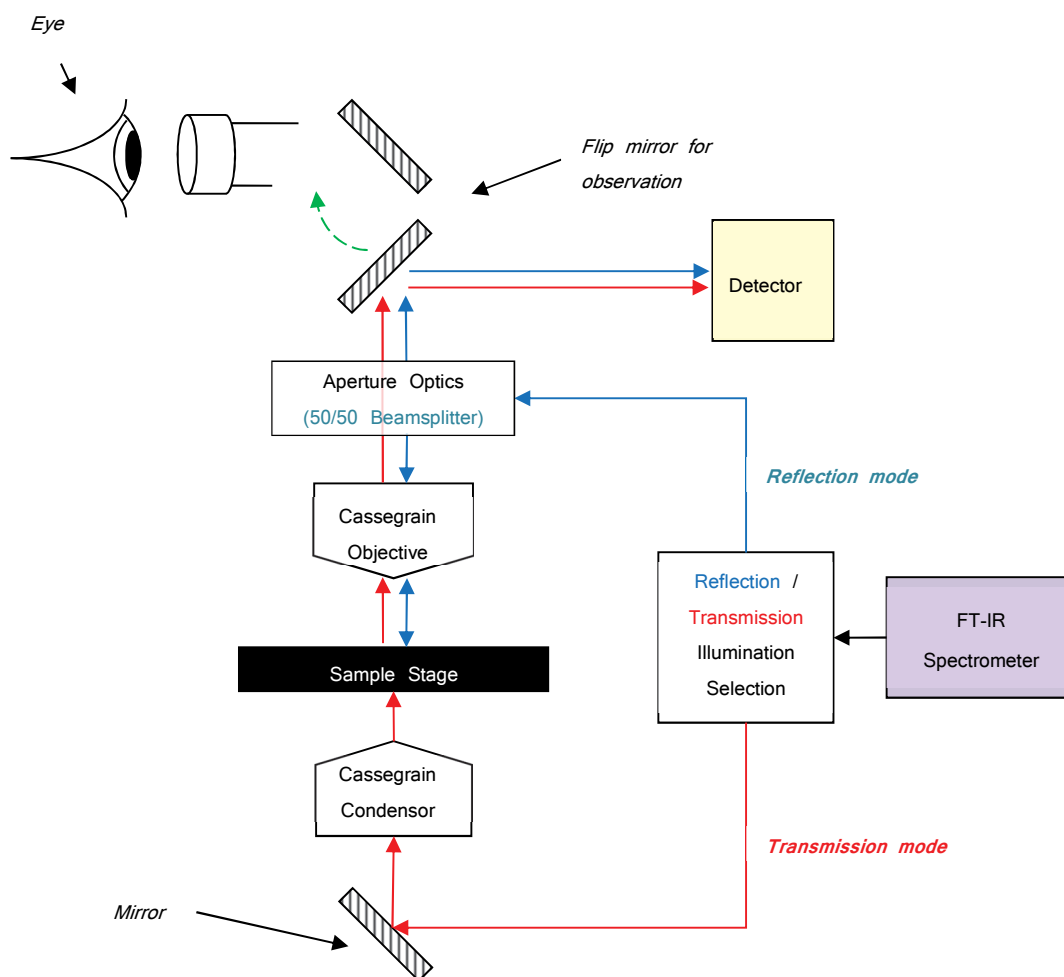


Figure 1-10: Diagrammatic representation of the construction of a FTIR microscope.

An advantage offered by FTIR microspectroscopy is its ability to enable microscopic pin-pointing of particular areas of interest within a heterogeneous sample matrix, such as those commonly found in biological tissue samples and materials analysis. FTIR

microspectroscopy is commonly employed in the forensic science arena, in such areas as fibre and paint chip analysis.^[22] The non-destructive nature of the technique, as well as the need for minimal to no sample preparation, makes FTIR microspectroscopy particularly suitable for microscopic quantities of samples and where subsequent testing may be required.

Ultimately, the most important feature of an optical system is its resolving power. The spatial resolution of any optical system is primarily controlled by the diffraction limit set by the wavelength of light employed. Hence, light with a shorter wavelength is capable of resolving finer detail than light with a longer wavelength. While mainly affected by the wavelength of light used and the numerical aperture of the microscope system employed, resolution capability is also influenced by the coherence of the light used for illumination, the degree of aberration correction, and even such factors as the specimen material and preparation methodology.^[23] If the optical system is well-constructed and capable of performing near its diffraction limit in the case of digital sensors and detectors, then the final image obtained is also affected by the spatial proximity of the pixels or sensors in the detector. Theoretically, the best spatially resolved image will be one in which at least one intervening pixel can detect the 'space', or difference in intensity value, between the two features of interest.^[24]

For example, a standard biological light microscope is generally capable of achieving a useful resolution capability of approximately 0.2 to 0.35 μm . With its longer wavelengths of light, an infrared microspectrometer with a standard globar source is capable of a theoretical spatial resolution (as defined by the Rayleigh criterion) anywhere from 5 μm upwards in the mid-infrared region, with the actual resolution achieved being highly dependent not only on the optics of the system, but the detector type (aperture or detector array) and response, spectrometer efficiency, wavelength range and the type of sample being examined. Synchrotron light sources offer better spatial resolution for the infrared region as its highly

collimated nature and brightness (100 to 1000-fold compared with a standard Global) enables the optical system to truly approach the diffraction limit, producing actual resolution values of around 3 - 5 μm in the mid infrared range with the added bonus of faster collection times and greater signal to noise ratios.^[25] However, synchrotron sources are, at this point in time, far from being considered as commercially viable for average research or hospital laboratory applications.

1.2.6 Applications of FTIR Spectroscopy to Biological Studies

Biological applications of infrared spectroscopy have ranged from lipid concentration determination in blood serum to the analysis of the interactions of various chemical mixtures with living tissue.^[26] The study of diseased states in various tissue types has been an area of considerable FTIR spectroscopic interest over recent decades. Pathology applications examined have sought to overcome the limitations of traditional visible light microscopic analysis. Examples of FTIR spectroscopic studies on diseased tissues have included the examination of human Alzheimer's diseased brain tissue,^[27] the detection of dysplastic and neoplastic changes in human cervical tissue,^[28] the examination of human prostate tissue for malignancy,^[29] diagnosis of abnormalities in resection margins in colon cancer patients,^[30] skin tumour diagnosis,^[31] and the imaging of the spatial distribution of various matrix components in cartilage samples.^[32] An excellent summary of FTIR spectroscopy applications to biological tissue analysis can also be found in an article produced by Movasaghi *et al.*^[33]

Biological tissues are by their very nature highly complex, and comprised of many different cell types, the interaction between which is mediated by various chemical pathways characterised by numerous chemical species. Infrared spectroscopy researchers such as Diem *et al* stress the necessity of understanding which spectral features are characteristic of particular cell-types.^[34] It is critical for accurate spectral interpretation that the spectroscopist

is aware that differentiation, maturation and natural stages in the cell cycle may also contribute to spectral variation. Spectroscopic variation between samples from the same tissue donor, as well as natural cell abnormalities present in healthy tissue, are yet further confounding variables which must be anticipated by the spectroscopist. Thus, minimizing variation and contamination due to sample preparation is of utmost importance.

Of potential concern for the spectroscopist is the method of sample preparation (i.e. frozen sectioning, paraffin embedding, etc.) chosen to enable the preservation, cutting and mounting of the tissue sections. Researchers such as Kidder *et al* and McIntosh and Jackson suggest that the use of traditional tissue processing techniques such as formalin fixing and paraffin embedding change the sample composition and may give rise to artefacts in the spectra, so the most suitable sample preparation technique is to employ frozen sections.^[35,36] Authors such as Dukor suggest that the routine removal of the embedding materials and fixatives prior to application of staining is sufficient for the removal of such potential contaminants.^[37] However, the spectroscopist must bear in mind the biochemical changes of interest in the tissue, and preserve and mount the tissue section accordingly.

Infrared microscopy of thin tissue sections is usually performed in either transmission or reflectance modes. Transmission mode requires that the thin tissue sections are mounted on an infrared-transparent substrate, such as calcium or barium fluoride. This technique, while providing good quality spectra, requires access to more expensive materials (the barium fluoride plates are considerably more expensive than standard glass microscope slides), which are also less robust when exposed to common pathology sample preparation solvents. An alternative suggested by Wood *et al*^[14] is the employment of glass slides coated with an infrared reflective material, with the tissue section mounted on the reflective coating and all infrared analysis performed in reflection mode. This collection method for infrared images is sometimes referred to as a 'transflection' experiment, as the incident infrared beam is transmitted through the tissue, reflected off the coating and transmitted

again through the tissue (see Figure 1-11). These slides are more cost-effective than standard barium fluoride plates, convenient to store, transparent to visible light, and resistant to many commonly employed biological solvents, permitting subsequent staining and standard pathological examination of the tissue.

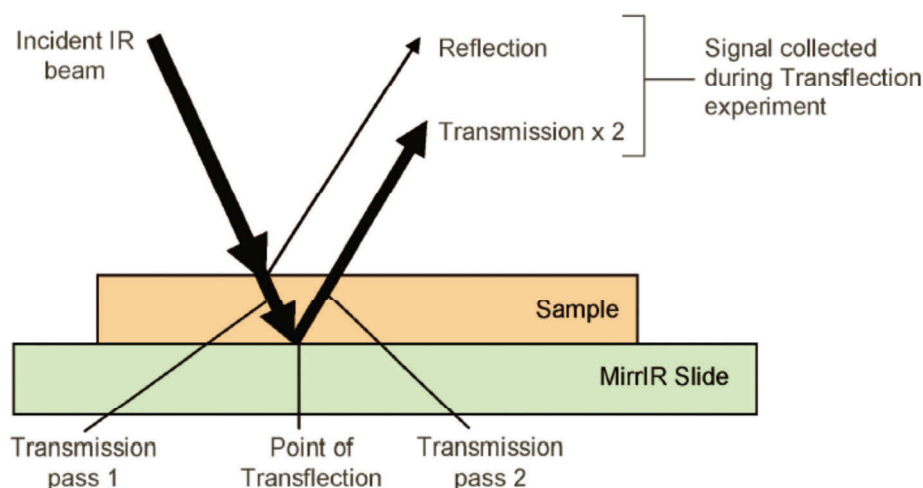


Figure 1-11: Diagrammatic representation of the 'transflection' sample analysis method (from Bassan *et al.*)^[38]

The majority of early studies performed using FTIR microscopes involved the sampling of a tissue specimen at a single point. However, the addition of a motorised microscope stage to the microscope made it also possible to obtain a chemical image using an approach referred to as spectroscopic mapping. In this method, the sample is moved at predefined intervals in an x - y plane, which then is used to compose a two dimensional image with an infrared spectrum at each point. Hence, a three-dimensional data array is constructed: two spatial dimensions, and a spectrum at each point on the sample. A schematic diagram of the three-dimensional array of data has been given in Figure 1-12.

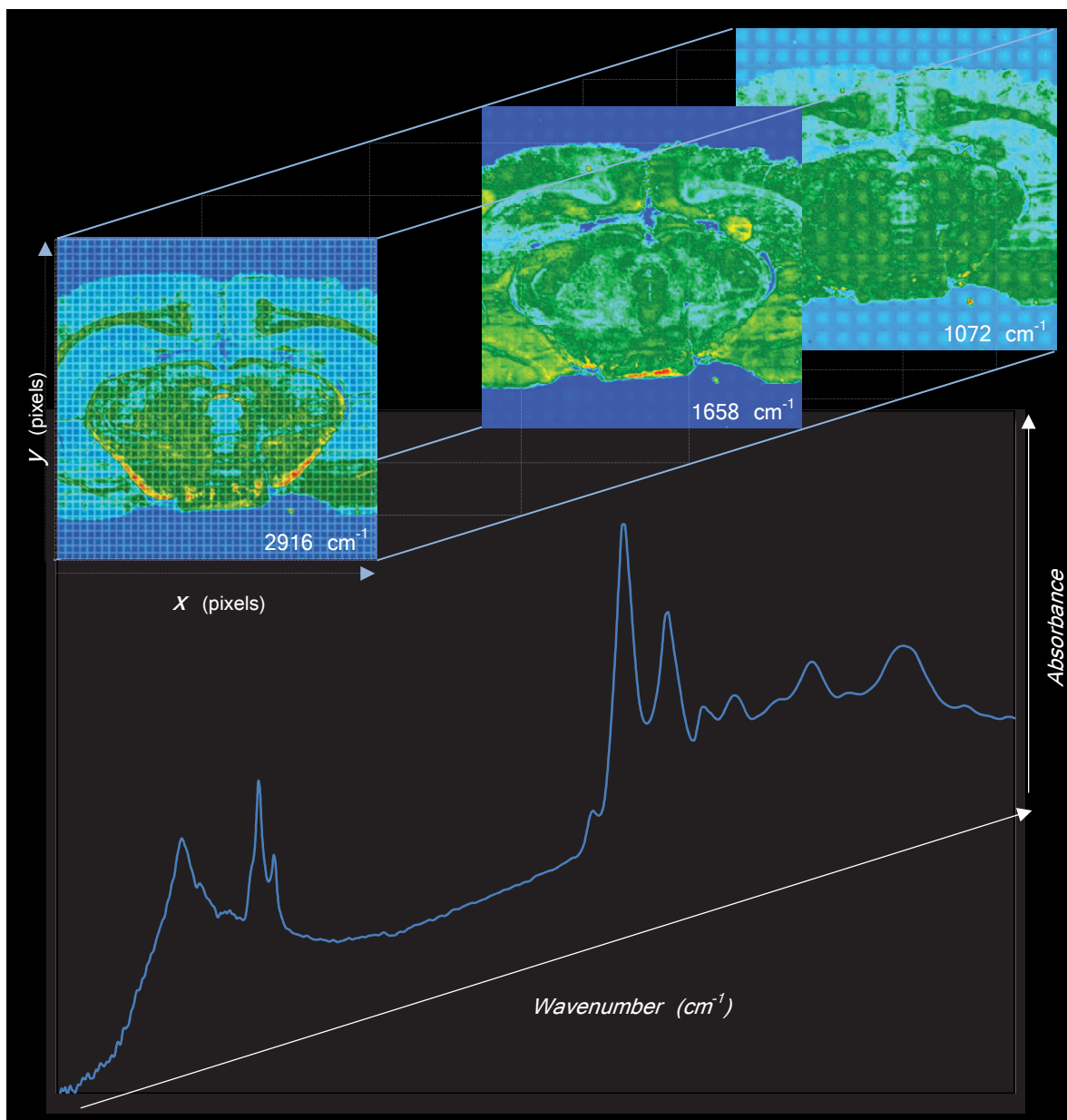


Figure 1-12: Diagrammatic representation of the three dimensional infrared image data array (sometimes referred to as a “data cube”).

There are a number of ways in which the infrared data array may be ‘viewed’ and information extracted from the image. One of the simplest means of examining the image data array is to construct a frequency map. For this approach, the measured value (e.g. absorption maximum) at a specified wavenumber in each pixel is assigned a particular grey

(if a grey-scale image is desired) or colour value, enabling a 'chemical map' to be constructed. Spatial distributions of particular chemical components within the sample image, and therefore structural information, may be quickly viewed. Grey level maps, false colour maps and contour plots are all examples of the means by which the sample image may be displayed in a two dimensional representation.

Alternatively, Beer's law states that functional group intensity values (e.g. peak intensity, integrated intensity, etc.) are directly proportional to the concentration of the compound producing that particular absorption value, and hence it is also possible to produce chemical maps displaying the spatial distribution of the selected functional group within the image (the images in the top of Figure 1-12 are examples of intensity maps at specific bands). Peak intensity ratios may also be employed for image construction. However, baseline fluctuations, instrument noise, sample thickness non-uniformity and overlapping bands of interest are all potential causes of error in the intensity values obtained for the individual pixels. In addition to these errors, the contrast obtained in the image may sometimes not be attributable to simple chemical differences and can arise due to non-chemical sources, such as refractive index variation and light scattering.^[20] The implementation of data treatment routines such as baseline subtraction, normalisation, and smoothing/derivatisation can assist in reducing or removing some of these non-chemical contrast effects. Caution must be exercised when employing such data treatment routines as one may also reduce or even remove the quantitative information present in the data. For example, the use of spectral derivatives, while accentuating narrow spectral features and assisting in smoothing the spectrum, can also negatively affect data with slowly varying backgrounds and potentially introduce noise.

While successful in application, spectroscopic mapping is time consuming, as the image is constructed point by point, spectrum by spectrum. An alternative method which has only become commercially available in the last decade employs the use of former military

technology - infrared detector arrays - which permit the simultaneous collection of spectra from numerous sample points, and is the subject of discussion in the next section.

1.2.7 FTIR Chemical Imaging

FTIR chemical imaging (FTIR CI), also sometimes referred to as 'spectral' or 'hyperspectral' imaging, is an instrumental technique by which the chemical composition of a sample may be determined in a spatially-resolved manner, with thousands of spectra being collected simultaneously across the sample. As with standard FTIR microscopy, the FTIR CI instrument is comprised of a spectrometer and an infrared-capable microscope with a specialised detector.

The key to this technology has been the development of the focal plane array (FPA) detector. The IR array is sometimes referred to as a "hybrid detector",^[20] which simply refers to the fact that it is comprised of two semiconductor layers: a photon detector layer (often made from mercury cadmium telluride (MCT or HgCdTe) or indium antimonide (InSb)) and a second layer which carries out the signal collection, multiplexing and amplification. These layers are sandwiched together and linked both mechanically and electrically by the presence of indium "bumps", with each bump representing a discrete detector or pixel.

Unlike standard infrared detectors which employ a single detector element, a HgCdTe (mercury cadmium telluride or MCT) FPA is comprised of numerous microdetector elements, mounted in a square two-dimensional grid formation. This array permits the simultaneous collection of thousands of individual infrared spectra, with the most common arrays consisting of 64×64 or 128×128 detector elements, producing images comprised of up to 4096 or 16384 individual spectra, with a spatial resolution in the range of 5 - 6 μm for a 64×64 array size.^[39,40] In the case of a standard MCT array, each detector element is sensitive in the 2.8 - 11 μm infrared fingerprint region. As with the point-mapping method, a four-dimensional array of data is again produced from a single sample, although

at a significantly faster rate. In addition to this, by placing the sample on a computer-controlled moveable stage it is also possible to obtain a mosaic of images, permitting an even larger area to be imaged.

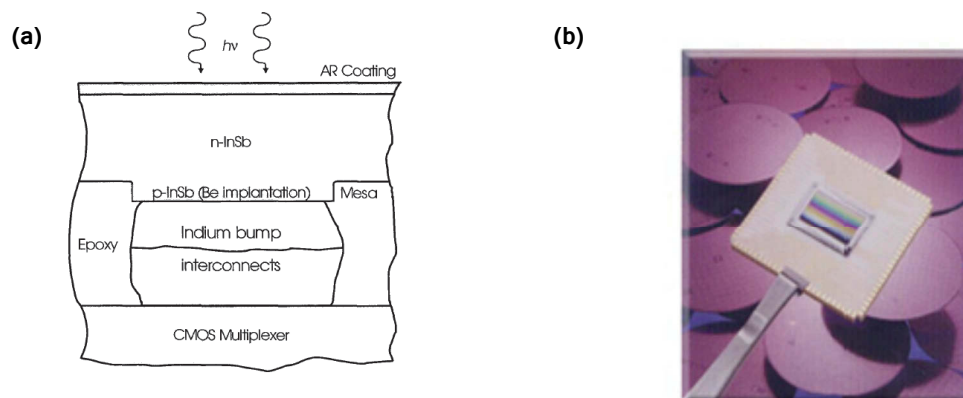


Figure 1-13: (a) Schematic of an indium antimonide (InSb) detector heterostructure,^[41] and (b) a complete focal array detector assembly.^[42]

FTIR CI offers the user the ability to collect large amounts of chemical data in a relatively short period of time, and it is this multichannel, multiplex advantage which has seen it employed in a wide variety of research applications. As was discussed earlier, forensic scientists are constantly seeking out new ways in which they may analyse evidence samples more rapidly and in the least destructive manner. FTIR CI has already been explored for the characterisation and comparison examinations of forensic evidence types such as vehicle multi-layered paint chips^[43,44], counterfeit bank notes,^[45] and bi-component fibres.^[46] The ability of FTIR CI to assist in determining the sequencing of intersecting lines on documents in fraud investigations^[47] and even to detect the presence of illicit drugs in human hair samples^[48] has been assessed. A considerable volume of work has also been published on the application of FTIR CI for the detection and enhancement of latent fingerprints (i.e. fingerprints not generally visible to the naked eye) on materials for which

current standard methodologies, such as powdering or chemical treatments, are known to produce poor results.^[49-52] An additional advantage of FTIR CI for fingerprint examiners is its simultaneous ability to produce a fingerprint image (enabling identification of the donor), distinguish between overlapping fingerprints, as well as give potential intelligence information in the form of the chemical contaminants within the latent deposit material, such as drug and explosive traces.^[53-56]

As with the forensic studies, the non-destructive nature of FTIR CI, combined with the chemical information that the technique provides, has attracted considerable interest from those undertaking the examination of historical cultural artefacts. For example, an object such as a painting may exhibit a complex, multilayered structure, and FTIR CI can be employed to identify each of the paint components used which can then assist in the understanding of the painting techniques used, state of conservation and any causes of material degradation. Paint cross-sections from historical objects have been examined using FTIR CI in a number of studies.^[57-59] FTIR CI has also been employed to identify the individual mineral components of ancient Maya paint^[60] as well as to assist in the characterisation of the organic compounds present in early 20th and late 19th century albumen photographic prints.^[61]

The ability of the infrared imaging approach to simultaneously capture microscopic sample images (and hence structural composition of cells and tissue), as well as to extract the associated chemical data, highlights the technique's biological application potential. FTIR CI offers a means by which changes in the cellular chemistry of biological samples may be monitored, and has led to an ever-growing number of studies in its application to tissue pathology. An early example of the power of this technique was the visualisation of silicone gel in human breast tissue,^[35] demonstrating the ability of this analytical approach to detect a foreign inclusion which could only be visualised and not identified using standard light microscopy. Biological samples examined in the literature using FTIR CI have included

bacteria,^[62,63] algae,^[64] wood,^[65] and numerous mammalian tissues such as brain,^[66-69] bone,^[70] cartilage,^[32] periodontal,^[71] skin,^[72-74] hair,^[75] heart,^[76] lung,^[77] cervical,^[14,78,79] colon,^[80] prostate,^[39,81-84] aortic,^[85] kidney,^[86] breast,^[87-90] oral,^[91] and lymph nodes.^[92,93]

Studies such as those conducted by Lasch and Naumann,^[94] Kidder *et al.*,^[95] and Walsh *et al.*^[96] indicate that FTIR chemical imaging may effectively be used to complement existing standard histopathological techniques, particularly assisting in the diagnosis of cancer. Of particular interest is the application of the imaging technology to cases which are difficult to conclusively diagnose using standard histopathology techniques, such as in samples that, while exhibiting a homogenous morphology, may in fact possess chemical heterogeneity indicative of a particular disease type or stage.

1.2.8 Limitations of FTIR Chemical Imaging

Although FTIR CI offers the opportunity to collect both spatial and compositional information from a sample, there are certain considerations which must be taken into account when assessing the viability of using this technique in a routine analysis schedule. Of most notable importance is the high cost of the FTIR CI equipment compared to a standard light biology microscope. Unlike standard microscopy, the collection of the image data may be time consuming, particularly in cases where a large region of sample requires imaging, and consequently tiling of images must be performed. Data storage may also present an additional challenge, particularly when file sizes are in the range of tens to hundreds of megabytes, although this problem may somewhat be alleviated by advances in data storage capabilities over time. Data interpretation itself is very complex as the spectrum obtained represents a combination of all of the infrared absorbing molecules in the IR path, and in the case of biological tissues most often requires the employment of multivariate algorithms and hence sophisticated computer software.^[97]

As a general diagnostic tool FTIR CI shares common limitations with that of light microscopy, such as the need for very thin samples and the fact that studies can only be generally conducted *ex vivo*. Also, IR sensitive detectors are subject to the effects of large dark currents, requiring cryogenic temperatures for operation. Another potential problem can be uneven light illumination across the sample area (which can lead to 'tiling' artefacts when performing image mosaic collection), as well as the mediocre light intensity typical of a global infrared light source.^[98] The use of high quality reflective optics, stabilised stages, scan co-addition and correctly aligned optical and mechanical elements, can reduce the effects of some of these problems somewhat. Alternative high intensity light sources, such as that derived from synchrotron radiation, are also an option, but their availability to operators is economically and geographically limited.

1.2.9 Advantages of FTIR Chemical Imaging

In spite of the disadvantages described above, FTIR CI offers a number of distinct advantages in the pathological examination of tissues. FTIR CI offers the pathologist an alternative means by which tissue samples may be screened for the presence of diseased states, as the "sample morphology can be probed by the quantitative spectroscopic parameters that highlight intrinsic biochemical boundaries and changes".^[20] Unlike current histopathological techniques, chemical staining of the tissue section is not required (reducing sample processing time), and examination of the physical sample is computerised, requiring minimal manual input. By selecting one or several spectral parameters the operator is able to control the image contrast (i.e. what features of interest are indicated), and effectively perform 'digital staining' of the sample image.^[74] Alternatively, more complex image processing may be performed using a wide range of chemometric techniques (e.g. hierarchical cluster analysis) to identify regions containing similar chemical properties. Compared with a standard pathology investigative approach which may involve numerous tissue samples, stains and histochemical techniques to search for a number of tissue

features, infrared images offer the ability to examine for numerous chemical species in the one tissue image.

One infrared image captured using either the mapping technique or a focal plane array can contain thousands of spectra, and depending on the spectral resolution selected, hundreds of points within each spectrum. While the resulting data cube requires computational assistance in the forms of data storage and mathematical processing (which is not a significant issue considering processor technology developments in the last decade), the mere fact that such large data sets are collected supports the validity of the statistical conclusions that may be drawn from the data. This is of particular importance when one considers the fact that the spectroscopic differences between tissue components or disease states may be extremely subtle.

Statistical evaluation of the collected data cube, using validated methods to classify the cell types present in the sample, offers a potentially objective and robust tissue screening technique. This can reduce the need for human interpretation and may potentially assist in reducing misdiagnosis rates. By establishing validated chemometric data treatment techniques, the examination of tissue sections becomes a non-subjective process and the potential for tissue assessment automation is clear.

In addition to these advantages, FTIR CI is a non-destructive analysis technique, permitting later examination of the same tissue specimen using alternative examination techniques. With advances in technology (particularly computer processor capability and speed, source and detector improvements), the infrared chemical imaging methodology may permit extremely rapid diagnosis times, resulting in earlier treatment initiation, as well as improving the detection and diagnostic sensitivity (i.e. detecting chemical change prior to any architectural manifestations of disease in the tissue).

1.2.10 Data Handling Methods

In an ideal world, the detection of change within a biological tissue using FTIR CI will simply involve the direct correlation of a measurable spectral feature (or number of features) with the biochemical changes occurring across the tissues or cells of interest. Earlier infrared tissue pathology studies, such as those conducted by Andrus *et al*^[99], Wong *et al*^[100] and Cohenford *et al*^[101] for example, focused on the possibility of measuring or ratioing peak height or area changes in various key functional regions of the spectrum, in an effort to discover the 'spectral profile' of a particular cellular material or disease state of a tissue. It was hoped that the mapping of specific functional group intensities and frequencies in the sample image would enable variation in tissue type to be clearly delineated. This approach has proven successful to some extent but may, in some cases, be of very limited value in disease states where the tissue morphology and biochemical make-up may vary only subtly across the imaged area.

In biological systems, there are often many biochemical changes happening at any one moment, which may in turn produce any number of confounding variables which the spectroscopist must take into account. Additionally, one sample may exhibit numerous cellular and tissue types, each composed of primarily the same major biological molecules, producing a large number of spectra that can appear grossly very similar. Compound this with the problems of potentially large, and quite biologically 'normal' intra- and inter-sample variations, and it becomes painfully apparent that the goal of finding one single spectral marker indicative of a particular tissue pathology is unattainable, particularly in the arena of cancer detection.^[39]

As may be observed in Figure 1-14, tissue samples are by nature highly variable in biochemical composition, and yield equally complex, and highly characteristic, infrared spectra. Consider a single infrared spectrum collected at 8 cm⁻¹ resolution, across a range of 900 to 4000 cm⁻¹. Such an infrared spectrum would possess 805 individual data points,

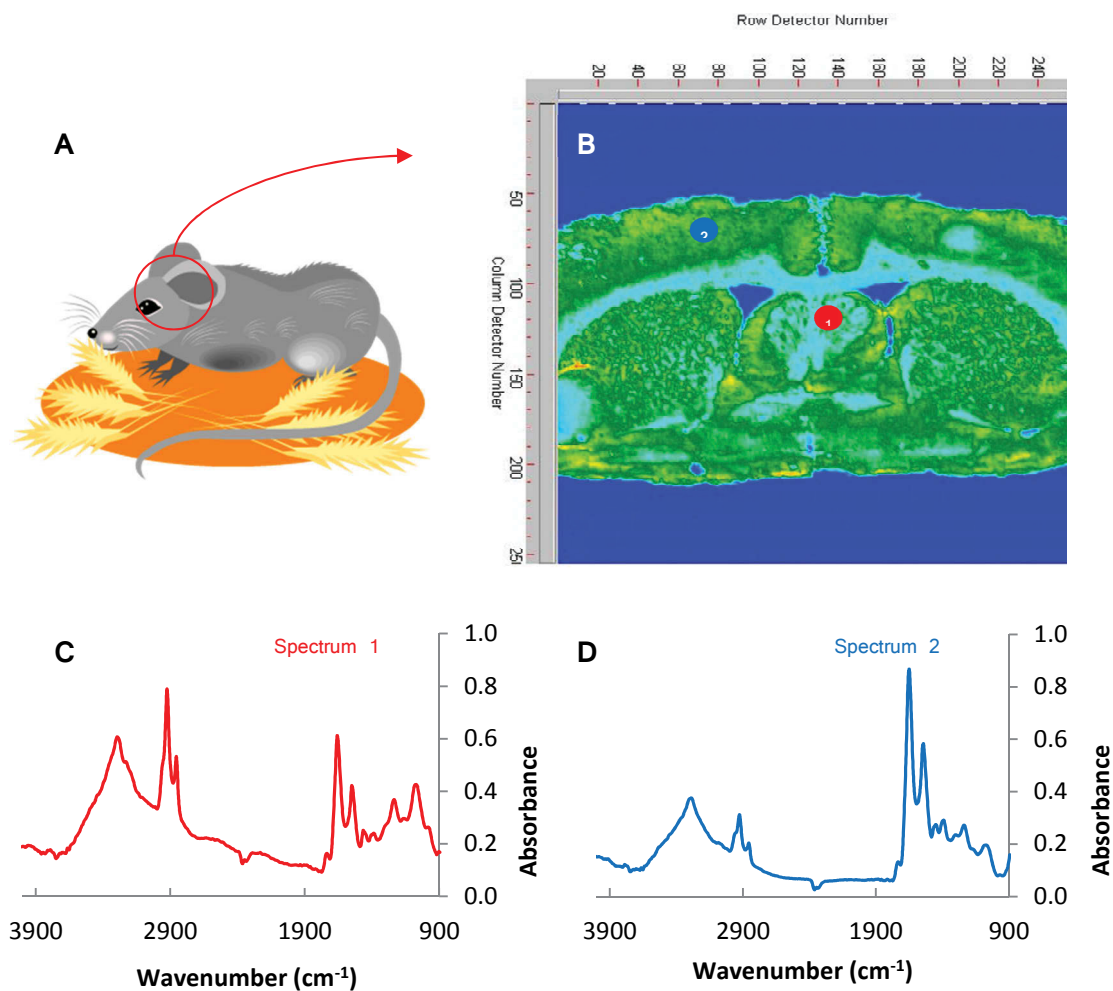


Figure 1-14: An example of the spectral complexity of a mammalian tissue sample - coronal mouse brain section. Panel B is a coronal section of brain imaged at 1650 cm^{-1} (amide I peak), 16 cm^{-1} resolution in reflectance mode. Panels C and D show the spectra obtained from two regions of the tissue sample.

or variables. To compare and analyse numerous samples consisting of such complex multivariate data, one requires sophisticated processing methods. To enable sample comparisons and data simplification, treatment of the resulting infrared data with computer-based statistical techniques is required. These techniques, referred to collectively as multivariate analysis, may be employed with FTIR CI data, to extract the characteristic

features of particular cell types, and could potentially be used to classify and/or identify different tissue types. The aim of multivariate classification techniques is to remove subjectivity from the interpretation of spectral image data. However, it is critical to select the most appropriate data pretreatment processes and classification algorithms to avoid drawing erroneous conclusions.

1.2.11 Data Preprocessing

Data preprocessing techniques may be employed to reduce inherent noise and other artefacts in the image spectra, as well as to remove redundant data and optimise the information available for subsequent multivariate analysis. Some common spectral preprocessing procedures employed by spectroscopists are described below.

1.2.11.1 *Baseline Correction*

The need for a flat, consistent baseline is particularly necessary in quantitative analyses where peak heights or areas are employed. This technique is employed to produce a flat baseline in spectra with sloping or curved baselines, which can be caused by sample scattering, instrument drift or inappropriate choice of background.

The simplest approach taken to correct baselines is to generate a function (such as a straight line or curve) that parallels the baseline shape and then subtract this function from the sample spectrum. Ideally, the user will employ a minimal number of line segments to generate the best baseline correction function, as spectral artefacts may be easily introduced with inappropriate function application.^[102]

1.2.11.2 *Spectral Smoothing*

All infrared measurement systems are subject to a varying degree of 'noise' contamination, which often arises from a variety of sources, both instrumental and sample-related. Noise

can be eliminated, at least partially, by employing spectral smoothing. Most smoothing functions are based on a convolution between the spectrum and a vector,^[19] with the user determining the degree of smoothing applied and hence the number of points of the vector. For example, a simple 5-point linear smoothing filter can involve looking at a data point and the two data points on either side along the spectrum, calculating the average intensity and using this as the new value at the original point. This process is repeated at each point along the spectrum (see Figure 1-15) to obtain a “smoothed” spectrum.

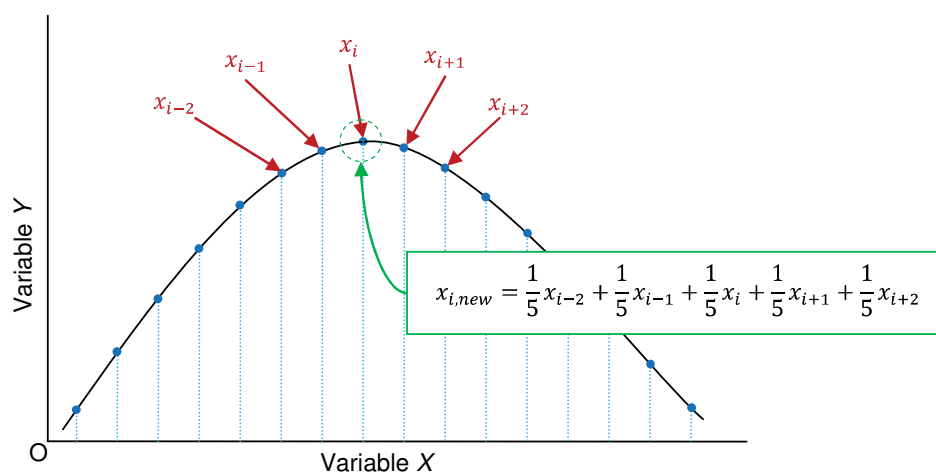


Figure 1-15: An example of how the data points are selected for a 5-point moving average.

There is a variety of mathematical routines to perform smoothing, such as the Savitsky-Golay filters, which use the same principles as the linear filter described above, but instead use a quadratic, cubic or even quintic approximation for the data. Other moving average smoothing filter options include the triangular smoothing functions,^[103] Hamming and Hanning windows (which attribute different weights to the various data points according to their location around the central value).^[104] Alternatively one may employ running median smoothing (which uses the median rather than the mean) to reduce the effects of ‘spikes’ (outliers) in the spectra.

In general, the smaller the smoothing value, the more subtle the alterations to the spectra. However, over-zealous smoothing, while seemingly improving the spectral shape and minimising noise, can also lead to a loss of features (resolution and peak intensity reduction) in the data, and should be performed with caution.

1.2.11.3 Derivatives

A derivative, by definition, is the determination of the slope of a curve at a particular point. The spectral first derivative calculates the slope of the curve around each point in the spectrum as a function of wavenumber, and the second derivative is the derivative curve of the first derivative spectrum. It is possible to take n -th order derivatives of a spectrum, but generally only the first and second are used in infrared hyperspectral analysis.

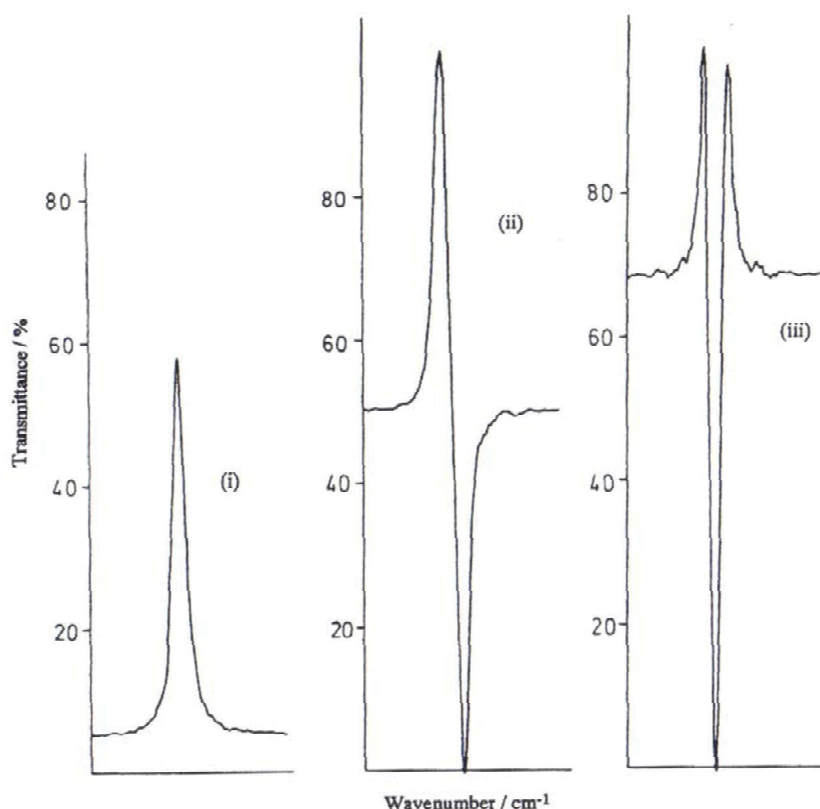


Figure 1-16: Spectral derivatives: (a) a single absorption peak in the original spectrum; (b) the first derivative and (c) the second derivative.^[105]

To reduce the computational complexity of the derivative function, a simpler method was developed by Savitsky-Golay in 1964, which employs the use of coefficients.^[106] The added advantage of using this calculation approach is that it incorporates a smoothing function. To perform the calculation, one simply has to decide the order of the derivative, along with the shape and number of points in the smoothing function, and obtain the corresponding coefficient values from established tables.^[106-108]

The advantage of taking the first derivative of a spectrum, aside from its smoothing effect, is that it improves resolution as it can detect the more subtle changes in gradient common in complex organic spectra. By taking the second derivative, closely overlapping peaks may be resolved, such as shown in Figure 1-17, and produces a sharp band with a minimum value at the same wavelength as the corresponding peak in the underlying original spectrum. However, derivatisation will enhance sharp bands at the expense of broader ones, and can potentially enhance noise in the spectrum or accentuate unwanted higher frequency spectral features.^[102,109]

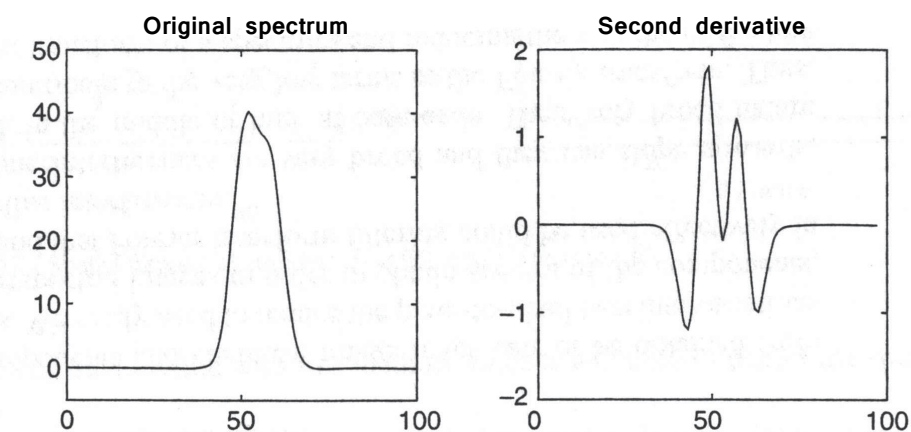


Figure 1-17: Example of how overlapping peaks may be resolved taking the second derivative (and multiplied by -1) of the original spectrum.^[103]

1.2.11.4 Normalisation

Normalisation is a data treatment method used to remove the variation in spectral intensities brought about by such factors as varying sample thickness or concentration. The investigator can choose to normalise spectra in a number of ways, but all tend to share the same idea of dividing each data value by a normalisation factor, f .

A few of these methods have been listed below:

- *Minimum/maximum (min/max) normalisation*

This method simply scales the spectra (which may be denoted as a row vector x), so each point on a spectrum will possess a value between 0 and 1 (according to the maximum and minimum intensity value in the selected spectral region). Hence, the min/max normalisation factor, f_i , of a spectrum for the i th sample with J variables will be:

$$f_i = \max(x_i) - \min(x_i) \quad (3)$$

The sample's minimum value is then subtracted from each value (at each variable), x_{ij} , and divided by the min/max normalisation factor:

$$x(norm)_{ij} = \frac{x_i - \min(x_i)}{f_i} \quad (4)$$

- *Vector normalisation*

Unlike the min/max normalisation approach, vector normalisation utilises the average value of the intensities for a selected spectrum or spectral region.

For vector normalisation, the factor f_i is given the following definition:^[110,111]

$$f_i = \sqrt{\sum_{j=1}^J (x_{ij})^2} \quad (5)$$

Using the method above means that the sum of squares of each of the data points in the spectrum (i.e. the elements of vector $\vec{x}_i$) following transformation equals one.

- *Normalising to a preferred peak*

This is much the same as the min-max calculation, but the value at a particular peak, n , is selected by the user. In cases where the peak of interest is the peak of maximum intensity, this will produce a similar result to the min-max approach.

$$f_i = x_{in} \quad (6)$$

1.2.11.5 Spectral Subtraction

Substrate contaminants or other known compounds in a sample may also be removed from the spectrum using a form of spectral subtraction. The most basic form of spectral subtraction involves the point-by-point subtraction of the absorbance values of the spectrum of interest (the reference, or subtrahend, of the compound we wish to remove) from the sample spectrum. It must be noted that by performing such direct subtractions one is not allowing for differing concentrations between the sample and reference material. However, this problem may be overcome to some extent by the employment of a *subtraction*, or *scale factor*.^[102]

$$(\text{Sample}) - (\text{Subtraction factor}) \times (\text{Reference}) = \text{Result} \quad (7)$$

For example, a potential biological tissue contamination issue in infrared spectroscopy is the unwanted presence of water vapour peaks in the sample spectra. Spectroscopists such as Diem^[112] and Lasch^[113] employ a form of spectral subtraction to minimise these effects.

Although the subtraction factor can assist in removing contaminants, caution should be exercised when performing such subtractions, particularly in cases where the contamination is not present in a consistent manner throughout the entire sample. Two common spectral artefacts that may appear in subtracted spectra are derivative shaped peaks and peaks arising from incomplete subtraction. Derivative shaped peaks are caused by the subtraction of one spectrum from another in which the two spectra do not overlap each other perfectly. Such differences in peak locations are often caused by wavenumber shifts or band broadening brought about by chemical interactions occurring in the sample. The second artefact mentioned above occurs where some of the peak heights obtained in the sample do not adhere to Beer's law and in turn produce a subtraction spectrum with peaks from the reference material. The subtraction factor cannot compensate for this effect, and alternative solutions may need to be sought, such as changing the sample concentration or pathlength to bring it in line with Beer's law for those peaks affected, or simply ignoring the bands affected (where possible).

1.2.11.6 Mean Centring

To simplify the visualisation of relationships between samples, mean centring can be performed. By subtracting the mean value for each variable j from the data values (x_{ij}) recorded for that specific variable (x_{ij}), it is possible to obtain the mean centred value $x_{ij(mc)}$:

$$x_{ij(mc)} = x_{ij} - \bar{x}_j \quad (8)$$

This data pre-processing step can be useful in reducing the influence that variables with intense magnitudes will have when one is attempting to study the variation in smaller variables. Mean centring does not alter relative inter-sample relationships nor does it usually significantly influence pattern recognition and classification results.^[111]

1.2.11.7 Minimum Noise Fraction

The maximum noise fraction and its modified form, the Minimum Noise Fraction (MNF) transform algorithms, are employed as a means of reducing noise levels in hyperspectral data, and have been used in hyperspectral imaging applications such as environmental remote sensing, materials analysis and the examination of biological tissues.^[114-121] By employing MNF, the user is offered a number of functions, including the segregation of noise in the data, as well as a means of analysing the inherent dimensionality of the image spectra, which may then in turn be used to identify the signal-rich parts of the spectra and to perform data reduction.^[122,123]

The MNF transform is comprised of two cascading principle component (PC) transformations.^[124] The first transformation obtains an estimated noise covariance matrix for the image data and then employs this result to decorrelate and rescale the noise present in the spectra to unit variance. These noise-whitened spectra are then subjected to a second transformation, a form of Principal Components Analysis (discussed further in section 1.2.13.1). To determine the inherent dimensionality of the data (i.e. how much of the spectrum contains information as opposed to simply noise), the user examines the final 'eigenvalues' (characteristic values as determined by the MNF routine) in reference to their associated images to find which spectral bands (frequencies) are responsible for the most coherent images. Once these bands are identified, the noise-dominated images may then be removed and data quality improved.

1.2.11.8 Additional Preprocessing Steps

Depending on the instrumentation and samples examined, there may be additional spectral-preprocessing steps that can be effectively employed to reduce various sources of error creeping into their analyses. For example, in the case of imaging studies where a focal plane array has been used, it is possible to record the locations on the detector of "bad"

pixels (pixels which are not recording properly) and remove these pixels from the subsequent image. Mathematical enhancement of the resolution of a spectrum may also be achieved using a method known as deconvolution, helping to determine the number and positions of several overlapping bands.

As with any preprocessing steps, the methods chosen may require some trial and error to correctly apply, and in some cases may seriously affect later quantitative analysis (e.g. deconvolution). Additionally, the preprocessing steps taken for one sample type may not produce the same improvements in a different sample, and hence the most suitable pre-processing approach used must be determined on a case-by-case, or application-by-application, basis. Once the need for data preprocessing is identified and a suitable method developed, greater confidence may then be had in the results obtained from any subsequent data exploration and classification studies.

1.2.12 Classification and Pattern Recognition

Multivariate data analysis methods are particularly useful when the investigator cannot easily determine if there is any direct relationship between a particular measured variable, or for that matter many combinations of variables, and a particular sample group. Initially, most investigations will commence with the analysis of known samples which possess the property(s) of interest - a training data set. Using the training data set, relationships between samples and groups may be assessed and a *pattern* potentially elucidated. Using the derived pattern, the investigator then hopes that a new, unknown sample, will be *recognised* and correctly classified into its relevant grouping based on its various component properties.

Multivariate analyses commence with the reduction of samples to more easily managed matrices and the removal of redundant information (e.g. noise). For example, a sample (such as a calibration standard) may be characterised by the measurement of two variables,

X and Y , which may be easily represented in a graphical form (see Figure 1-18a). However, such graphical representations become impossible when the number of variables exceeds three dimensions, which is quite common in spectroscopic analysis. While the simultaneous visual depiction of numerous measurement variables for humans is not possible, the numerical analysis (with the aid of computers) of such data is, and the results may then be used to construct simplified representations such as a table or a two dimensional image.

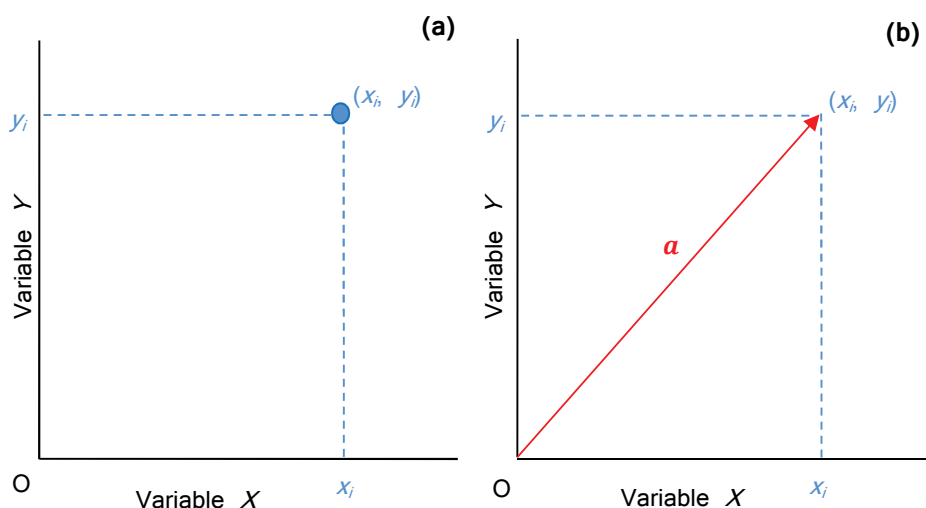


Figure 1-18: (a) Basic two-dimensional Cartesian coordinate representation of measurement (x_i, y_i) . (b) The same measurement as in (a), but represented as a data vector, $\vec{t}_i$ (adapted from Miller and Miller).^[125]

What the investigator is ultimately seeking is a means by which they may distinguish between different samples, which will then later enable the potential classification of new, unknown samples. In general, pattern recognition schemes are often divided into two groups: supervised and unsupervised methods. Supervised classification systems are employed when the investigator is aware of the sample groupings prior to the data analysis being performed, and wishes the system to find the relationships between samples of the

same groups which differentiate them from the other groups. Unsupervised methods, on the other hand, are performed with no prior information regarding the sample groupings included with the data for analysis. As a result, the final decision regarding the number of sample groupings can be quite subjective. Once the relationships between the known data are determined, be it by supervised or unsupervised means, the relationship can be then tested on new samples of known grouping (although treated by the classification system as of unknown origin), and if found robust, potentially applied to truly 'unknown' samples for classification or even identification purposes.

A brief description of a number of frequently encountered pattern recognition and classification approaches detailed in FTIR literature is given below.

1.2.13 Common Multivariate Analysis and Pattern Recognition Methods

1.2.13.1 *Principal Component Analysis (PCA)*

Principal Components Analysis (PCA) is often described as an exploratory data analysis tool as it may be employed to examine complex data for interesting descriptive features and inter-relationships even in the absence of prior information. The fundamental task of PCA is to find the number of significant, or dominant, independent patterns present in a data set. The number of significant patterns, or 'principal components' found using PCA will be ideally equal to the number of significant variables. For example, if we have two drug formulations which differ in concentration of three active compounds, but also all possess the same amount of another two compounds, one would expect PCA to reveal that the first three of the five principal components derived have a much greater impact on the variation between formulations (the first principal component always describes the greatest variation between data, followed by the second and so on).

Refer to the two-variable data array in Figure 1-19. Neither variable X or Y gives a clear way of distinguishing one sample from another. However, if one projects a new principal coordinate axis, labelled PC1 in the diagram, suddenly the relationships between data points may be more easily visualised. A second principal component axis, PC2, is then selected which is positioned at a right angle (orthonormal) to the first principal component axis to describe the remainder of the variation in the data, and is independent of PC1. As can be seen from the projections of the data points onto these new axes, PC1 describes more of the variation between samples than PC2.

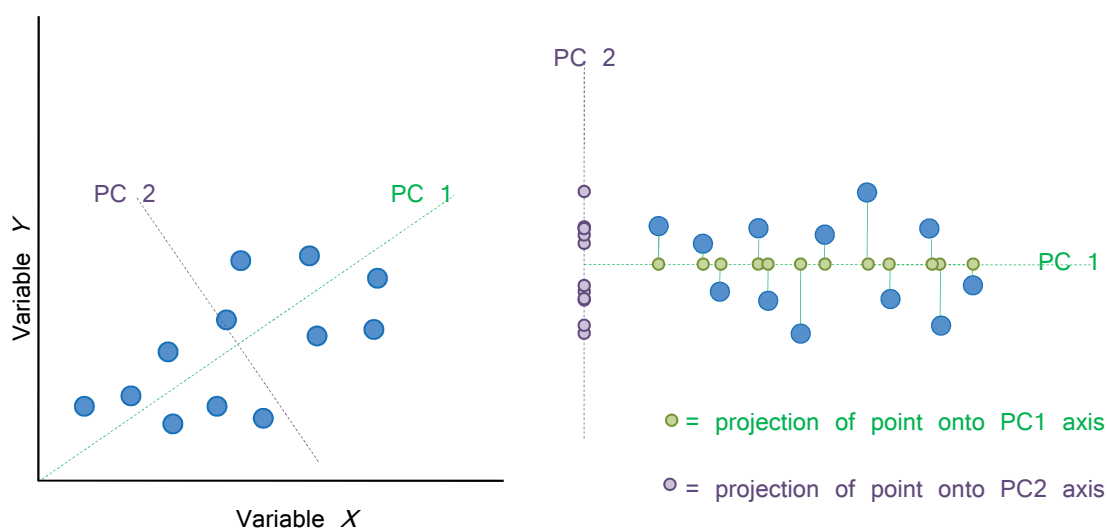


Figure 1-19: Simple two-dimensional representation of the determination of principal components.

In multi-dimensional (multi-variable) situations, such as found in infrared spectral data, the computer will weight each of the variables appropriately to determine the first principal component coordinate axis. PCA is useful in that it can be used to reduce the amount of data which needs to be handled at the next stage of analysis. In addition to data reduction, PCA is also an effective data exploration tool in that it may be employed to study the variance inherent in a data set (between groups) and possibly assist in the

identification of, in the case of spectral data, the spectral regions or peaks that are characteristic of a particular sample type.

The mathematical process behind PCA can be described using matrices. Consider a data matrix X (usually mean-centred) comprised of I rows (the total number of samples) and J variables (the number of spectral bands or wavenumbers measured). PCA reduces this X matrix into a smaller space:

$$X = T \cdot P + E \quad (9)$$

where T is the scores matrix (and can possess as many rows as the original data matrix); P is the loadings matrix (which possesses the same number of columns as the original data matrix); and E is the ($I \times J$) residual matrix (see Figure 1-20). The residual matrix is also referred to as the error matrix and accounts for 'imperfections' in the data such as those due to instrument noise or measurement error.^[111]

Also note that the number of columns in the matrix T will be equal to the number of rows in matrix P . For example, in the case of a 4 x 4 original data matrix, no more than 4 non-zero principal components may be calculated (i.e. the same number as the number of original variables).

Using the principal components derived for the data set, a model for each element of X may be determined:

$$x_{ij} = \sum_{a=1}^A t_{ia} p_{aj} + e_{ij} = \hat{x}_{ij} + e_{ij} \quad (10)$$

as matrix T is comprised of a series of column vectors that may be denoted by t_a , and matrix P consists of a series of row vectors denoted by p_a .

PCA may be performed using either a correlation or covariance matrix, although using the

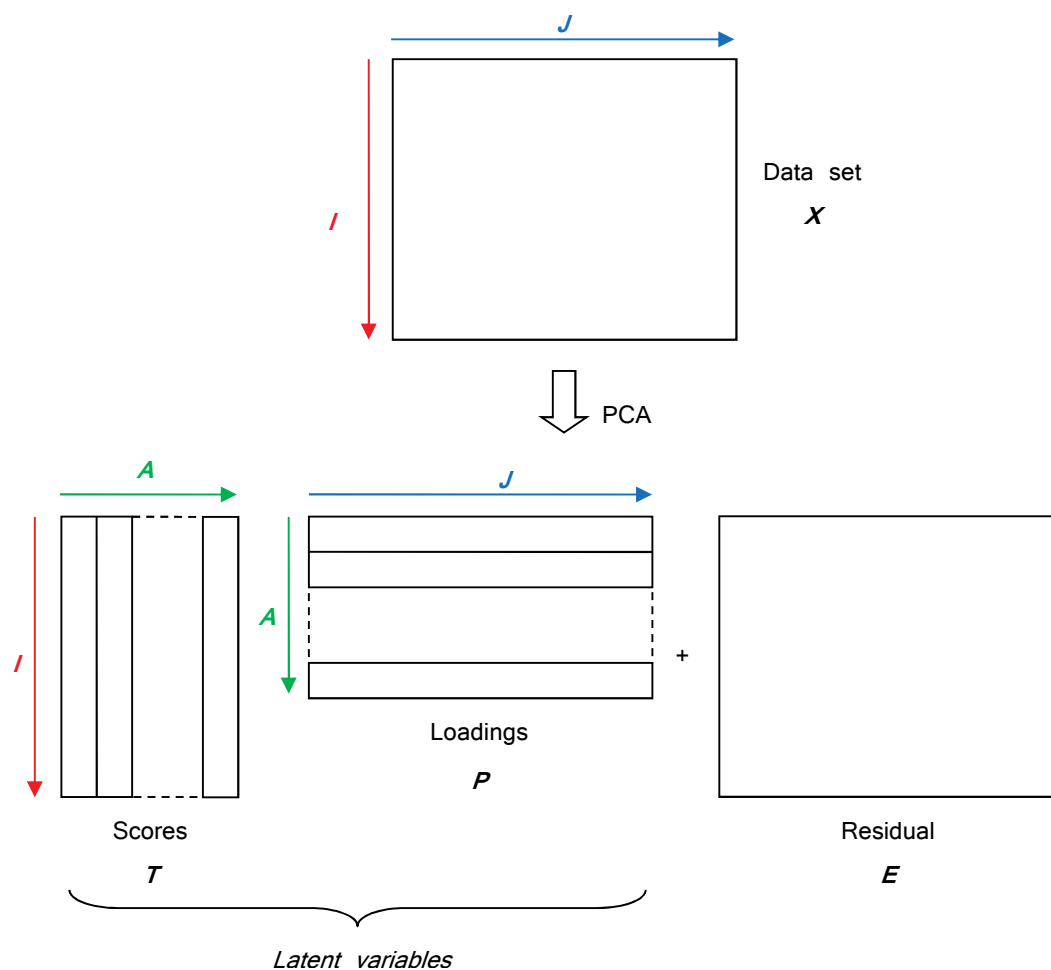


Figure 1-20: A diagrammatic explanation of Equation 9 (adapted from Brereton).^[104]

latter can result in the PCs being very sensitive to the units of measurement and to the relative variance in each variable, giving more weight to the larger and more variable measurements.^[126,127]

As may be understood from Equation 9, there is one score vector and one loading vector for each principal component calculated, with the first pairing of vectors for each principal component referred to as an *eigenvector*. This term should not be confused with an *eigenvalue* (notated by g), which is a measure of the variance in the data set ascribed to that principal component:

$$g_a = \sum_{i=1}^I t_{ia}^2 \quad (11)$$

Eigenvalues are often plotted as a visual guide to assist in the investigator's decision as to how many principal components they wish to retain; a chart commonly referred to as a 'Scree plot'. Graph A in Figure 1-21 is an example of a Scree plot.

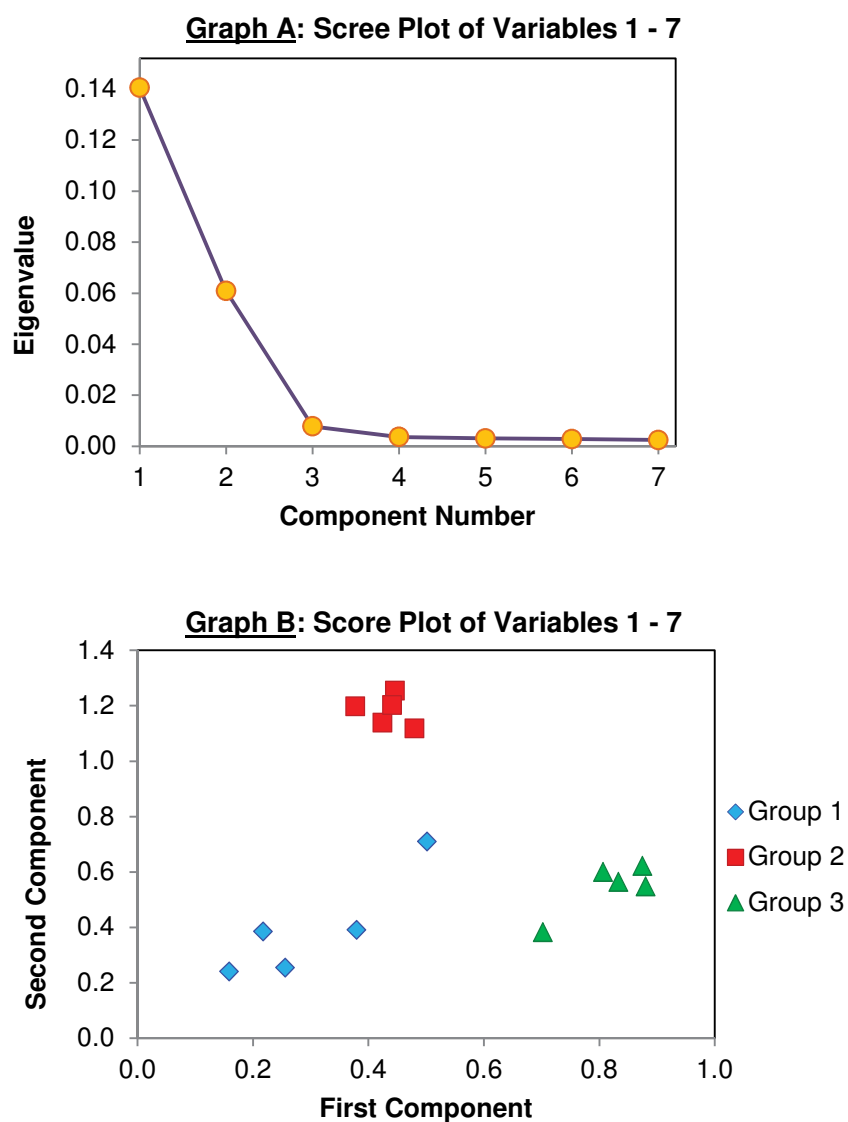


Figure 1-21: Examples of an eigenvalue (Graph A) and score plot (Graph B) obtained using Principal Components Analysis with a covariance matrix.

Another use for the PC data is in the form of scatter plots. Often plotting one principal component against another can be of great assistance in visualising data relationships, as groups of data points with minimal variation between them will appear as dense clusters (refer to Graph B in Figure 1-21). Score plots in particular can be quite useful in identifying outliers as well as assisting the investigator to determine which PCs provide the best source of variance and thus separation of sample groups.

The most difficult process when using PCA is often determining the significant number of PCs to be employed for the reduced data set. Brereton^[104] suggests that a simple approach can be to reject those PCs whose cumulative eigenvalues account for less than a particular percentage of the total data. However, as Brereton advises, this can be dangerous, particularly in situations where the first PC is highly influenced by the magnitude of the data values using non-standardised data values. Often the best method to determine if one has selected a suitable number of PCs is to test the system using cross-validation.

In relation to a hyperspectral image data set, PCA analyses all of the spectral channels (variables) in the image and searches for the variables which can explain the maximum of the remaining variance in the data.^[20] This method is unsupervised, and the technique may attribute the principal component to chemical, physical or instrumental variations. Often, when an experiment produces large volumes of data composed of numerous variables, there will possibly be a portion of the data which is effectively redundant (collinear), in that it doesn't contribute significantly to the variance between data points (e.g. noise). The use of PCA in such cases, particularly where the amount of redundant data collected is unknown or difficult to reduce, allows the analyst to obtain a new data set generated from the orthogonal-axes system derived from a select number of principal components (i.e. those believed to describe the most variance in the original data set). A potential drawback with PCA is that it assumes that all of the data is continuous and normally distributed, and the method can be highly influenced by noise present in the data. Also, as the PCA model

is, in a sense, performing a least-squares analysis on the data points, the presence of significant outliers may have a disproportionate effect on the calculation of the principal components, altering the location of “true” maximum variance.^[128]

PCA has been used, either on its own or in combination with supervised classification methods, for a range of infrared applications utilizing both imaging and individual spectral data. The analysis of organic materials in paint cross sections,^[58] as well as the discrimination and classification of various cancer-affected cells and tissues^[91,94,129-135] are some examples of the assistance PCA offers for information gathering and sample classification.

1.2.13.2 Cluster Analysis

While exploratory data analysis techniques such as PCA can be used to determine the relationships between data, the actual grouping of data points is not the main purpose of such methods. Consider the data in Figure 1-22. In this example, the first principal component, while effectively showing the maximum variance between all data *points* does not show good discrimination between data *groups*. Cluster analysis, on the other hand, is wholly concerned with the grouping of similar objects in a data set.

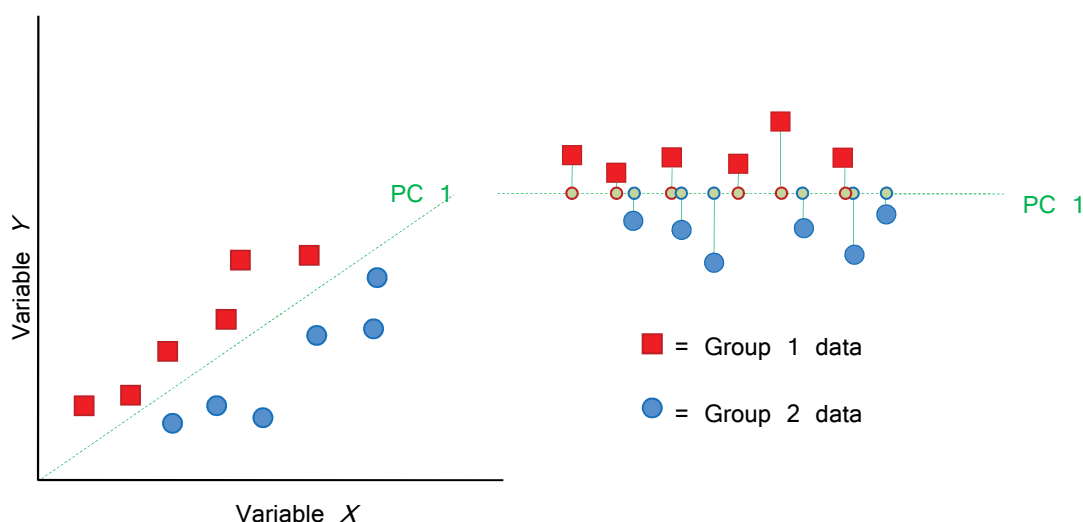


Figure 1-22: An example of a data set where PCA does not give group separation of data.

Cluster analysis was originally employed by biologists as a means to study the similarities between organisms, and is sometimes referred to as 'numerical taxonomy'.^[104] Like PCA, cluster analysis is an unsupervised multivariate statistical method, grouping spectral on spectral features alone. Cluster analysis does not require the provision of any *a priori* information as to the groups that may be present in the data set, and it makes no assumptions regarding how the variables of the data set are distributed.^[125]

There are two main steps in the cluster analysis process:

Step 1: Perform distance calculation (similarity measure) between objects

For the sake of simplicity, the following discussions will be concerned with hyperspectral data comprised of a number of samples (be they single spectra from a number of different samples, or spectra from pixels comprising a hyperspectral image) with corresponding spectra measured at a number of wavelengths.

In this first step of the clustering process, a matrix is derived based on the measurement of the similarity between each possible pair of sample spectra. How this similarity is measured will be determined by the algorithm chosen, as there are many to select from. A few of the more common methods have been detailed below.

Similarity measures are the mathematical relationships used by pattern recognition and classification schemes to discriminate between spectra. Fundamentally there are two categories of similarity measures: deterministic (there is a direct relationship between variables without random phenomena being involved) and stochastic (there is an element of randomness or probability in the analysis).^[136] Deterministic measures may be further subdivided into three major groups: angle based, distance based and correlation based. Some examples of commonly employed measures of similarity have been defined below:

- *Spectral Angle Mapper (SAM)*

In order to compare the spectra, each pixel spectrum is considered as a vector. This approach is possible when one considers that at each pixel in an infrared image there is an associated spectrum. Each spectrum possesses a number of data points or variables (which will be defined by the spectral resolution and wavenumber range recorded) and an intensity value at each data point. The spectrum at a pixel may then effectively be represented by a row of numbers which represent the intensity values at each wavenumber. This row of numbers can in turn be represented by a vector, or a point in space (see Figure 1-18b). Hence, each pixel is characterised by a vector, the value of which may then be mathematically compared to that of other sample pixels.

The Spectral Angle Mapper (SAM), also referred to as the spectral angle measure, like many other similarity measures, considers each pixel spectrum to be a vector and then calculates the spectral similarity between the image pixel spectrum in comparison with a selected reference spectrum (see Figure 1-23).^[137,138]

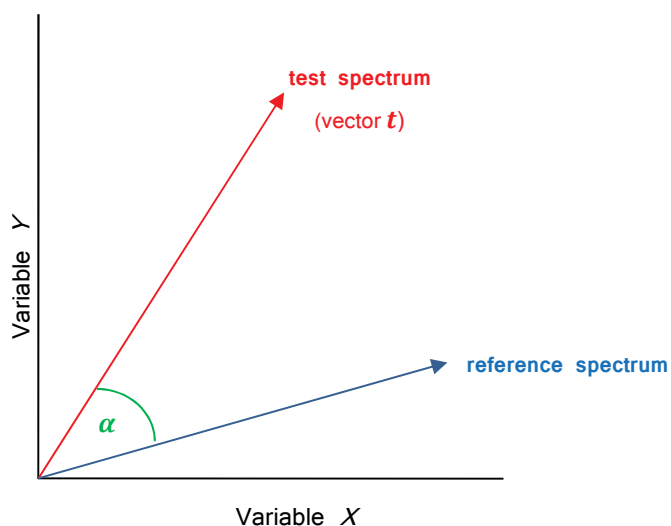


Figure 1-23: Two vectors t and r representing two spectra. α describes the angle between the two vectors.

Consider the two spectra in Figure 1-23, in this case a spectrum taken from the sample being examined and a reference spectrum to be compared against, represented by the vectors $\mathbf{t}$ and $\mathbf{r}$ respectively. The SAM calculation takes the arccosine of the dot product between the two vectors.^[137]

$$\begin{aligned} \text{SAM}_{\mathbf{t},\mathbf{r}} = \alpha &= \cos^{-1} \left(\frac{\sum_{i=1}^L t_i r_i}{(\sum_{i=1}^L t_i^2)^{\frac{1}{2}} (\sum_{i=1}^L r_i^2)^{\frac{1}{2}}} \right) \\ &= \cos^{-1} \left(\frac{\vec{t} \cdot \vec{r}}{\|\vec{t}\| \|\vec{r}\|} \right) \end{aligned} \quad (12)$$

where L = the number of spectral bands (variables).

SAM is a deterministic measure (i.e. it does not take into account the potential randomness in a system),^[139] which calculates the angle between two spectra to determine their similarity to each other.

Aside from its use in remote sensing applications, SAM has been utilised in a number of multispectral and hyperspectral imaging studies such as the classification of routine H&E stained clinical histopathological images,^[140] identification of intercellular space in diabetic retinal tissue,^[141] identification and enhancement of vascular structures and haemoglobin oxygen saturation in skin,^[142,143] tongue colour analysis,^[144] breast tissue analysis,^[140] and the classification of contaminated poultry carcasses.^[145,146] In regards to FTIR CI applications, Ng *et al* employed SAM to search for and identify pixels in a fingerprint secretion image contaminated with illicit substances such as drugs and explosives.^[54]

- **Euclidean Distance (ED) measure**

Aside from considering each sample spectrum as a vector, it is also possible to represent each pixel in an image as a point in Cartesian space, with the number of

coordinates describing the point being equal to the number of variables measured in the spectrum (e.g. wavenumber values). In its simplest form, the Euclidean Distance (ED) measure between two data points requires the calculation of the sum of squares of the differences between the corresponding data values.

For the measure of the ED between two spectra represented by vectors $\mathbf{a}_i = (a_{i1}, \dots, a_{il})$ and $\mathbf{a}_j = (a_{j1}, \dots, a_{jl})$ (as depicted in Figure 1-24 for two dimensions) the calculation may be defined as:

$$\begin{aligned} ED_{\mathbf{a}_i, \mathbf{a}_j} &= \sqrt{(a_{i1} - a_{j1})^2 + (a_{i2} - a_{j2})^2 + \dots + (a_{il} - a_{jl})^2} \\ &= \sqrt{\sum_{l=1}^n (a_{il} - a_{jl})^2} \end{aligned} \quad (13)$$

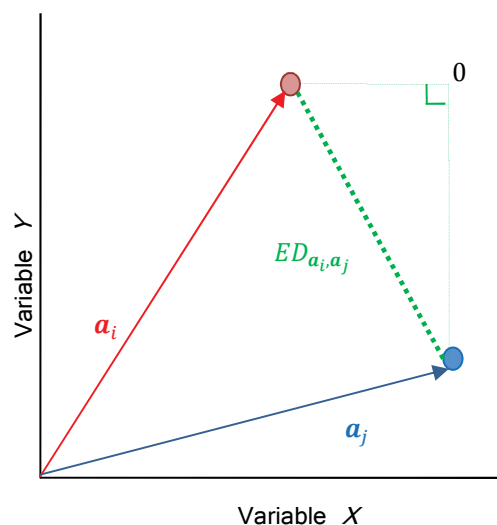


Figure 1-24: Two-dimensional graphical representation of the Euclidean distance measure between two vectors.

Equation 13, however, does not take into account large variations in magnitude among measurement variables. Because of this, some workers prefer to normalise the data

first, using methods such as min/max normalisation^[136] or dividing by the standard deviation.^[113]

According to van der Meer, there is also a form of the Euclidean distance (ED) measure which may be derived from the spectral angle measurement function, and unlike SAM, it takes into account the magnitude difference or absorbance intensity (what van der Meer refers to as the 'brightness') between the two vectors:^[147]

$$ED_{a_i, a_j} = 2 \sqrt{1 - \cos \left(\text{SAM}(a_i, a_j) \right)} = 2 \sin \left(\frac{\text{SAM}(a_i, a_j)}{2} \right) \quad (14)$$

- *Spectral Correlation Similarity (SCS), D-value and the Spectral Similarity Value (SSV)*

Correlation, in its simplest definition, is a means by which the strength and direction of the relationship between two vectors may be determined. The Spectral Correlation Similarity (SCS) is actually a form of the Pearson product moment correlation (r),^[136] which compares the relative shapes of the two spectral vectors as well as the spectral match.^[147]

Using the spectral vectors as outlined in Figure 1-24, the following may be derived:

$$\sum_{l=1}^n \left((a_{il} - \mu_{a_i}) (a_{jl} - \mu_{a_j}) \right) = \sum_{l=1}^n (a_{il} \cdot a_{jl}) - n \cdot \mu_{a_i} \cdot \mu_{a_j} \quad (15)$$

And

$$\sigma_{a_i} \sigma_{a_j} = \sqrt{\left(\sum_{l=1}^n a_{il}^2 - n \cdot \mu_{a_i}^2 \right) \cdot \left(\sum_{l=1}^n a_{jl}^2 - n \cdot \mu_{a_j}^2 \right)} \quad (16)$$

So

$$r_{a_i, a_j} = \frac{1}{n-1} \left(\frac{\sum_{l=1}^n (a_{il} \cdot a_{jl}) - n \cdot \mu_{a_i} \cdot \mu_{a_j}}{\sqrt{\left(\sum_{l=1}^n a_{il}^2 - n \cdot \mu_{a_i}^2 \right) \cdot \left(\sum_{l=1}^n a_{jl}^2 - n \cdot \mu_{a_j}^2 \right)}} \right) \quad (17)$$

$$r_{a_i, a_j} = \text{correlation}_{a_i, a_j} = \frac{\text{covariance}_{a_i, a_j}}{\sigma_{a_i} \sigma_{a_j}} = \frac{1}{n-1} (z_{a_i} \cdot z_{a_j})$$

$$= \frac{1}{n-1} \left(\frac{\sum_{l=1}^L ((a_{il} - \mu_{a_i})(a_{jl} - \mu_{a_j}))}{\sigma_{a_i} \sigma_{a_j}} \right) \quad (18)$$

where μ and σ are the mean and the standard deviation values for the vectors respectively.

To obtain a 'distance' value, (which is sometimes referred to as the 'D-value'),^[113,148] rather than a correlation coefficient, the following conversion may be performed:

$$D_{a_i, a_j} = (1 - r_{a_i, a_j}) \cdot 1000 \quad (19)$$

Alternatively, one can combine the intensity-sensitive Euclidean distance with the spectral shape measurement capability of the SCS, to obtain the Spectral Similarity Value (SSV).^[136]

$$SSV = \sqrt{ED_{a_i, a_j}^2 + (1 - r_{a_i, a_j})^2} \quad (20)$$

While the SSV offers the best of both worlds by incorporating two spectral measures into one, it will potentially be more computationally demanding.

Step 2: Linkage of objects

Once all of the similarity measures between the samples have been conducted, the two samples which have been determined to be the most similar are grouped together to form a cluster. This cluster is then compared to the remaining samples and a new similarity matrix constructed. The sample which is determined to be the most similar to the cluster is then grouped with it, forming a cluster now of three spectra. This new cluster is compared to the remaining samples and so on, until all of the samples have been

clustered together. The approach described above is often referred to as *agglomerative* clustering, which is more commonly labelled *hierarchical* clustering, and is depicted in Figure 1-25.

Prior to performing cluster analysis of any data, the analyst must also decide how the cluster formed at each step is to be compared to the remaining samples. Consider item (b) in Figure 1-25. How should the newly formed cluster of points 2 and 3 be represented in further similarity measurements? There are three common approaches taken when linking clusters and objects:

1. Nearest Neighbour (NN): this linkage method looks at the similarity of each of the objects in the Cluster A (point 2 and point 3), to all of the remaining unclustered data points, and uses the highest similarity measure (shortest distance) of the two points in the cluster as representing the whole of Cluster A. For example, if point 3 had a correlation coefficient of 0.90 with point 1, and point 2 a value of 0.88, the correlation coefficient of Cluster A would be 0.90.
2. Furthest Neighbour: the opposite of NN, so in this case the similarity measure for Cluster A to point 1 would be 0.88. This is a method to determine the similarity measure between Cluster A and points 1, 4 and 5 and does not change the fact that the next point clustered with cluster A will be the one that is most similar to it (i.e. possesses the 'highest' similarity value).
3. Average linkage: in the above case, the similarity value as determined by average linkage would be 0.89. However, the method for determining the average will vary depending on how many objects comprise the groups being combined. There are two methods:^[104]

Weighted:

$$s_{AB} = \frac{(s_A + s_B)}{2} \quad (21)$$

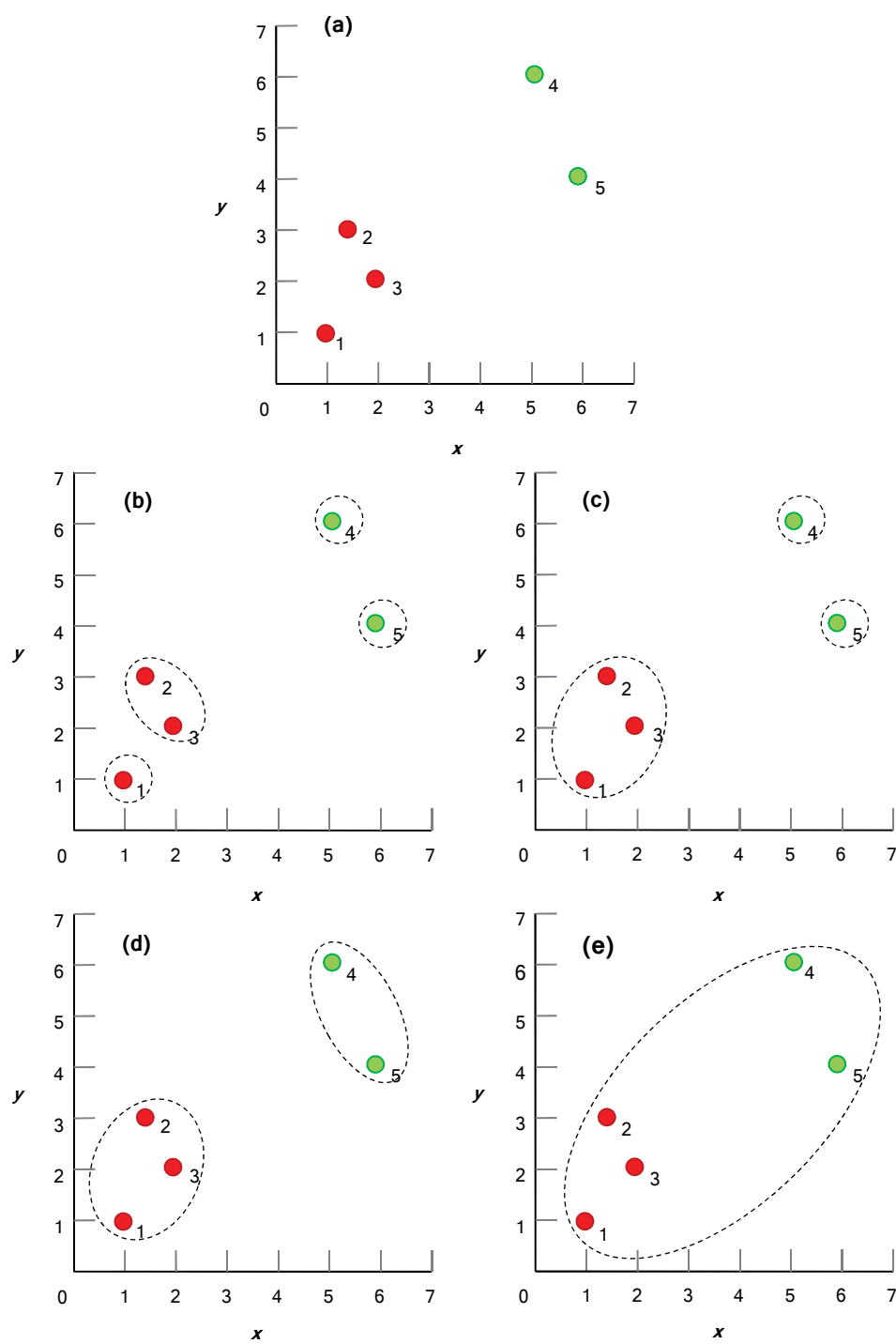


Figure 1-25: A simplistic diagram of the clustering process adapted from Miller and Miller.^[125] Figure (a) depicts the original data, and Figures (b) to (e) show the sequential clustering steps performed.

Unweighted:

$$s_{AB} = \frac{(N_A s_A + N_B s_B)}{(N_A + N_B)} \quad (22)$$

Where N_A and N_B are the number of objects in groups A and B respectively.

There are a number of clustering methods available to the spectroscopist to discriminate between samples and the pixels within an image. A few have been discussed below:

- *Hierarchical Cluster Analysis (HCA)*

This chemometric technique considers spectra with n wavelengths as single points in n -dimensional space; the distance in this space between each pair of spectra is then calculated using one of the distance metrics discussed earlier. Numerous small groups of 'similar' spectra are formed, with the algorithm gradually merging the groups based on similarity until only one group, or cluster, containing all of the spectra remains (similar to biological taxonomy schemes).^[104]

This mathematical grouping process can be displayed in the form of a schematic diagram known as a dendrogram (refer to Figure 1-26). A chemical image can then be 'coloured' to show the regions of the tissue sample determined as similar or different according to the clustering performed. Hence, the demonstration of two clusters - the simplest sorting of the component spectra into two groups - would be given as only two colour tones in the image. The number of clusters employed is chosen by the spectroscopist, as well as the method of distance calculation. Comparison of the cluster images with those obtained using standard histological staining protocols may assist in the assessment of the clustering method chosen.

Examples of unsupervised cluster analysis being applied to FTIR tissue and cell imaging data have included the examination of samples such as cervical tissue,^[14] lymph nodes,^[93,149] human breast cells,^[87] esophageal tissue^[150] and skin sections.^[151,152]

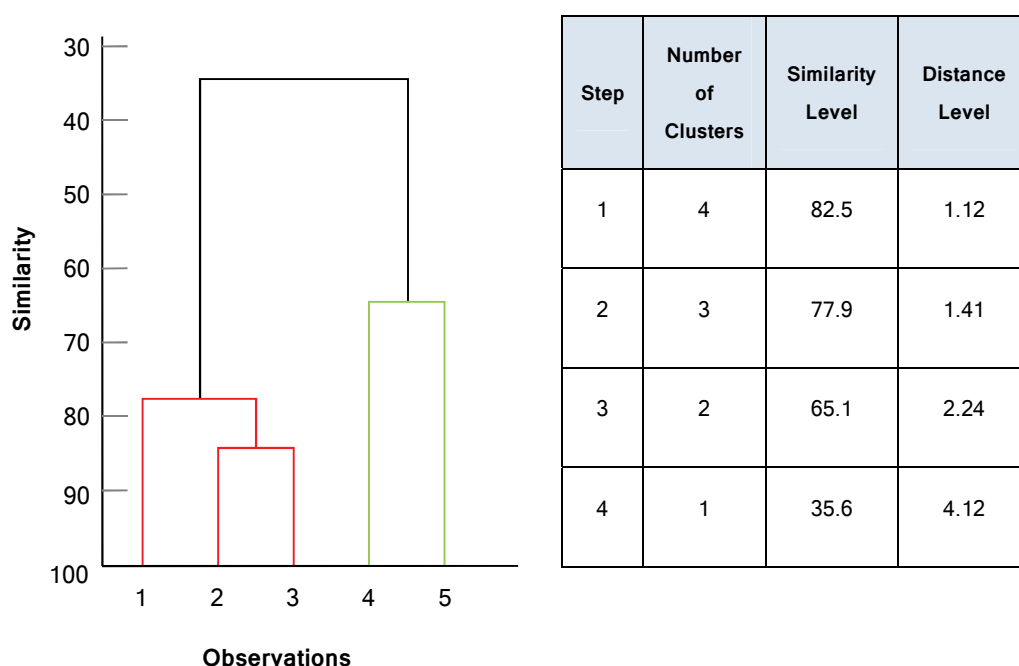


Figure 1-26: A dendrogram for the clustering performed in Figure 1-25. The table to the right of the dendrogram lists the corresponding amalgamation step data from Minitab™ using the Euclidean distance measure and single linkage.

- **K-means clustering**

Unlike hierarchical clustering, where the number of clusters required does not need to be defined prior to analysis, *k*-means actually assigns each data point (spectrum) to a user-determined number of clusters, *k*. Initially the data may be divided randomly into *k* groups, or data points can be directly assigned to a particular cluster. The method then looks at each data point and decides, based on a distance measurement, whether the point is closest to the centre (centroid) of the cluster it is currently in, or if it should belong in a different cluster. If the data point is moved, the centroids for the affected clusters (i.e. the one losing the data point and the one gaining it) are re-calculated and the process begins again. The centroid is simply the arithmetic mean for each

dimension (variable) separately over all points in the cluster. The method iterations continue until no further points are moved between clusters.

The disadvantage of this method lies in the fact that the user must decide the value of k in advance, and that the final groupings (particularly when there are small sample sizes involved) achieved are dependent on the original choice of how to cluster the data points. Due to the predetermination of the number of clusters, the algorithm is computationally simple and quick, allowing clustering to be performed on large data sets quite efficiently. An overview of the k-means clustering work flow has been given in Figure 1-27. In relation to biological sample classification utilising spectroscopic analysis methods, K-means clustering has been employed in the study of a wide variety of mammalian cells and tissues such as those from the colon,^[80,153,154] breast,^[87] aorta,^[85] lung,^[77] and skin.^[155]

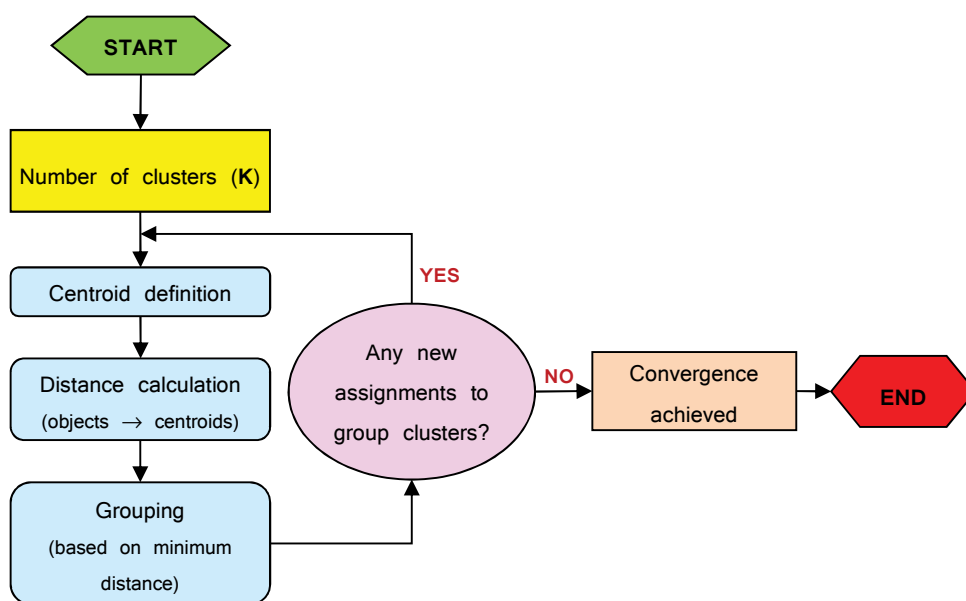


Figure 1-27: Diagrammatic depiction of the k-means clustering work flow (adapted from Brereton).^[156]

- *Fuzzy C-means clustering*

Similar to k-means clustering in that it is a non-hierarchical clustering method, fuzzy C-means clustering begins with the designation of the number of clusters (k) and each data point belonging to a cluster. In the case of fuzzy C-means clustering, the classification output actually defines the degree of membership of a pixel to all of the clusters, not just one. Each pixel will have a degree of membership in every cluster, with data points towards the centre of a cluster in a sense belonging to that cluster to a higher degree than a data point on the outer edges of the cluster.

Fuzzy C-means clusters data in such a way that the differences between the spectra within a cluster are minimised, and the intercluster spectral differences maximised, simultaneously. Note, this process is iterative and eventually converges to a solution. The number of iterations through which the fuzzy C-means algorithm proceeds to reach its solution is predetermined by the user in the form of a threshold value. Once the magnitude of the change in the cluster membership values drops below the selected threshold, the iterative process ceases.^[157]

Examples of the application of Fuzzy C-means to classification of biological materials based on sampled spectroscopic data have included its use on near-infrared spectroscopic imaging data obtained from dried blood,^[157] the measurement of blood flow through tissues,^[158] and the pathological diagnosis of skin tumors based on infrared images.^[74] Fuzzy C-means clustering of images has also been successfully applied to infrared spectra collected from colorectal adenocarcinoma^[159] and human basal cell carcinoma^[74] samples.

While clustering provides a truly objective imaging technique in that no prior knowledge of the sample composition is required, the computational requirements for the calculations are

high, particularly when one considers that each image will be comprised of thousands of pixels, each with its own spectrum of numerous data points. In addition to this, the number of clusters chosen for display is usually obtained after comparison of the clustered image with that of an H&E stained section. Further comparison of the spectral characteristics of each cluster must then be performed to assess what each cluster is in fact representative of in terms of the tissue composition.

The detection and analysis of cancer in numerous tissues,^[14,92,149-152,159-161] fungi in wood,^[65] pathogenic processes in periodontal tissue,^[71] along with the identification and classification of various bacteria,^[62,162] are all biological studies in which HCA has been an integral part of the data analysis process.

1.2.13.3 Linear Discriminant Analysis (LDA) and Quadratic Discriminant Analysis (QDA)

A supervised pattern recognition system, Linear Discriminant Analysis (LDA) seeks to find a rule to describe the variation between sample groupings that may then be used to allocate a new (unknown) object to its correct group. As with all supervised learning procedures, LDA commences by employing data with known group membership, referred to as a 'training' or 'learning' data set. LDA then examines the data and searches for a latent variable known as a linear discriminant function, Y , that describes the largest difference between data groups based on a linear combination of the original data variables X_1 , X_2 , etc:

$$Y = a_1X_1 + a_2X_2 + \cdots + a_nX_n \quad (23)$$

where a_n is the coefficient for the n th data variable X_n . In general, the larger the value of a , the better the discrimination achieved by its corresponding variable.

The coefficient values chosen ensure that there is as much difference as possible maintained between data points from different groups. As may be seen in Figure 1-28, the

LDA is, in a sense, forming a new set of axes, called canonical variates, to best define the variation between data groups. LDA employs the Mahalanobis distance calculation to determine the class membership of a data point.

Once the linear discriminant function has been determined, the model is then used to determine the classification of a new, unknown sample, using its Y value. It is logical to expect that the newly determined Y will lie closest to the average Y value of one of the pre-determined sample groups. Note that the number of groups involved in the study must not exceed the number of variables chosen to describe the study's data set.

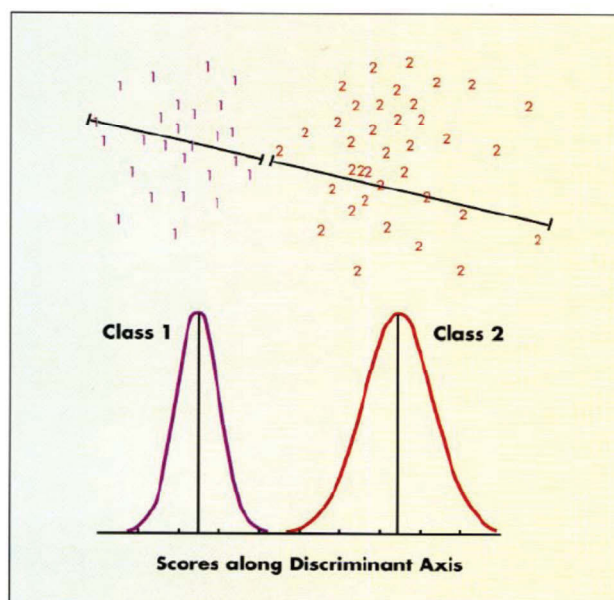


Figure 1-28: A diagrammatic representation of how LDA may be used to distinguish between two classes (taken from Brown^[163]).

Ultimately LDA seeks to derive linear discriminant functions that will minimise the amount of variance between samples (distance within groups) from the same group and yet also maximise the ratio of between-class variance (separation between groups).^[164] LDA assumes that the groups possess similarly shaped multivariate normal (MVN) distributions. However,

when the groups are not similarly shaped, Quadratic Discriminant Analysis (QDA) relaxes the MVN assumption somewhat, as it assumes that the groups still do possess MVN distributions but with differing variances. Unlike the linear decision boundary produced by LDA, that of QDA is curved.

LDA and QDA can be used for both classification and data analysis (i.e. to assist in the determination of the variables which contribute most to the variation between samples and groups), although they are most often used as classification tools. As with all supervised pattern recognition and classification schemes, the discriminating function derived needs to be checked using either a training set or cross-validation approach (see section 1.2.15) to determine if the classifier is in fact correctly identifying the variables which contribute to group differences.

LDA has been used for a variety of infrared spectra and imaging classification studies. Biomedical applications of LDA to infrared spectra have included the diagnostic classification of prostate,^[165] brain,^[166] and colon cancer,^[135,167] as well as for cutaneous neoplasia screening^[168] and to differentiate BCC from normal skin excision specimens.^[169] Colour-coding of infrared images by assigning pixels to various classes based on an LDA-based classification algorithm has been successfully applied in a number of FTIR CI tissue studies also.^[155,169-171]

1.2.13.4 *Soft Independent Modelling of Class Analogy (SIMCA)*

Soft Independent Modelling of Class Analogy (SIMCA) is a supervised data classification method which employs a form of principal components analysis to construct the statistical model to differentiate between groups, or 'classes', of data. The SIMCA data analysis method can be divided into two stages, the disjoint class modeling stage (where a PC model for each class is derived) followed by the classification stage (where samples are examined for their group membership).^[172]

The first part of the SIMCA technique involves the centering (often to the mean of the data values) and scaling of the input data set, which is then followed by a local PCA being performed to model the variables responsible for variation within the classes (and not the whole data set).^[163] Because a PCA is being performed on each class, the data set is, in a sense, reduced as only a sufficient number of PCs are kept to describe the variation within the class. Often cross-validation is performed within the method to determine the number of PCs to retain for each class, and may result in the number of PCs employed for each class model differing,^[128] as shown in Figure 1-29.

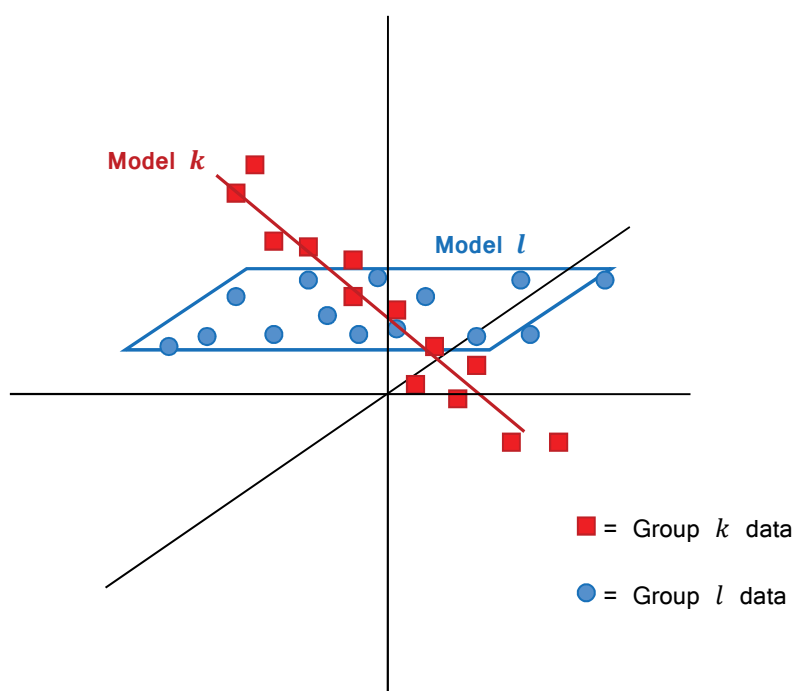


Figure 1-29: Example of how two models obtained using SIMCA may use a different number of principal components to describe a given group of data. The group k data is modelled using one principal component, whereas group l uses two principal components.

The PC model for each object in a class k , symbolised by x_{ij}^k , can be described using the following relationship:^[163]

$$x_{ij}^k = \bar{x}_{kj} + \sum_{r=1}^{p_k} v_{jr}^k u_{ir}^k + e_{ij}^k \quad (24)$$

where:

p_k is the number of PCs needed to explain the variance in class k ;

v_{jr}^k is the loading of variable j on PC r in class k ;

u_{ir}^k is the score of object i on PC r in class k ;

$\bar{x}_{kj}$ is the mean of feature j in class k ; and

e_{ij}^k is the unexplained variation of object i on feature j in class k .

To determine if a sample belongs to a particular class, an F-statistic is used which compares the residual variance (the variation of the data not explained by the PC model) of the sample with that of the mean value of the residual variance for the class. A probability of class membership for each sample may also be determined using the F-statistic and a pre-determined confidence interval, which can calculate the upper limit for the residual variance of the samples in a class, and detect outliers of the class model. An alternative method is to calculate the Euclidean distance of the samples from the PC model. Effectively, for each class model those samples from the same group should ideally lie close together on the line (1 PC retained), plane (2 PCs retained) or hyperplane (3 or more PCs retained) described by the PCs selected for that class. The choice of using geometric class distances or probabilistic measures is up to the investigator. Regardless of the measurement method used, when a sample's variance or distance is compared to that of the class, and the residual error or distance from the model is above the statistical limit set for the class, it will be considered an outlier by the system and removed from that class.

The 'soft' component of SIMCA refers to the fact that the method accepts the fact that classes can overlap, and that an object may belong to two classes simultaneously. The fact that SIMCA can allow for that fact that some sample groups may overlap, and as a consequence may assign a sample to two or more groups, gives SIMCA a distinct advantage over other statistical methods which would forcibly assign an object to one particular class.^[128] Samples which are considered outliers from all of the classified groups may either represent a new, unexpected group, or in fact just may suggest that it is a sample containing bad data.^[163] To assist in the examination of the impact each of the variables is having on the classification ability of a model, the modeling power, M_j , of each variable, j in a class k can be assessed using the following relationship:

$$M_j = 1 - \frac{s_o^k}{s_j^k} \quad (25)$$

Where s_j^k and s_o^k are the standard deviation values for the variable in the raw data and residual values respectively. Note also that $0 \leq M_j \leq 1$, and that using variables which produce an M_j of less than 0.5 is generally ill-advised.

To gauge the ability of the chosen models to discriminate *between* classes, (for example two classes k and l) the discriminatory power, D , can be determined using the following relationship:^[163,173]

$$D_{kl} = \sqrt{\frac{s_{kl}^2 + s_{lk}^2}{(s_o^2)^k + (s_o^2)^l}} \quad (26)$$

Here, s_{kl} is the standard deviation of the residual value matrix when the samples in class k are fitted to the model of class l , s_{lk} is the standard deviation of the residual value matrix when the samples in class l are fitted to the model of class k , and $(s_o^2)^k$ and $(s_o^2)^l$ are the residual variance values obtained when the samples in classes k and l respectively are fitted to their own class models.

Because a probability of class membership is provided for an unknown, it is also possible to investigate misclassifications during the testing phase of the model's construction, as well as to identify possible outliers in the training data set. Classifying an unknown sample into the SIMCA system involves finding which is the best fit of the various class models to the data. A probability of class membership for the unknown to each of the classes is then determined based on the distance of the class models to the unknown data sample.

In the case of pattern matching of infrared spectra, SIMCA offers a means of data reduction, by mapping the spectra into a lower dimensional subspace, to make classification an easier process. Unlike LDA, SIMCA can be used with a small sample set and unrestricted numbers of measurement variables (it is not affected by the problems of collinearity which many statistical methods face). Due to the fact that different models are being constructed for each specific class, SIMCA can also be used to recognise the fact that each class might be related to very different properties. SIMCA also allows the investigator to examine the results of the analysis graphically, which assists the investigator in visualizing the data structure behind the classifications achieved. An example of a useful graphical tool using the SIMCA data is the construction of Cooman's plots, which is a means of displaying the class distance values for the data for any two models simultaneously.^[164,174] An example of this has been given in Figure 1-30.

When selecting a reference data set for SIMCA, the investigator must be careful to seek out samples which reflect the homogeneity and variation of the groups, without risking the inclusion of incorrect samples for the sake of having a large representative training set. Unfortunately, SIMCA is very sensitive to the quality of the original data, so it is always wise to check the modeling and discriminating power of the models generated to assess if this method is suitable for the data generated or if perhaps data pre-treatment may be required.

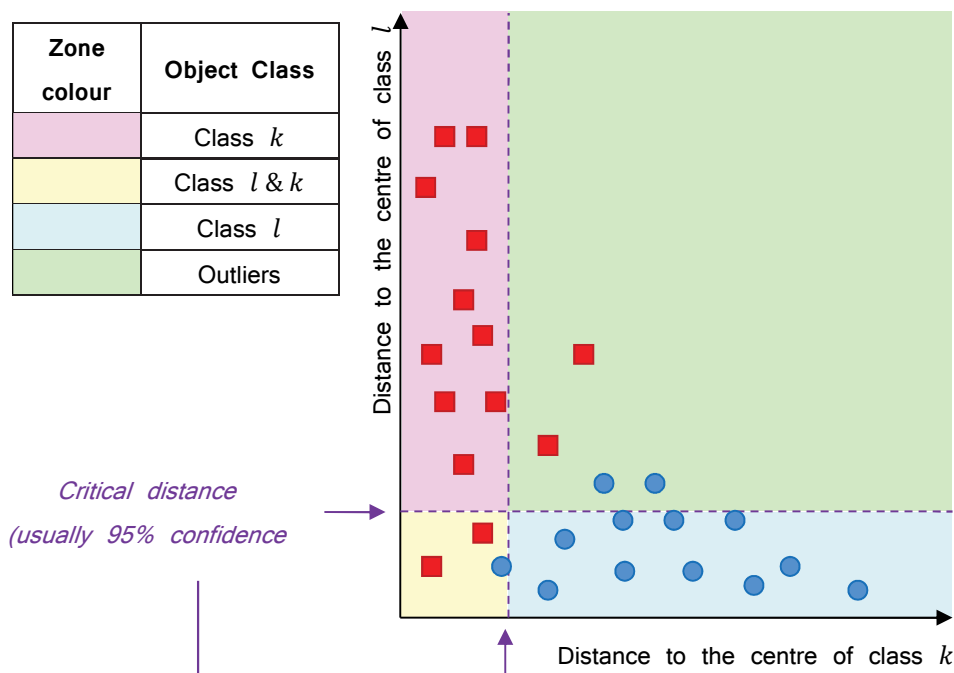


Figure 1-30: Example of a Cooman's plot with data groups k and l .

Examples in which SIMCA has been applied to spectroscopic data includes the identification of primary tumors of brain metastases based on infrared images^[175] and the discrimination of pharmaceutical tablets containing varying active and excipient concentrations using near-infrared spectra.^[176]

1.2.13.5 Artificial Neural Networks (ANNs)

Developed to imitate the operation of neurons in the human brain, artificial neural networks (ANN) offer a supervised, non-linear statistical data modeling technique. The 'network' component is a description given to the modeling function, $f(x)$, that is comprised of a number of neurons (other functions $g_i(x)$) which in turn may be comprised of further functions.^[177]

The basic unit of the network, known as a neuron or node, has numerous inputs but only one output. The input values may be from raw data, or from other neurons, and are referred to as connections. Neural networks can possess a variety of 'architectures', such as feed-forward and feedback or recurrent. In feed-forward neural networks, the signals only proceed one way, whereas in feedback networks, signals can travel in both directions using loops in the network. In feed-forward neural networks, they can be simply composed of one input layer and one output layer, or they may have an input layer, output layer, and a hidden layer, which sits between the two (as seen in Figure 1-31). In pattern recognition applications, the input layer represents the raw data which is being fed into the network, such as infrared spectra. The values from the input unit are then weighted and processed via the neurons of the hidden layer.

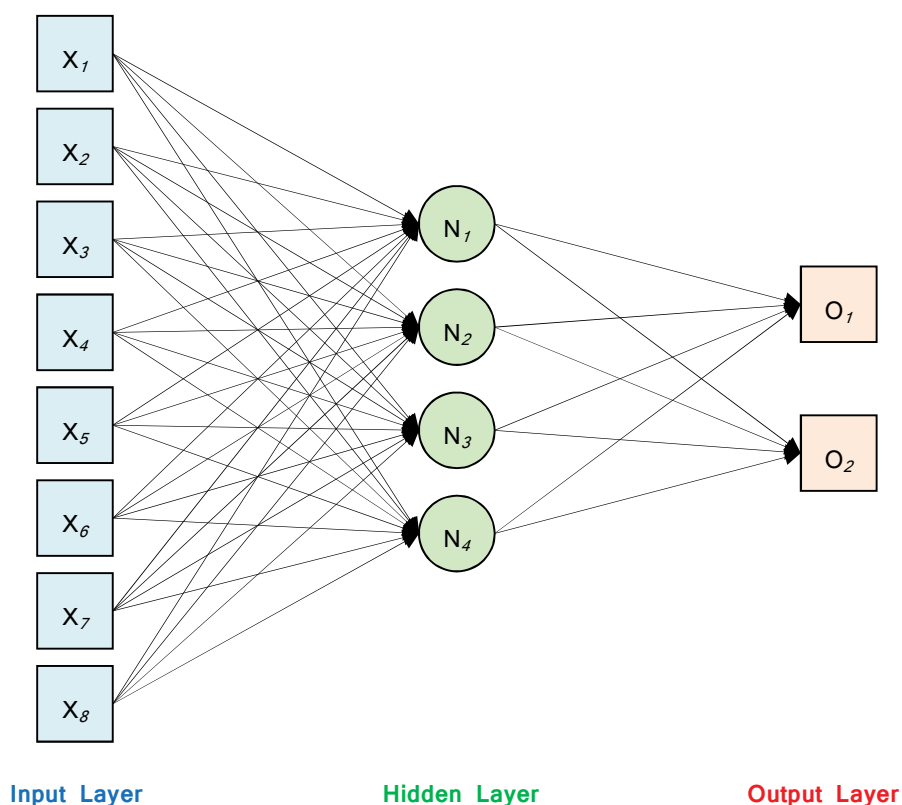


Figure 1-31: An example of a feed forward neural network. The flow of data through the network structure is depicted with arrows to show the dependencies between variables.

In effect, the hidden layer is assessing which spectral information provided by the input layer is of most discriminating value and characteristic of the desired output.^[149] The number of hidden units required will be dependent on numerous factors, such as the number of training patterns, the number of input and output units, how much noise is present, and even the training algorithm, just to name a few. Too few or too many hidden units leads to various errors, such as underfitting of the model due to the former and poor generalisation (overtraining) in the latter.^[178]

When using ANN for pattern recognition, the process begins with the training phase, during which the neuron is 'taught', using a particular rule (function) to fire, or not to fire, for particular input patterns, and weighting values may be assigned to particular inputs. Following this, new data of known sample group membership is then used to check the performance of the ANN. If this step reveals a difference, or error, between the desired and computed classification that is considered too large, the weighting or threshold values may be adjusted until the error is minimised. After the ANN is established, a sample of unknown group membership may then be fed into the network, and the model then gives an output value that it determines is most similar to one of the given patterns used during training.

ANNs in a sense memorise the patterns of interest and will recognise any new samples that share the same features. It is possible for the network to over-train itself, and find itself also classifying the noise or other redundant data present in the original training samples. This makes the second task of generalizing to new samples by the neural network difficult, as it will look for the noise in the new input which may not be consistently present. Performing various data pre-treatments, such as spectral smoothing and data scaling, can be used to further minimise errors. As with any classification method, it is vitally important to use a training and test data set prior to analysis of any new, unknown samples, to ensure that over-training has not occurred.

Figure 1-32 shows example data sets where conventional linear modeling methods may render unsatisfactory classification boundaries. ANN may also be used in an unsupervised manner, such as that of the Kohonen self-organizing network model, which learns to form its own classifications of the training data based on the fact that the samples in the same groups possess common features.

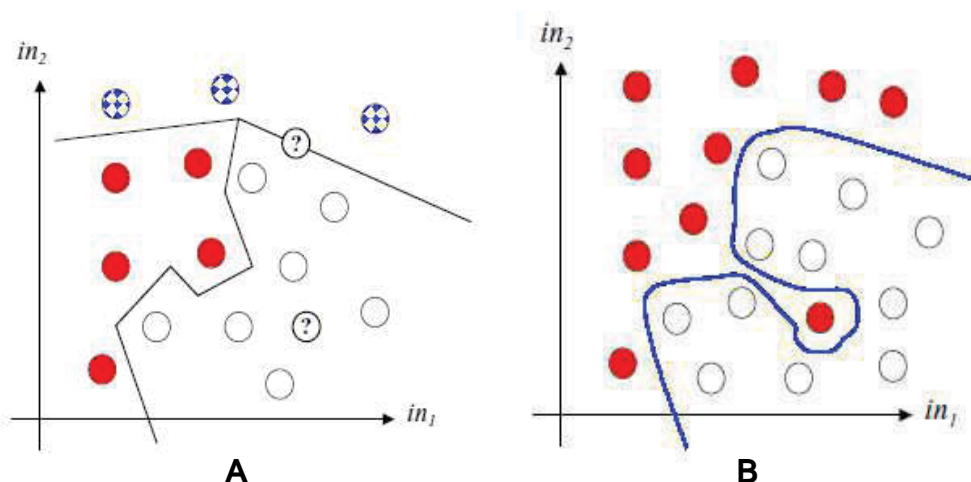


Figure 1-32: Graphs A and B are examples of data for which neural networks can provide complex decision boundaries.^[178] A distinct advantage with ANNs is their ability to recognise patterns in data that more conventional statistical algorithms may fail to perceive.

Advantageous features of ANNs include (i) their ability to learn and adapt; (ii) nonlinearity, allowing better fitting of the data; (iii) fault tolerance ability (particularly in relation to hardware failure and processing capability); (iv) relatively excellent noise insensitivity (in comparison to other pattern recognition tools); (v) uniformity of analysis and design; (vi) generalisation capability and (vii) ease of implementation to operating systems.^[164,179] The fact that causal knowledge of the relationship between the input data (e.g. spectra) and the output values (i.e. group membership), is not required is also a distinct, but not unique, characteristic of ANNs.

ANNs have been used in infrared studies to assist in the investigation of biological processes and tissues such as examining metabolite overproduction using bacterial strains,^[180] and cancer detection in pap smears^[181] and colorectal tissue sections.^[182] Imaging-specific applications have included the examination of normal and diseased oral mucosal tissue,^[91] lymph nodes,^[92,149] colorectal tissue,^[183] squamous cell carcinoma (SCC)^[91] and human melanoma studies.^[130]

Self Organising Maps (SOMs)

As a part of this research project, the application of a particular form of neural network, known as a Self Organising Map (SOM), to infrared spectra for pattern recognition was explored. SOMs are useful in that they enable the user to easily visualise high-dimensional data by producing simpler, low-dimensional views of that same data while preserving the topological relationships within the data.^[184] A vector quantisation method, the SOM approach possesses both vector quantisation and projection algorithm properties.^[185] During the initial stages of training the network, neurons, represented by prototype vectors, are placed on a generally regular, low dimensional grid (although higher dimension grids are possible). Each neuron is connected to its adjacent neurons according to a neighbourhood relation which in turn determines the structure of the map.

A training process then takes place in which each learning step consists of the input data, in the form of a sample vector, being presented to the individual nodes. The node with the prototype vector closest in weight to that of the input data is found and is known as the Best-Matching Unit (BMU). The prototype vectors in the neighbourhood of the BMU are then moved towards the sample vector. This process is iterative and continues until the vector weights associated with the nodes fall below a pre-set threshold.^[186]

Because the prototype vectors of neighbourhood SOM units are also neighbours in the data space, the topology of the input data is preserved and can in turn be visualised.^[187] A

distance matrix (also known as a U-matrix) can be easily constructed to show, using colour scales, the similarity of each unit to its neighbours and hence any potential clusters in the data.^[111] An example of a three dimensional data set and a resulting U-matrix following SOM analysis has been given in Figure 1-33.

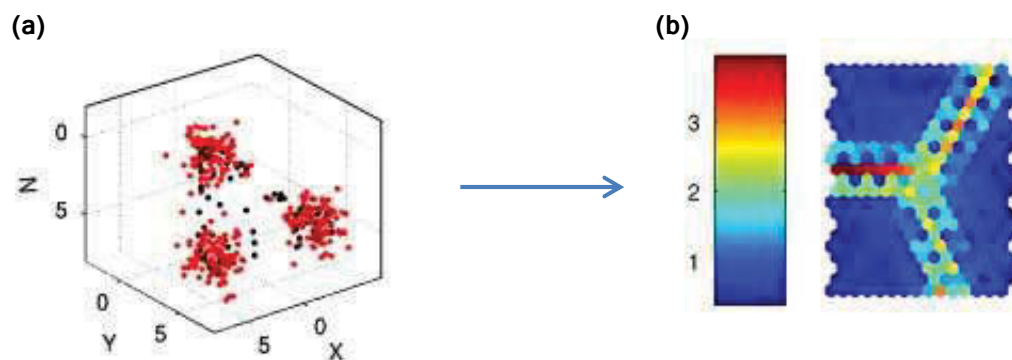


Figure 1-33: SOM application example adapted from Köhler *et al.*^[187] In this example a simple three-dimensional data set (a) has been analysed (the red symbols represent data points, the black prototype vectors) using SOM to produce (b) a U-matrix. The colour scale indicates the prototype vector distances of directly connected units. Accordingly, cluster borders are indicated by the presence of warmer colours (larger mean distances between vectors). In this diagram the three data clusters are clearly visible.

1.2.13.6 Vertex Component Analysis (VCA)

Vertex component analysis (VCA) is a multivariate data analysis technique that employs a form of spectral unmixing - a technique used to decompose mixed pixels into distinct, unique spectra reflecting the constituent substances, or endmembers, that make up each spectrum, as well as calculating the extent (abundance) to which each constituent spectrum contributes to the overall pixel spectrum.^[188,189]

Unlike HCA, the VCA algorithm seeks out the most dissimilar spectra in a data set and assumes that any mixed pixel is a linear combination of the most dissimilar spectra.^[189]

The VCA algorithm, like HCA, is unsupervised. VCA is also premised upon two facts: 1) the endmembers are the vertices of a simplex and 2) the affine transformation of a simplex is also a simplex.^[190] Quite simply, the algorithm first performs dimension reduction by initially representing each spectrum as a vector in N-dimensional space and decomposing it into intensities along each of the wavenumber values collected (refer to Figure 1-34).

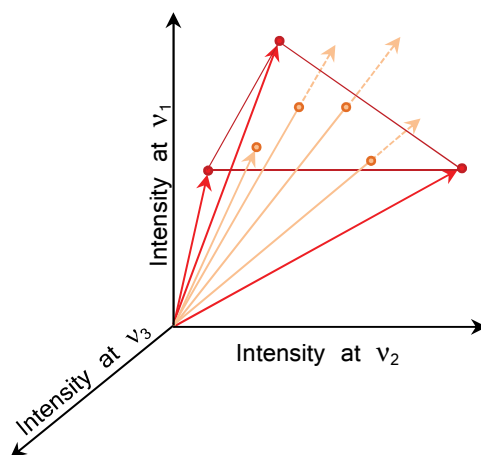


Figure 1-34: VCA algorithm diagram depicting the vector space for three wavenumber values. Note that all of the vector endpoints lie in a triangle. Adapted from Matthäus *et al.*^[189]

Accordingly, if two spectra have the same “composition” but different total intensities their vectors will be parallel.^[189] VCA also assumes the presence of pure component pixel spectra or endmembers in the image, which are represented as extreme vectors in the original representation. Dimension reduction is then performed using an iterative process which reduces the simplex by projecting data onto a direction orthogonal to the previously determined subspace (spanned by the previously determined endmember signatures). This process continues until the number of endmembers reaches a user-determined level and

endmember determination has been achieved. Inversion is then performed to estimate the fractional abundances of each endmember at each pixel, based on the pixel's spectrum, and an abundance map is generated for each endmember.

Compared to HCA, VCA is not a computationally intensive process and offers considerable speed advantages. However, Nascimento and Biouca Dias demonstrated that the performance of VCA is reduced when the image being examined is composed of very noisy spectra, although a lack of pure pixels can be a more serious problem.^[190] Examples of VCA being employed in spectral imaging studies have included the analysis of satellite images for geological information as well as cells and drug delivery systems using Raman spectra.^[189-191]

1.2.14 Data Reduction and Feature Extraction

In general there are two different types of multivariate data analysis approaches: clustering and ordinal methods.^[149] Clustering methods employ algorithms which attempt to divide a group of individual samples into a number of classes which are comprised of similar samples. Ordinal methods on the other hand seek to reduce the complexity of multivariate data into a set of new coordinate axes which describe the variance (the measure of the extent of differences between samples) or covariance (measure of the differences which samples have in common) within the data set, thus leading to a reduction in data dimensionality.

Although computer processors are becoming faster and more powerful, often it is desirable to reduce the large amount of data present in an infrared image into a more manageable, and consequently much faster to process, size. There are numerous methods by which this data reduction may be performed and the procedure used will highly depend on the initial reason for the image collection and the need to preserve the spatial relationships within the data and/or sample heterogeneity information. However, where data reduction is

performed, great care must be taken to preserve the information within the samples and the relationships between the groups of data.

If it is the reduction of a number of images representing the same sample, or sample group, into one descriptive representative value (feature vector) of that sample or group, there are numerous methods to choose from. In their 2009 paper, Rutledge and Reedy discuss a number of methods of data reduction, such as calculating score vectors for each image based on either HCA-derived or self-organising feature map (a form of ANN) image classifications, the creation of feature vectors based on PCA scores, or even by simply taking a combination of statistical measures such as the median-interquartile range (MIQ) and average-standard deviation (ASD).^[192]

If one is simply seeking to reduce within-image processing times or file-sizes, methods such as spectral subsetting, pixel aggregation and feature extraction can be useful. Often researchers will use a combination of chemometric techniques when analyzing their data. For example, Fernandez *et al*^[83] extracted a number of what they referred to as spectral 'metrics' (e.g. the ratio of different characteristic functional group absorbance peaks within a spectrum) which they in turn used for their pattern recognition method - a combination of a learning algorithm and a probabilistic measure - to relate the metrics of the sample to its pathologically determined tissue classification. Ly *et al*^[154] employed PCA to remove the paraffin contribution from formalin-fixed paraffin-embedded colon and skin cancer lesion spectral images, and then performed *K*-means clustering to highlight particular areas of interest in the tissue, such as tumourous growth.

It is clear from the number of hyperspectral imaging studies performed to date, particularly those regarding pathology applications, that the effective sequential application of multiple statistical data treatment and multivariate data analysis models can have significant advantages.

1.2.15 Test Sets and Validation of the Pattern Recognition Model

The most critical task when attempting to use a chemometric technique to perform pattern recognition is to ensure that the model chosen is correctly classifying the input data. As shown in Figure 1-35, the spectroscopist or chemometrician must attempt to discover a model which balances Type I (false positive) and Type II (false negative) errors for their given application.^[193]

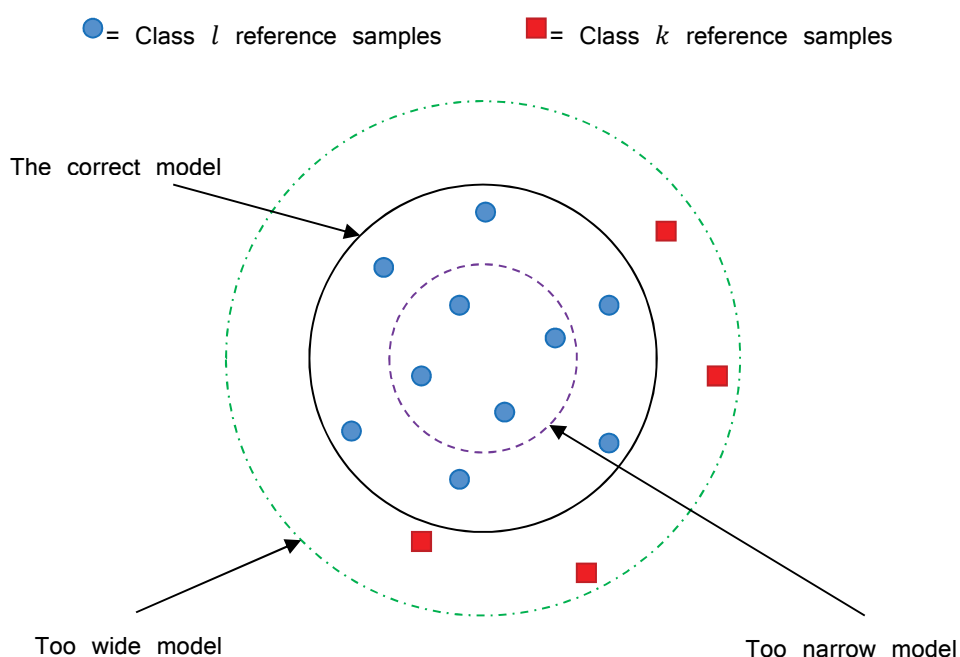


Figure 1-35: Three possible models for a data set (with non-overlapping groups) are represented by the three circles, with the locations of the training and test samples indicated. While the innermost circle has the lowest standard error, some of the reference samples from that group are not included. The outermost circle is too wide a model as it includes samples from a different group (adapted from Flåten).^[193]

Please note that validation is sometimes employed for different purposes in statistical studies. Aside from determining the quality of a classification method (the topic of discussion for this section), validation is also employed to determine the optimum operating parameters within a model, such as the determining the most suitable number of PCs in SIMCA.

In general, the construction of a pattern recognition procedure should follow the process given in the example flowchart of Figure 1-36. While the process given seems straightforward enough, there are many ways, each with their proponents, of how to most effectively validate the model developed. The most logical step is to construct a test set of data of known group membership, separate from the original training data set, and then input these values into the model as unknowns and check that they are classified correctly. However, often the investigator does not have the luxury of access to a large enough number of samples to enable the construction of separate training and test data sets with statistically significant replicates in each group. Hence, re-sampling methods have been developed for small sample set situations, as well as a means for statistical model refinement and evaluation in some situations (e.g. SIMCA).

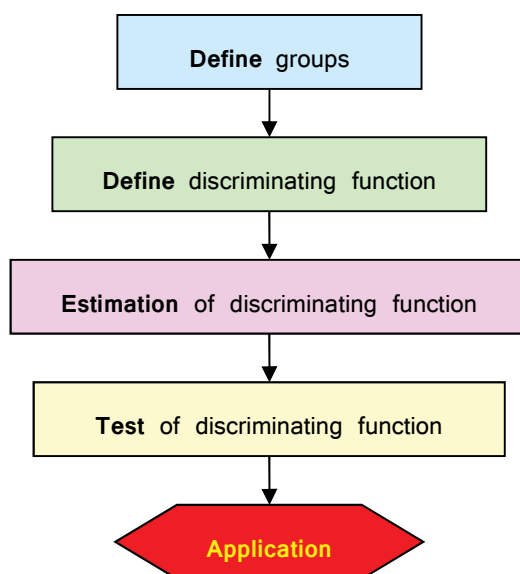


Figure 1-36: Example of a data analysis process workflow which employs LDA.

Commonly employed methods include cross-validation, bootstrapping and jackknifing. A brief explanation of each validation method has been provided below.

- ***Cross-validation***

In cross-validation the training data set is initially divided into two components in a pre-determined ratio. One component of the training data is employed for the model construction. Once the model is established, the second component is treated by the model as 'unknowns' or test data, and the predictions of class membership obtained and compared against their known group membership. This process is then repeated, using the same ratio of subdivision, but with a different combination of samples representing the training and test data set. The procedure is repeated again and again until all samples have been part of both the testing and training data sets.

A common approach found in literature is the use of the Leave One Out (LOO) cross-validation method. In LOO, literally one sample is left out for each iteration, until all samples have been left out in turn. After all of the iterations have been completed, the percentage of correctly classified (%CC) samples using cross-validation is calculated. In general, the percentage of correctly classified samples using cross-validation is less than that obtained for the training data set, so obtaining a similar %CC value for the training and test set, as well as the cross-validation is a reassuring sign that the modeling method chosen for pattern recognition is suitable. However, when the %CC for the cross-validation is significantly low compared to that obtained from the training set, further investigation into the suitability of the chemometric model proposed is highly recommended.^[104]

- ***Bootstrap***

The bootstrap method uses only a portion of the original training data set to form an internal, or 'pseudoreplicate' test set,^[194] but possesses the same number of samples as

the original training data set. Hence, some samples will occur more than once in the pseudoreplicate test set, and some not at all. The pseudoreplicate data set is examined using the chosen statistical model (e.g. HCA) and the classifications for the samples obtained. Following this, another pseudoreplicate test set is then resampled from the original data set, and the process of classification repeated.

Hundreds to millions of reiterations of this resampling process may be performed, with the predictive ability of the selected classification model determined by averaging the percentage of correctly classified samples for each iteration.^[173] The bootstrap method is considered as providing good agreement with normal (Gaussian) distributions, but it is often described as 'overly optimistic' as it tends to underestimate the errors.

- ***Jackknife***

More alike to cross-validation, jackknife analysis involves the construction of a limited number of test sets by the systematic removal of one sample from the training set, to produce a test set with one less elements than the original training data set. This procedure is repeated until all samples have been left out once. Therefore, if the original data set contained n elements, each test set will contain $n - 1$ elements, and there will be n pseudoreplicate test data sets available for processing.

Instead of simply looking at the predictions for the 'unknown' sample for all iterations, the jackknife may be used to examine the changes in the model brought about by the variation in the training set. Like bootstrapping, jackknifing is a technique used by an investigator to determine when to stop analyzing their data, and offers assistance in the identification of the stage in the statistical analysis beyond which sampling error, and not the underlying sample characteristics, is more greatly influencing the results.^[194]

If the model produced is yielding poor validation results there are a number of options open to the user. One can attempt to modify the method being used by perhaps reducing the number of variables (i.e. removing redundant data or performing some form of feature selection to reduce the data set), or employ a different algorithm within the method (e.g. use a different distance calculation metric). Ultimately the investigator may always change to another pattern recognition technique. However, it would be well-advised to make an attempt to understand more about the data being examined, and to attempt to understand why a particular statistical method is not working well, so as to improve one's chances of selecting a more appropriate method the second time around.

1.2.16 Selection of the Pattern Recognition Method

The ideal for routine pathological analysis will involve the construction of an automated analysis scheme, relying on human interaction only for the initiation of program sequences, to produce a completely objective classification result for comparison with the diagnosis obtained by the pathologist using standard histological examination techniques. Therefore, the choice of a suitable pattern recognition algorithm will prove to be a critical step in the automation of result analysis stage.

As described earlier in Section 1.2.15, whichever pattern recognition method is chosen must be assessed, with the principles of the algorithm suitability testing based on the same fundamental principles of pathological tissue analysis, the so-called 'Gold Standard'. Put simply, a sample of unknown tissue quality is examined in comparison with a library of known cell or tissue classification. For spectral imaging, computerised pattern recognition techniques process the test sample image by comparing the spectrum comprising each pixel against a reference spectral data set of known histopathological diagnosis. What varies between one pattern recognition method to the next will be the mathematical 'matching' process.

To select potentially viable pattern recognition methods, the most obvious first step is to examine the literature and explore what approaches other research groups have attempted using similar data. Excellent example papers which compare and discuss the effectiveness of a variety of multivariate statistical methods in field applications such as those of archaeometry,^[195] food analysis,^[164] remote sensing,^[147] and forensic science,^[54] can also prove extremely insightful.

It is apparent from the information provided earlier that there are numerous chemometric methods at the spectroscopist's disposal to perform data reduction, feature extraction, automated pattern-recognition and model validation using multivariate data. Each method has its advantages and disadvantages as each has a class of target functions for which it is best suited. The selected applications of the various chemometric methods discussed above demonstrate how opinions differ in the spectroscopy community as to the best approach for data analysis. Hence, there appears to be no clear answer or definitive approach as to how to select the most appropriate pattern recognition method for one's data.

It is inherently obvious that the determination of the best approach to be taken will depend on time, hardware and sample availability. A good study might include multiple classification methods being attempted and their performance measured and compared using methods such as ANOVA (analysis of variance) and 'area under the curve' (AUC).^[196,197] The first and most critical step to be performed prior to the employment of any pattern recognition method is to determine as much about the statistical characteristics of the data on hand, and to assess the methodology employed by individuals examining similar sample materials or data output as published in the literature. From this simple founding step it may then be possible to discover the most suitable pattern recognition and overall data analysis technique.

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CHAPTER 2:

MELANOMA STUDY PART 1

(BACKGROUND AND METHOD)

2.1 MELANOMA THEORY

2.1.1 Normal Cell Growth

In order for a complex, multicellular living organism, such as a human, to grow as well as maintain tissue functionality, the various cellular units that comprise the organism and its tissues must be able to differentiate, replicate and in some cases undergo programmed cell death, in a controlled manner.

When tissue growth is required, the cells involved are signalled to begin replication. In eukaryotic cells (i.e. cells that possess a nucleus), the cell first undergoes a period of growth and DNA replication, known as interphase. On a simple level, interphase itself consists of three steps: 1) G1, or 'Gap 1', in which the cell grows and produces RNA and proteins, readying itself for DNA replication; 2) DNA replication is then performed to enable two daughter cells to form, known as the S phase; followed then by 3) 'Gap 2' (G2), in which the cell continues to produce more proteins and grow in readiness for mitosis.

Once the nuclear material has been duplicated and sufficient nutrients accumulated, the cell then undergoes mitosis, in which the enlarged cell splits itself into two separate, or "daughter" cells (known as M phase). The cells may then enter a resting state (G0) which may be brief or permanent. This series of events (interphase and mitosis) that describe the division and replication of a cell is known as the 'cell cycle'.

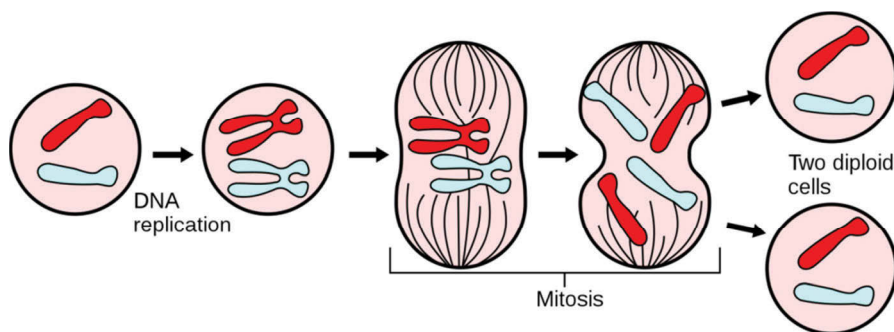


Figure 2-1: A diagrammatic representation of the cell cycle.^[1]

To progress through the various phases of the cell cycle, there is a sequential expression and activation of cyclin/cyclin-dependent kinase (CDKs) complexes,^[2] and in particular, specific cell cycle control mechanisms occur during the G1, G2 and M phases to regulate the process. These control mechanisms are genetically encoded in the cell. For example, there are genes for DNA repair, oncogenes for growth acceleration and tumour suppressor genes to slow cellular growth.^[3]

In certain cases, the biochemical cascade necessary for the regulation of the cell cycle has been disrupted by some means and the cells undergo uncontrolled cell division. This state of uncontrolled cell growth in a living organism is referred to as cancer.

2.1.2 Disorders of Cell Growth

There are over two hundred different cells types in the human body,^[3] the majority possessing a nucleus responsible for ensuring that the specific functions required of that cells are performed in a predictable manner. A cell may undergo a range of physiological changes to perform its various duties (e.g. differentiation, replication, etc.). Any deviation from these programmed changes does not necessarily produce cancer, but it may result in tissue changes that are undesirable to the host organism. Figure 2-2 provides an overview of the various cellular abnormalities that may occur in tissues.

It is possible for an individual to exhibit more than one of the abnormalities described in Figure 2-2 in a single tissue sample, and it is the extent to which the abnormalities are expressed that will assist in determining a prognosis. For example, the presence of dysplastic tissue does not necessarily imply that the individual will develop cancer. However, in the case of tissue exhibiting dysplasia, a greater risk of developing invasive cancer has been observed to vary with the grade (severity), growth duration and the actual site of the abnormal cell growth.^[4] The correct identification of the cellular changes occurring in the tissue is the responsibility of the pathologist.

- Abnormal differentiation
- Replacement of mature cells of one type with cells of another
- Regular organization of tissue maintained
- Abnormal differentiation and maturation
- Partial loss of control and organization
- Slight increase in cell number
- Cytologic abnormalities
- Abnormal differentiation and maturation
- Marked increase in cell number
- Complete loss of control
- Variable loss of organization

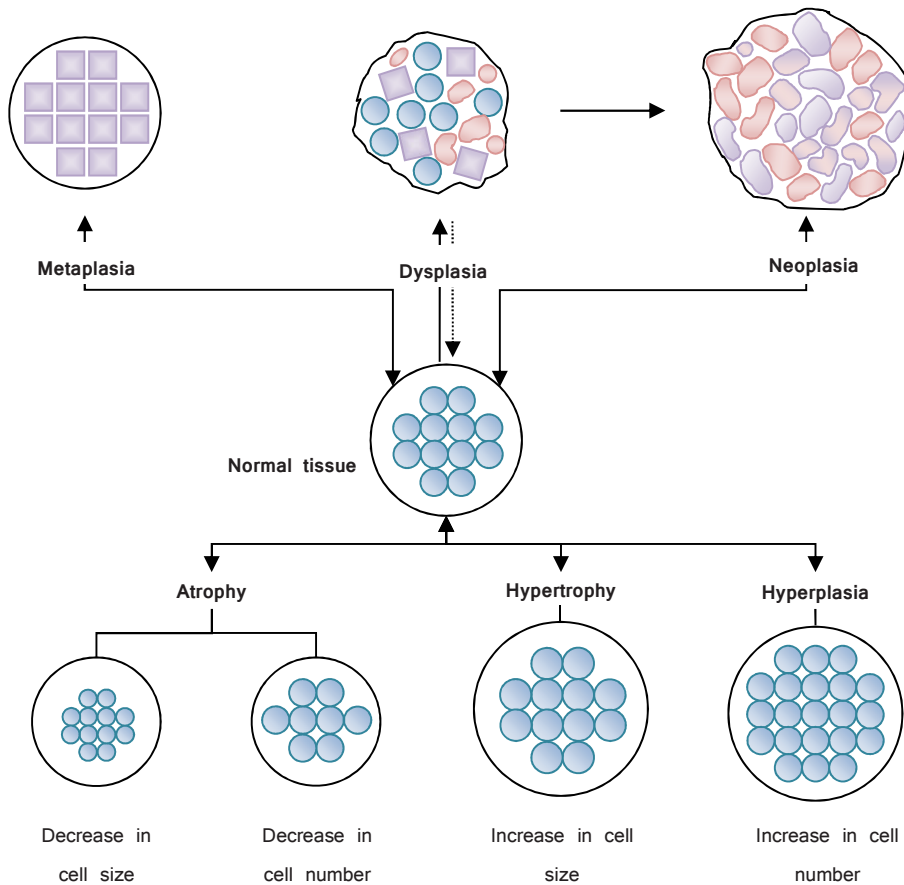


Figure 2-2: Examples of the abnormalities of cell growth and maturation that may be observed in tissues (adapted from Chandramosa and Taylor).^[4]

2.1.3 Cancer

Cancer describes a range of diseases in which abnormal cells proliferate and spread out of control, invading healthy tissue and potentially taking over various organs of the body to the point where the sufferer can no longer survive. Unlike dysplasia, the abnormal cellular proliferation of cancer results in an invasion of the basement membrane and then a spread from the primary growth site. A mass of cancer cells is referred to as a **tumour** or **neoplasm**, and **malignancy** describes the spreading of abnormally growing cells into adjacent tissue and/or to distant sites (metastasis). A summary of the physiological characteristics typical of cancer is given in Figure 2-3.

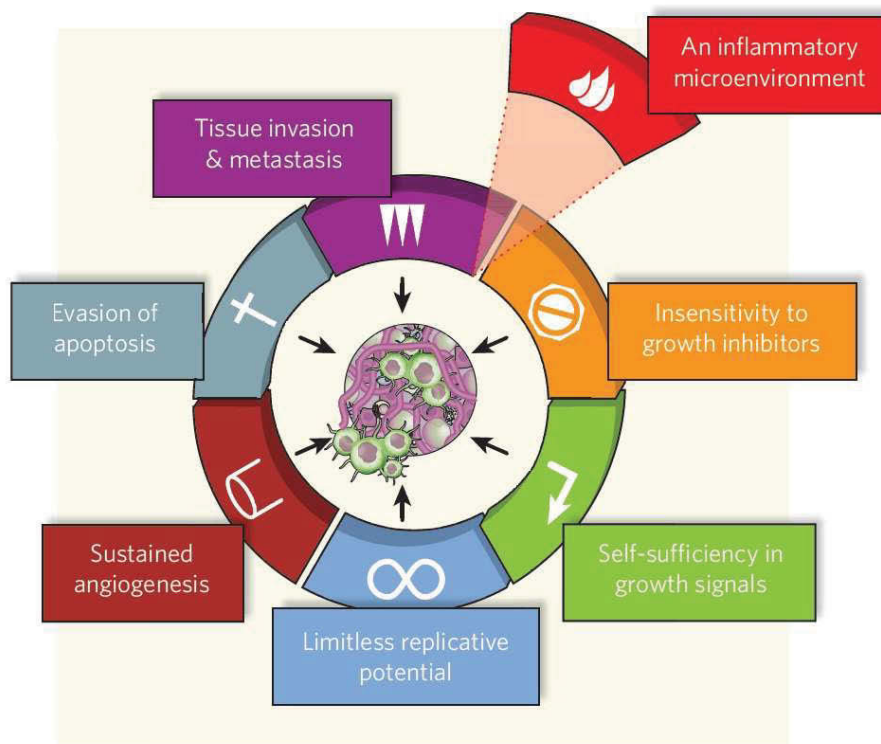


Figure 2-3: A recent revision of the hallmarks of cancer. Traditionally there were six recognised hallmarks of cancer, but some researchers argue that inflammatory cells and molecules are important in influencing cancer progress and should also be included (from Mantovani).^[5]

There are a number of terms used to describe the extent of the cancer upon diagnosis.^[6]

Localised cancer refers to a malignancy found only in the organ of origin which has not spread beyond the boundaries of that affected organ. Disease described as being at the **regional** stage means that the malignancy has managed to invade into the surrounding organs and/or adjacent tissues or spread to nearby lymph nodes (LNs). If, at diagnosis, it is observed that tumour cells have travelled to other parts of the body (via the local tissues, lymphatic or cardiovascular system), after breaking away from the primary malignancy, and managed to start growing, the stage is referred to as **distant metastases**, **metastatic**, or **secondary disease**.

The causes of cancer vary dramatically, with the formation of a tumour arising due to a range of possible reasons, such as the inheritance of genetic mutations, or perhaps exposure of the individual to damaging environmental conditions or chemical agents (carcinogens). At the most basic level, cancer occurs when the DNA of a cell is damaged, which then results in a series of mutations to produce a cell which is no longer subject to the normal control mechanisms. In addition to this, not all cancer cells are capable of metastasizing, with factors such as the tumour microenvironment, as well as intrinsic properties of the tumour cells themselves, affecting tumour progression.^[5]

Tumours are generally classified into one of two types: **benign** or **malignant**, although a tissue may exhibit a spectrum of cellular behaviour between the two. Benign neoplasms are slow growing and do not display any invasion into the surrounding tissues or metastasis. Whilst rarely life-threatening (often cured by a simple excision of the tumour), benign neoplasms may cause physiological complications in the sufferer, including death, due to a disruption in normal biological processes, such as excess hormone secretion or physical pressure on adjacent tissues (e.g. nerves).^[4] Malignant neoplasms are characterised by their rapid growth, adjacent tissue infiltration and destruction, as well as metastasis. The prognosis for an individual with a malignant neoplasm is much less optimistic in

comparison to a benign growth. In between the malignant and benign classifications is that of an **intermediate** neoplasm which possesses a variable growth rate, signs of local invasion but low metastatic potential. Whilst the prognosis for such neoplasms is not quite as grim as that for malignant neoplasms, surgical removal may not remove all of the growth and a local recurrence is possible.

2.1.4 Skin Cancer

Skin cancers are neoplasms on the skin which may arise for a variety of reasons and from a range of different cells in the skin. The main types of skin cancers are basal cell carcinomas (neoplasms originating from the basal layer of the skin), squamous cell carcinomas (neoplasms originating from skin epithelial cells exhibiting squamous cell differentiation) and melanoma (neoplasm originating from melanocyte cells) (see Figure 2-4).

Basal cell carcinoma (BCC), the most common skin cancer in Australia, is only locally invasive and its metastasis is rarely ever documented.^[7,8] BCCs are often located on the sun-exposed areas of the face and may be cured by surgical removal. Squamous cell carcinomas (SCCs) are relatively slow-growing cancerous lesions which can metastasise, but are often diagnosed in the earlier growth stages and are hence usually effectively treated by simple excision. SCCs, like BCCs, are often found on sun-exposed areas of the skin such as the backs of the hands, head and lower lip, although they can occur in the mouth, bladder, prostate, vagina, cervix and lungs. In contrast to BCC and SCC, melanoma can spread quite rapidly through the body at the earlier stages of the tumour invasion process and cause death within months of metastatic disease detection. Melanoma can occur anywhere on the body where there is pigment.

BCC and SCC are often collectively referred to as cutaneous carcinomas or non-melanoma skin cancers (NMSC). NMSCs are the most common cancers diagnosed and treated in Australia, with an estimated incidence of 433,613 cases in 2008 of either BCC or SCC^[9]

although exact figures regarding their occurrence are difficult to gather as they have not been generally reported to cancer registries in the past (they are not reportable by law). NMSC was also responsible for approximately 95,000 hospitalisations in Australia for the 2010-2011 financial year.^[10] Whilst the incidence of NMSCs is staggering (BCC and SCC are each more common than all other cancers combined),^[11] only a small percentage of NMSCs result in death (1.0% of total cancer deaths in Australia in 2010).^[10] In contrast to NMSCs, melanoma is the least common type of skin cancer (representing approximately 5% of all skin cancers)^[12] and in 2010 represented 3.4% of all cancer deaths in Australia.^[10]



Figure 2-4: Images of various skin cancers (A) melanoma (B) nodular melanoma (C) squamous cell carcinoma and (D) basal cell carcinoma.^[13]

2.1.5 Melanoma Epidemiology

Although melanoma is one of the least most common skin cancers, it is considered the most deadly. Across the world the incidence of cutaneous malignant melanoma is increasing at a rate faster than any other cancer, particularly in fair-skinned Caucasian populations,^[14] with Australia possessing an age-standardised incidence rate 13 times higher than the average world rate.^[15] A comparison chart for international melanoma incidence

rates has been provided in Figure 2-5. Melanoma is one of the most common cancers in people aged 15 to 44 years of age in Australia,^[16] with males from Queensland, Western Australia and New South Wales representing the top three melanoma incidence regions of the world.^[17] In Australia melanoma is currently the third most common cancer in men (after prostate and bowel cancer) and women (after breast and bowel cancer).^[10]

The five-year relative survival for individuals diagnosed with melanoma in the period 2006 - 2010 was 92% in Australia,^[10] a considerable improvement considering that the value was only 86% for the 1982 - 1986 diagnoses period.^[11] For the New South Wales population alone, the five-year relative survival rates for melanoma in the 2002 to 2006 period (i.e. were diagnosed in the 2002 to 2006 period and whose deaths were recorded until 2007) was 88% for men and 93% for females,^[18] although this value varies depending on the age and disease stage (e.g. malignant melanoma has a lower relative survival rate compared to superficial spreading melanoma).^[17] In the 1999-2008 period, melanoma mortality rates in New South Wales increased by a statistically significant value of 11.9% in men, and incidence rates rose by 14.9% in men and 11.2% in women.^[18] The median survival rate of individuals with metastatic melanoma, unlike that of early stage primary melanoma, is quite poor, with many studies reporting a survival period following diagnosis of only 6 to 9 months.^[19]

Melanoma's death rate continues to increase faster than that of most cancers (excepting non-Hodgkin's lymphoma, lung cancer in women and testicular cancer), although at a slower rate compared to that of melanoma incidence.^[14] One study has calculated that melanoma would represent over 15,000 years of 'healthy' life lost due to premature mortality in 2012 in the male Australian population (based on incidence and mortality figures available in 2003)^[10] and is the sixteenth leading cause of mortality burden in the Australian male population.^[20] In 2010, melanoma represented 10.1% of all new cases of cancer diagnosed in the Australian population.^[10]

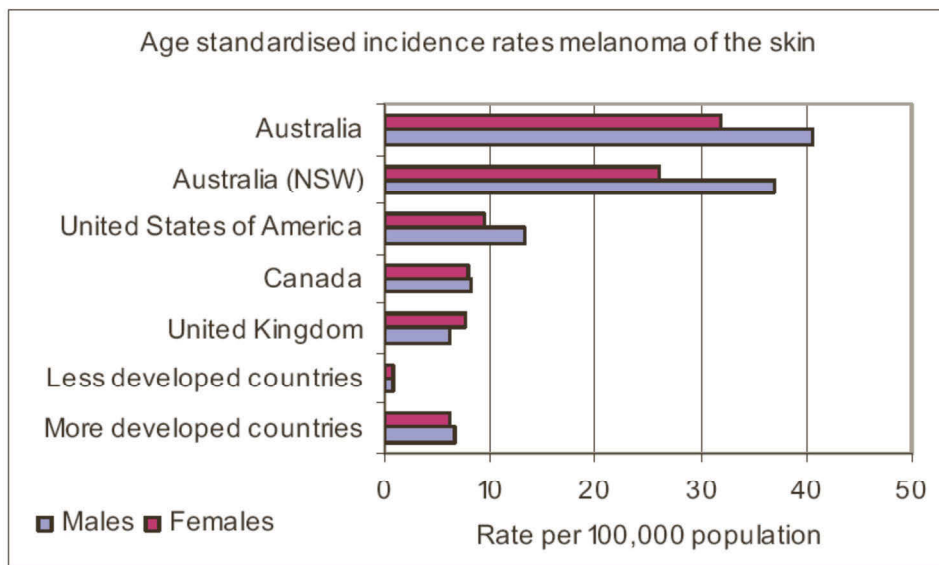


Figure 2-5: A graph showing the international variation of melanoma incidence from Tracey *et al.*^[6]

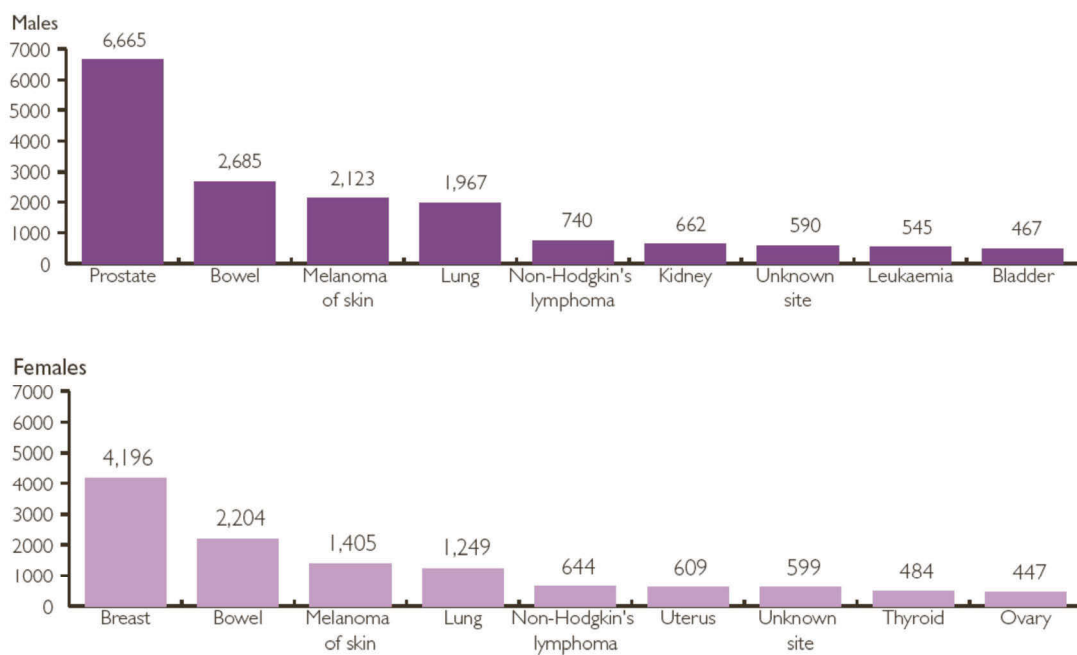


Figure 2-6: The most common types of cancer in the Australian NSW population in 2007 from Tracey *et al.*^[17]

Superficial spreading melanoma was the most common melanoma cell type encountered in NSW (45.9% of total cases) in 2007, and as a proportion of all melanoma cases it is also increasing. Long term predictions (up to 2021) for melanoma incidence and mortality in NSW suggest that the number of cases per 100,000 individuals will continue to increase for the former but remain relatively constant for the latter.^[17] This trend, according to many researchers, would suggest that people are being diagnosed at an earlier stage (lower median tumour thickness), possibly as a result of skin awareness campaigns.^[14,17]

2.1.6 Melanoma Pathogenesis

A melanoma is a malignant (cancerous) mole which may arise from a pre-existing mole (also referred to as a naevus) or from a particular type of single skin cell (epidermal melanocyte), the cells responsible for the production of photoprotective melanin pigments. Melanocytes are found at the dermo-epidermal junction (basal layer) in the skin, but they are also found in other sites throughout the body, such as the hair follicles and eyes.^[21,22] Melanoma development can be traced to a pre-existing naevus in about 95% of cases, but sometimes the original site may not be determined.^[23] A diagram of the distribution of melanocytes in normal skin and in a melanocytic lesion is given in Figure 2-7.

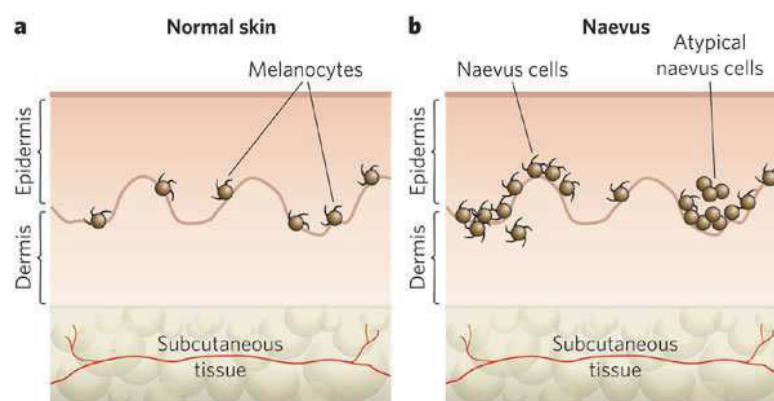


Figure 2-7: A diagrammatic comparison of normal skin (a) to a melanocytic naevus lesion possessing a dysplastic component (morphologically atypical melanocytes) (b). From Gray-Schopfer *et al.*^[22]

If restricted only to the lower layers of the epidermis, the melanocyte proliferation is referred to as a **junctional naevus**. If the growth is restricted to the dermis it is referred to as a **dermal naevus**, and if it overlaps both the epidermis and dermis it is referred to as a **compound naevus**. Although generally benign, a naevus may start to exhibit the radial growth-phase (RGP) of melanoma and develop into an intra-epidermal lesion with melanocytes migrating into the upper dermis and displaying upward growth into the upper epidermis (pagetoid spread) (see Figure 2-8).^[22] From there, the lesion may then progress to the vertical-growth phase (VGP), a stage at which the cells are considered to possess dangerous metastatic potential.^[24] Both RGP and VGP are capable of arising from naevi or isolated melanocytes, and each phase is capable of progressing into metastatic malignant melanoma ^[22]. A melanoma may not necessarily pass through each of these phases.

In adults, melanocytes are only intermittently mitotic, with their homeostasis regulated by epidermal keratinocytes, but given certain events, such as exposure to ultraviolet (UV) radiation or wound healing, the skin requires the mitotic rate of the melanocytes to increase temporarily to produce additional protective melanin pigments - the tanning response.^[25] UV radiation and chemical carcinogens, amongst other factors, can damage the melanocyte DNA, suppress the immune system and alter the fine balance of cytokines in the skin. This alteration can produce an imbalance in the cell cycle regulatory signals required for the proliferative response and may result in the uncontrolled cell division of melanocytes and the subsequent development of a naevus and/or melanoma.

The precise biological pathway taken by cutaneous melanoma is not fully understood, particularly when one considers the fact that not all melanomas form on sun-exposed areas of the body. This has led to the theory that melanoma may arise via two pathways.^[26,27] The first pathway suggests that the epidermal melanocytes of the host, who has been exposed to particular phenotypic and environmental conditions, are initiated by sunlight exposure (usually during early life) and become neoplastic without the requirement of further

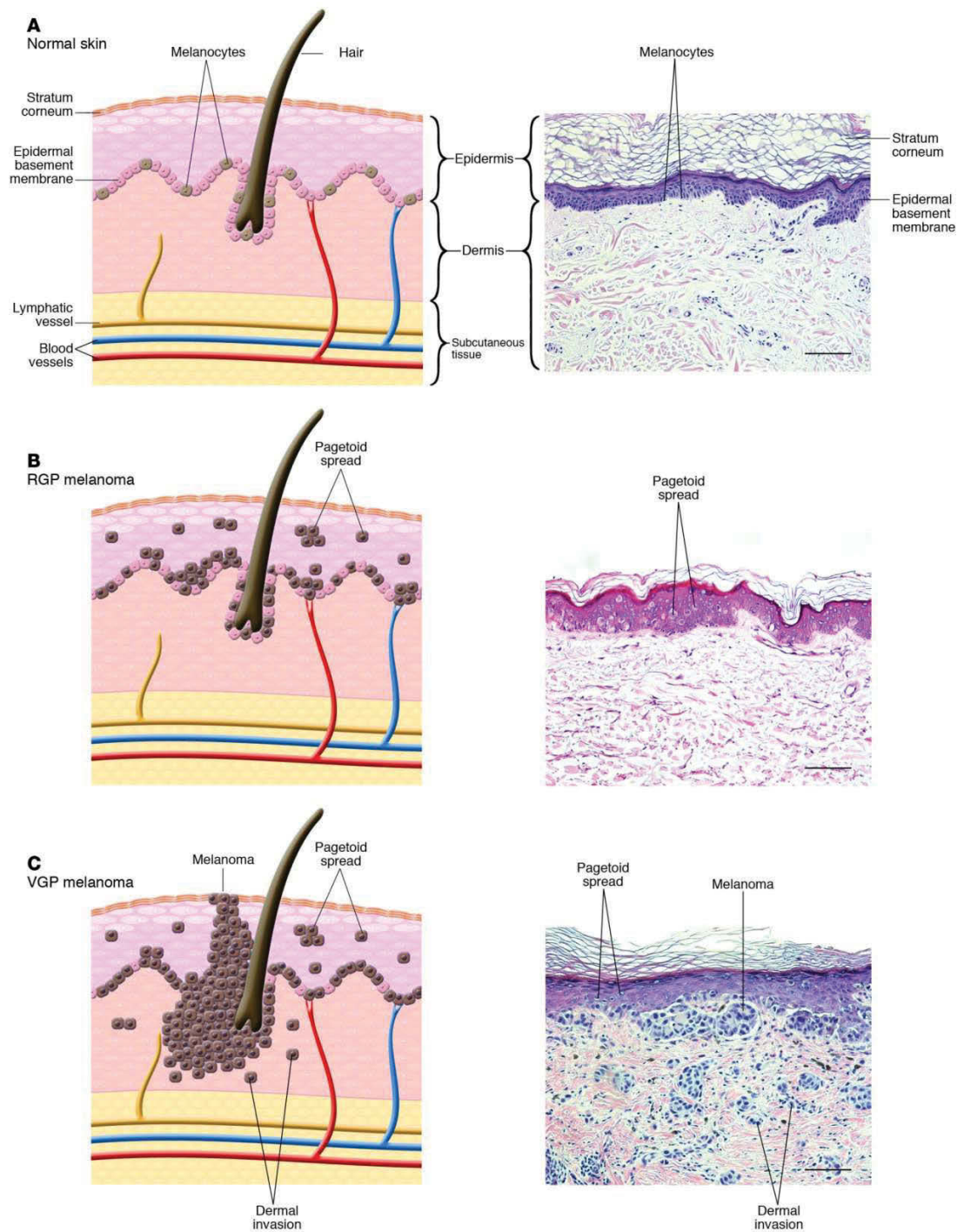


Figure 2-8: The phases of histologic progression of melanocyte transformation using a pictorial (left) and a corresponding H&E-stained section (right) at magnification x20 (scale bar: 20 μ m).^[28]

sun exposure. This pathway explains the evidence observed in numerous studies linking melanoma development, particularly in naevus-prone individuals, with sunlight exposure in their early years. The second pathway explains more the occurrence of melanoma in people with a low tendency to develop naevi, who tend to tan rather than burn, suggesting that the initiation of melanocytes of such individuals requires not only early-life sun exposure, but also chronic exposure to sunlight during later years. A summary of the divergent pathway model is given in Figure 2-9.

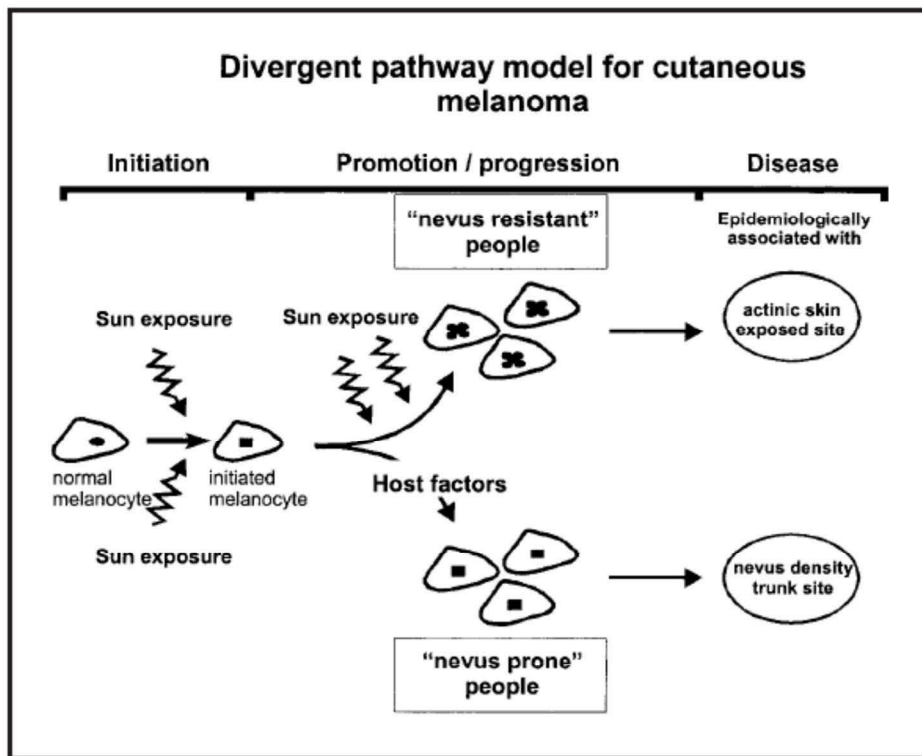


Figure 2-9: Diagrammatic representation of the divergent pathway model for cutaneous melanoma from Whiteman *et al.*^[26]

Most often encountered in the skin, melanoma may also occur, somewhat less frequently, in the mouth, nose lining or genitals and is not restricted to areas only exposed to the sun. As the cancer spreads, or metastasises, it will move initially from the primary tumour site

to the closest lymph gland through normal lymphatic drainage. From the lymph node the metastatic cells may then spread through the lymphatic system and into the bloodstream to almost any part of the body including the lungs and brain.^[3] A diagram depicting the lymphatic system network, and the structure of a lymph node, is provided in Figure 2-10.

2.1.7 Melanoma Aetiology

Cutaneous malignant melanoma possesses a multifactorial aetiology in that an individual's risk of melanoma development is the result of a number of factors such as host-related, genetic and environmental.^[14,29]

1) Host-related factors

Phenotypic features associated with increased melanoma risk include those individuals with fair skin (which tends to burn and not tan), red or blonde hair, light-coloured eyes (e.g. blue or green) and freckles. Individuals with multiple, large or unusual (non-dysplastic) moles also exhibit another independent risk factor.

2) Genetic-related factors

A genetic susceptibility to melanoma has also been observed based on familial clusters, but these genes have only been attributed to a small proportion of the hereditary component.^[30] Inherited genetic mutations (referred to as 'constitutional') have only been found in the tumour suppressor CDKN2A gene and to a lesser extent the CDK4 gene. The research regarding the genetic risk factors surrounding melanoma development is continually being gathered. Whilst there appears to be a higher risk in individuals developing cutaneous malignant melanoma where a family history of such disease exists, particularly in first-degree relatives, finding the specific gene or genes responsible for the melanoma development has so far proven difficult. At present, mutations in the cell cycle regulatory genes CDKN2A and, less commonly, CDK4 are believed to be linked to a higher risk of developing melanoma.^[31]

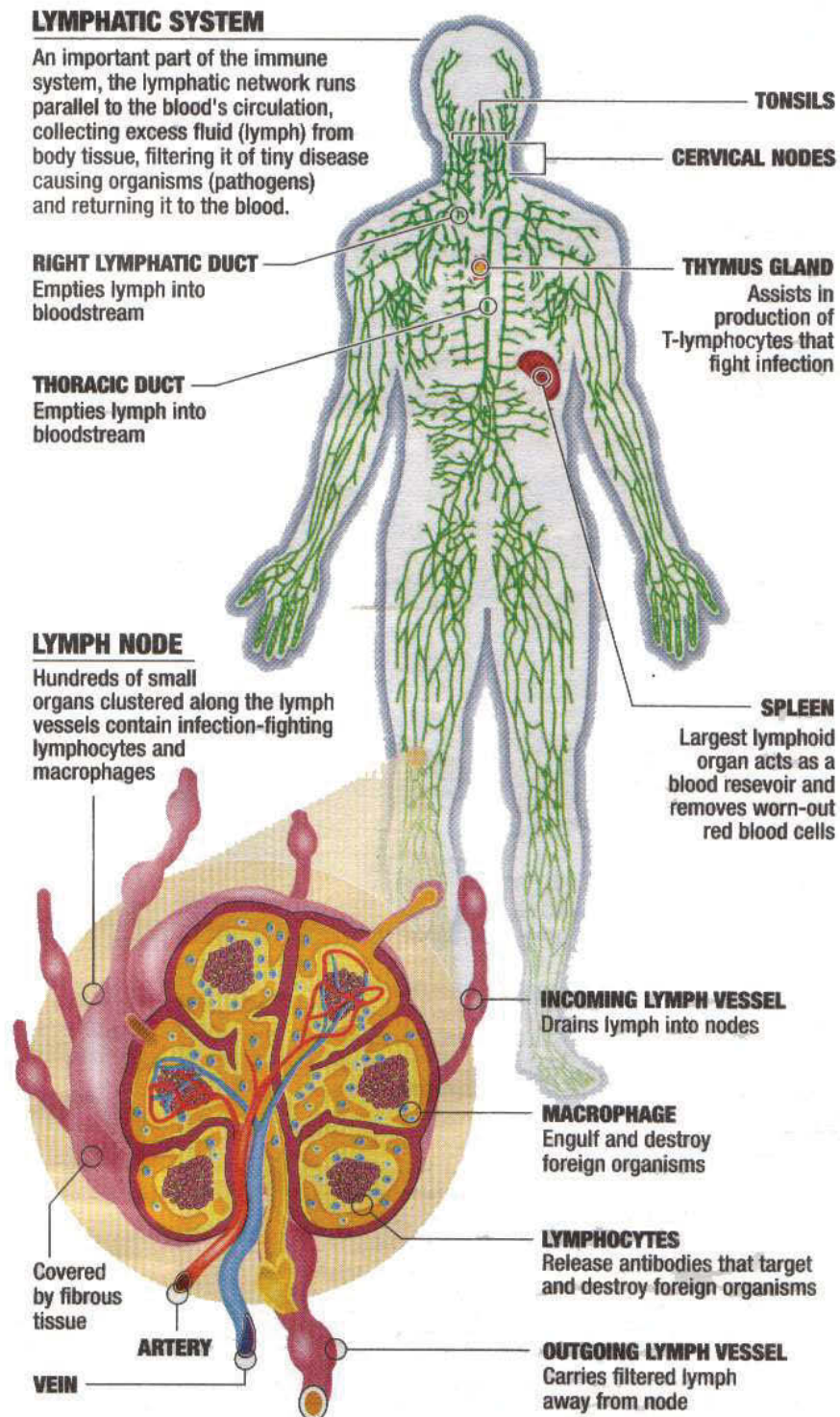


Figure 2-10: The lymphatic system (from Clark and Grant).^[32]

Features associated with genetic susceptibility to melanoma include individuals who have had:

- a) two or more close relatives who have had melanoma, particularly on the same side of the family;
- b) previous primary cutaneous melanoma;
- c) multiple naevi (including both atypical and common acquired melanocytic naevi);
- d) earlier than average age at the onset of cutaneous melanoma
- e) suffered from other cancers, and in particular pancreatic cancer.^[30]

3) Environmental factors

Sun exposure is the only well-established environmental factor in melanoma incidence and has been directly linked to cutaneous and non-melanocytic skin cancer.^[33] Put simply, UV radiation can damage (mutate) DNA, although it is not known which specific wavelengths are the most influencing on melanoma development. Effectively, the UV radiation alters the melanocyte in such a way that the accumulated genetic abnormalities promote cell proliferation, and the normal apoptotic (cell death) mechanisms which would normally occur in response to the DNA damage are prevented.^[31] Unfortunately, it is not a simple link between the total dose of solar radiation an individual may suffer over their lifetime and melanoma development. Factors such as the pattern of sun exposure (i.e. a lifetime of exposure, intermittent, but intense, sun exposure resulting in burning, or a combination of both) and the age of an individual at exposure have been linked to melanoma induction. A seasonal variation in melanoma diagnosis has also been observed in some studies, with the suggestion being that the increased UV exposure associated with the summer months may even be promoting the progression of early melanomas.^[29]

Melanoma is higher in incidence amongst white-skinned individuals living in regions nearer the equator than those closer to the poles, although in Europe this relationship

is less clear when one considers the high incidence rates amongst the Scandinavians and Swiss compared to those residing in France or Italy.^[29] Earth's elliptical orbit of the sun also means that the southern hemisphere is brought closer to the sun during its summer months, resulting in countries such as Australia experiencing more intense UV values in January compared with those recorded in the northern hemisphere in July during its summer.^[34] Stratospheric ozone depletion is also a potential point of concern considering that the ozone layer is responsible for absorbing the shorter ultraviolet wavelength component of solar radiation. A 1% increase in UVB levels measured at ground level is predicted to produce a 0.5 to 2% increase in melanoma incidence, and a 1% decrease in ozone is believed to increase ground UVB levels by 2%.^[29]

Aside from solar UV exposure, the use of solariums (tanning booths and sunbeds) has also been brought into question. Based on the available data obtained from numerous studies conducted in Australia and elsewhere, it may be concluded that there is a strong causal association between indoor tanning devices and melanoma, particularly in individuals who participate in such procedures before the age of 35 years.^[35,36] Findings from solarium use studies have led to the Clinical Practice Guidelines for the Management of Melanoma in Australia and New Zealand recommending that caution should be exercised by younger users, and that the risks associated with their use should be explained.^[37] A report compiled for the Queensland Institute of Medical Research estimated that 12 - 62 new cases of melanoma, and less than 1% of UV radiation-attributable melanoma deaths, each year in Australia may be attributed to indoor tanning devices.^[36] A study published the following year by the same author, estimated the harmful impact of UV radiation associated with solarium use in younger people (aged less than 45 years of age), based on data obtained from the five most populous Australian states, to be in the region of 281 new melanoma cases, 43 melanoma-related deaths and 2,572 new cases of SCC per year.^[38] Based on such

studies, the NSW Government has recently announced a ban on all solariums by 2014.^[39]

The relationship between skin cancer risk, UV radiation exposure, the above aetiological factors and lifestyle factors has been given in Figure 2-11.

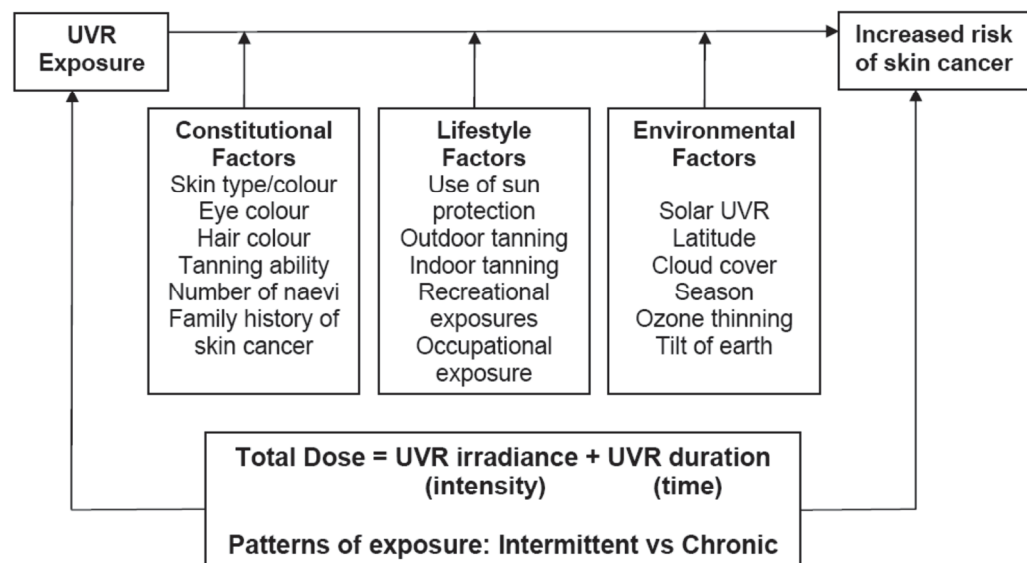


Figure 2-11: A diagrammatic representation of the relationship between the risk of skin cancer (melanoma, BCC and SCC) and ultraviolet radiation exposure (from Gordon and Hirst).^[36]

2.1.8 Melanoma Diagnosis and Prognosis

Diagnosis of melanoma often commences with the examination of a mole, or naevi, which the patient or consulting doctor has observed to possess an abnormal feature or number of features, such as an asymmetrical shape, jagged borders, irregular colouring, large diameter or a recent change (observed over weeks or months) from its known normal appearance. Whilst good lighting and magnification may enable a physician to identify clear cases of

melanoma, additional tools which may be employed to assist in the detection and diagnosis of melanoma include high-resolution photography (particularly effective in combination with computerised monitoring systems) and skin surface microscopy (epiluminescence).

It should also be noted that there are a number of varieties of melanoma, not all displaying the classical recognisable features. For example, approximately 5% of all melanoma lesions are amelanotic (melanin is absent) and are sometimes misidentified as basal or squamous cell carcinomas.^[31] A table summarizing the various types of melanoma, along with a brief description of each form, is provided in Table 2-1.

In cases where the diagnosis cannot be obtained using good lighting and magnification, and where the additional diagnostic tools mentioned above are not available to the patient, the physician may choose to perform an excision biopsy (preferable) or wait to review the lesion again in 2 - 3 months.

When performing an excision biopsy, the physician is advised to remove the lesion (down to the underlying muscle fascia) along with a 2 mm clearance margin of the surrounding tissue. In cases of larger lesions, an incision biopsy or punch biopsy (and in some cases a shave biopsy) may be performed to minimise the wound size and later complications to the patient's health and treatment options. However, if only a portion of the lesion is biopsied, the subsequent pathological examination, whilst able to perhaps diagnose the presence of melanoma cells, will be potentially missing other critical prognostic information (e.g. tumour thickness) and consequently partial biopsies are highly recommended against.

Table 2-1: Recommended terminology for common forms of malignant melanoma.^[17,28,40-44]

Recommended Terminology	Description
Mucosal melanoma	A rare form of melanoma (<1% of all melanoma) which occurs in the nose, mouth, oesophagus, gall bladder, urethra, anus, vulva and vagina. It is usually asymptomatic and is generally detected late, thus producing a poor diagnosis.
Subungual melanoma	Forming under the nail bed, this melanoma is often difficult to diagnose and is usually deeply invasive by the time it is. Signs to assist in diagnosis include Hutchinson's sign (pigmentation development in the skin around the nail), and in cases of paler-skinned individuals, the presence of a dark band in the nail. Subungual melanoma is most common in the great toenail and thumbnail.
Desmoplastic melanoma	Characterised by a spindle cell pattern with thickening of the dermis, this uncommon variant of melanoma may even invade nerves. Commonly found on the head and neck. Often misdiagnosed because it is usually amelanotic and possesses histological features not commonly observed in melanoma.
Superficial spreading melanoma	The most commonly occurring melanoma (approx. two thirds of all melanomas), it generally grows within the outer layer of the epidermis and is often found on the trunks of men and the legs of women. Also referred to as <i>malignant melanoma with an adjacent component of superficial spreading type</i> or <i>pagetoid melanoma</i> .
Lentigo maligna melanoma	Associated with chronic sun exposure, it is a flat, slow growing lesion commonly found on the sun-exposed areas (heads and necks) of the middle-aged and elderly. It is rarely invasive. Also referred to as <i>malignant melanoma with an adjacent epidermal component of lentigo maligna melanoma</i> or <i>malignant melanoma of Hutchinson's melanotic freckle type</i> .
Acral lentiginous melanoma (ALM)	This melanoma forms in the thickened skin found on the nails, palms and soles (subungual and acral regions). It is the most common melanoma of non-white people (30-70% of melanoma in dark-skinned individuals) and is not believed to be related to sun damage. Sometimes described as <i>malignant melanoma with an adjacent epidermal component of acral lentiginous type</i>
Mucosal lentiginous melanoma	Malignant melanoma with an adjacent component of mucosal lentiginous type
Nodular melanoma	Usually presenting as a smooth pigmented nodule (sometimes with bleeding and may be amelanotic), these tumours are raised above the surface of the skin, grow downwards and are generally thicker than other melanomas. Commonly found on the trunks of men and the legs of women they can form anywhere on the skin. This is a malignant melanoma with no adjacent component (lacks radial growth phase).
Unclassified melanoma	This is a malignant melanoma of unclassifiable histogenetic type.
Uncommon Melanoma Variants	This category includes desmoplastic melanoma and a number of controversial and provisional melanoma variants such as malignant blue naevus (melanoma resembling or arising in a blue naevus), melanoma in congenital naevus, minimal deviation (naevoid) melanoma, animal type melanoma (pigmented epithelioid melanocytoma) and primary dermal melanoma.

Pathological Examination

To ensure that the most effective treatment of the melanoma may be offered to the patient, an accurate histological assessment must be performed by a pathologist experienced in melanocytic lesion examination. Such specialised pathologists are trained to recognise the numerous different diagnoses that may occur in a lesion, such as pigmented spindle cell naevus, dysplastic naevus, halo naevus, recurrent naevus, Spitz naevus and cellular blue naevus, just to name a few.^[45] Initially, the pathologist must examine the biopsied tissue and identify any features which are not consistent with that of normal, healthy tissue. For example, a change which may arouse suspicion of cancer to the pathologist would be the presence of cells with inappropriately large nuclei. Although cells undergoing cell division will possess a very large nucleus relative to the amount of cytoplasm, the presence of such dividing cells may be unexpected in the tissue under examination. Unlike normal, specialised cells, cancerous cells will often possess nuclei that vary in size and shape (nuclear polymorphism) and exhibit dense-staining, 'clumped', DNA material (nuclear hyperchromatism).^[46]

To determine how much cancer is present in the body of the patient and where, cancer staging is employed. Staging involves examining the tumour (which may employ a variety of methods both when the cancer is in situ and after biopsy) and assessing the extent of the primary tumour and its spread in an effort to describe the extent of the patient's cancer.^[47]

Local tumour growth (T), as well as the spread of cancer to the regional lymph nodes (N) and metastasis (M), if any, are the three significant events in the progression of a cancer that are used to assess the extent of disease, with TNM staging considered the gold standard of tumour assessment.^[6] Staging is generally classified as being clinical (determines extent of cancer based on physical examination, imaging and biopsies) or

pathologic (this method combines the information from the clinical staging with those of the examination of the excised tissue and any observations made during the surgery). Restaging may also be performed if a cancer returns in order to assess treatment options.

To perform pathological staging the pathologist must first microscopically examine the component cells within the naevus tissue and confirm the presence of cancerous melanocytic cells. This is usually performed by taking a thin cross-section of the naevus and surrounding tissue, preferably a microtomed section of the excised tissue specimen. Whilst using frozen sections enables rapid histological of the tumour (i.e. whilst the patient is still on the operating table), cytological details in the section are generally poor, therefore it is recommended that formalin-fixed, paraffin-embedded tissue sections are used for a more accurate diagnosis to be made. In the case of paraffin sections, the tissue is then mounted on a slide and stained with haematoxylin and eosin, or any particular specialised stains for more difficult cases, and examined using a biological microscope.

Whilst this morphological interpretation method may sound quite straightforward, it can take many years and exposure to hundreds to thousands of tissue samples, exhibiting a range of cell and tissue growth states, for a pathologist to attain expertise in melanoma diagnosis. It should also be noted that there is no one specific histological criterion that definitively distinguishes between malignant and benign melanocytic lesions, as features observed in benign naevi have also been observed in melanoma samples, and vice versa.^[44]

Additional tissue examination techniques which may need to be employed by the pathologist to identify or discount melanoma include:

- Immunohistochemistry: to assist in differentiating melanoma from epithelial and other tumours, staining a melanocytic lesion with polyclonal antibodies that specifically target the S-100 protein, can be employed. However, S-100 protein immunostaining may also yield positive results for a variety of non-melanocytic tumours.^[48,49] The monoclonal

antibodies Melan-A (MART-1) and HMB-45 also may be used to label melanoma (see Figure 2-12). Unfortunately Melan-A is useless for differentiation of benign naevi from malignant melanoma, and HMB-45 can occasionally appear negative in melanoma.^[44,50]

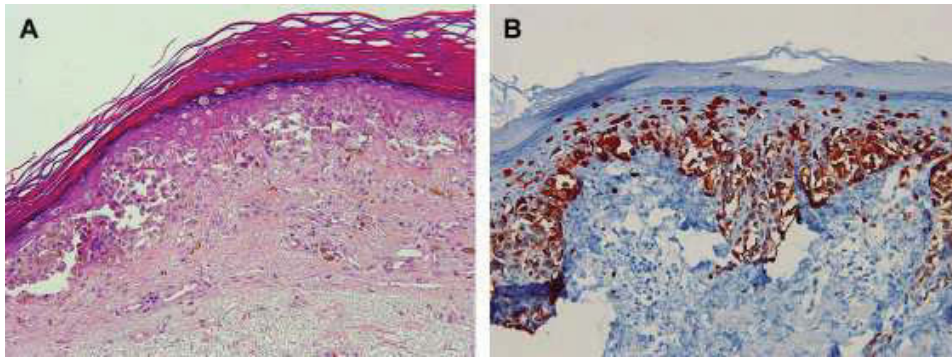


Figure 2-12: Examples of haematoxylin-eosin staining (A) and MART-1 immunostaining (B) on a section of tissue exhibiting superficial spreading melanoma. Original magnification x100. Image from Forman.^[51]

- AgNORs staining: this technique uses silver (argyrophilic) nitrate staining of nucleolar organiser regions (NORs) (ribosomal DNA), which acts as an indicator of the level of transcriptional activity occurring within a cell and may assist in distinguishing between melanoma and benign naevi.^[52]
- DNA microdensitometry: a technique which measures the DNA nuclear profile of cells; its ability to distinguish melanoma from benign naevi rests on the concept that the former cell type possesses an abnormal number of chromosomes, unlike the diploid latter.^[44]
- Nuclear morphometry: the invasive growth stage of a pre-malignant precursor lesion, on its way to malignancy, is often associated with an increase in cytological abnormality.^[53]

This microscopic technique can be used to measure the associated nuclear polymorphic changes such as area, length and perimeter to detect melanoma cell maturation.^[44]

Where the examination of excised tissue from the primary tumour site indicates malignancy and adjacent skin invasion, an examination of the sentinel lymph node (SN - the node receiving direct lymphatic drainage from the primary tumour site) is usually then performed. Initially, the physician will physically examine (palpate) the patient to check for any lymph nodes which may feel hard to the touch. A computed tomography (CT) scan may be of assistance in assessing the extent of lymph node involvement and possibly detect the presence of systemic metastatic disease.^[31] Any affected nodes may be biopsied using a fine needle to aspirate some of the node's contents, or removed via excision.

In cases where there are no physical indicators of lymph node involvement, or the investigating physician prefers additional information regarding lymphatic drainage from the primary melanoma, a technique known as sentinel lymph node mapping (also known as lymphoscintigraphy) is performed. In this method, a blue dye and a radioactive tracer are injected intradermally into the area around the excisional biopsy wound to monitor the drainage of lymph from the tumour site, and hence locate potential sentinel lymph nodes.^[45] Any nodes exhibiting the presence of the blue dye are removed (a process known as sentinel lymph node biopsy - SLNB), pathologically examined for the presence of metastases (using serial sectioning, see Figure 2-13), and the need for further lymph node removal (completion regional lymph node dissection, CLND) assessed.

Examination of the excised lymph nodes is usually performed using H&E stained sections of the tissue under a standard light microscope. In theory, if the SN does not contain tumour cells, then it is believed that the other nodes in the node field will also be unlikely to possess tumour cells.^[31] However, the discovery of even a small amount of metastatic disease in a SN is an extremely important prognostic factor for melanoma.^[55] Additional immunohistochemistry markers, e.g. S100 protein, HMB-45 and Melan A may also be

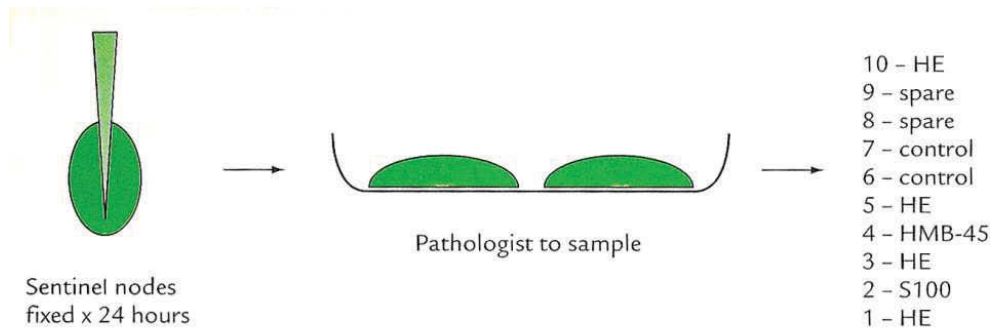


Figure 2-13: Recommended method for sampling a lymph node for histological analysis (the numbers on the right indicate the required serial full face sections).^[1,54] Note that larger nodes (> 10 mm thick) may require additional parallel slices to be cut.^[49]

employed to assist in evaluation of melanoma infiltration of the lymph node.^[56]

Whilst the value of one technique may be great for a particular application, such as the use of immunohistochemical stains to assist in the detection of melanoma micrometastases in SNs, it may be found that the same approach fails to increase the detection rate in alternative samples, such as CLND specimens.^[57] Tyronase polymerase chain reaction (PCR) analysis of lymph nodes has shown some promise, and in some cases detected micrometasis in non-sentinel lymph nodes which had not been revealed using the standard techniques.^[58] Unfortunately none of the above tissue examination methods are 100% accurate in all cases, even between similar sample types, particularly as they require analysis by trained, but very human, pathologists, and the need for further research into alternative diagnostic procedures of a less subjective nature is ever present.

Additional Lymph Node Investigation Techniques

The occurrence of substantial false-negative results associated with erroneous histological evaluation has led to the development of additional melanoma investigation techniques:

- Carbon Dye Examination - the employment of carbon dye examination in histological sections has been explored by Haigh *et al.*^[59] This approach involves the injection of carbon dye (India ink), which has been combined with the blue stain and radioactive colloid mixture, during the routing sentinel lymph node mapping procedure. The excised lymph nodes are subsequently sectioned and examined for the presence of carbon particles. The low false negative results obtained using this method appeared to support the intraoperative use of carbon dye as an adjunct to assist in the histological examination of SNs removed as a result of sentinel lymph node mapping. A problem with this technique, highlighted by Dawson *et al.*,^[60] is that the large, dense carbon particles may obscure the identification of metastatic tumour cells with the SN, and produce false negative results during subsequent standard pathological examination.
- Molecular Testing - reverse transcriptase-polymerase chain reaction (RT-PCR) is an assay method which identifies a specific type of genetic material, such as tyrosinase messenger RNA,^[61] which may be used as a marker for the presence of melanoma cells in tissue samples such as sentinel lymph nodes.^[62] This method permits the detection of submicroscopic quantities of melanoma in lymphatic tissue, and potentially may be used to predict melanoma recurrence, particularly when used in combination with standard pathological examination results. A drawback of this method is the large quantity of sample examined, and the destructive nature of the testing procedure. Nevertheless, good sensitivity of RT-PCR has been demonstrated on archival tissues using thin sections,^[63] and when combined with routine testing methods such as H&E and immunohistochemical stains, it has shown excellent promise as a positive prognostic factor for overall survival.^[64]
- Proton Magnetic Resonance Spectroscopy - examination of benign and malignant lymph node percutaneous fine needle aspiration biopsies (FNBs) by proton magnetic resonance spectroscopy has been shown to be capable of providing a rapid, accurate and

relatively cost-effective (in comparison to surgical excision of the SN) technique to distinguish between uninvolved nodes and those containing metastatic melanoma.^[65] The advantage in this technique is that it can detect changes in the chemical composition of a cell during tumour development that would not be observed using a morphological analysis technique.

- Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) - inductively coupled plasma mass spectrometry (ICP-MS) may be employed for the analysis of technetium 99 m-labelled antimony trisulfide colloid ($^{99m}\text{Tc-Sb}_2\text{S}_3$) which is deposited in sentinel lymph nodes during lymphoscintigraphy. In theory, the antimony particles used for the lymphoscintigraphy procedure are only deposited in the SNs, and because human tissue contains negligible amounts of antimony, this method may be used to confirm that a true SN was sampled and the correct diagnosis of lymph node involvement in metastatic disease obtained.

ICP-MS produces results indicative of a method possessing great sensitivity and specificity, without obscuring the identification of metastatic cells.^[60] However, a drawback to this sample examination method is the necessary destruction of the tissue section, and the need for initial tissue processing. A successful alternative sampling method explored using this analysis technique has been the use of fine-needle aspirates obtained from lymph nodes, providing a potentially rapid intraoperative diagnostic method for SN identification to measure the antimony concentrations in FNB samples from SNs which had been localised by preoperative lymphoscintigraphy.^[66]

- Fluorescence *in situ* Hybridisation (FISH) Microscopy - is a cytogenetic analysis technique which labels DNA sequences in chromosomes with the goal of detecting and measuring the structural and numerical chromosomal abnormalities observed in tumour cells.^[67] Using this technique, chromosome-specific probes may be employed to assess chromosomal copy numbers within a cell. Alternatively, smaller locus-specific probes

can be used to detect the loss, gain, or disruption of specific regions of the genome. The various probes involved in an analysis are directly labelled with fluorochromes which enables the target genomic sequences to be identified using fluorescence microscopy. Based on the high sensitivity and ability of this technique to discriminate the malignant and benign components of melanomas, it has been recommended by some researchers as a potential adjunctive tool for microstaging^[68] and has been used to examine histologically ambiguous melanocytic lesions.^[69,70]

None of the above techniques are in routine use by the majority of melanoma centres as they are still undergoing error rate assessment. Continual research is being conducted to validate these methods and the development of routine rapid, accurate, and cost-effective sentinel lymph node identification and early metastatic disease detection methods for clinical practice may be a reality in the not too distant future.

Prognosis

When an excised tissue sample is confirmed as primary melanoma, tumour staging is then performed in line with the American Joint Committee on Cancer (AJCC) system with the pTNM (primary-tumour-node-metastasis) classification system.^[37] A summary of the skin melanoma classification scheme is given in Table 2-2.

The depth of skin invasion of the primary melanoma, measured from the granular layer of the epidermis to the deepest point of invasion (a measurement referred to as Breslow depth of thickness), is accepted to be the most important prognostic feature of a primary melanoma.^[74] The tumour thickness of a primary melanoma can also be directly related to the risk of metastasis to lymph nodes. For example, a thin melanoma (< 0.75mm) has a 5-year survival rate of 95-99%,^[75] and possesses only a 5% risk of nodal involvement compared with a 40% risk for melanomas > 4.0mm thick.^[37] Evidence of ulceration is also

Table 2-2: The AJCC staging system for melanoma of the skin.^[71-73]

Staging Variable	Level				Further sub-classification	
T <i>Primary Tumour</i>	X Primary tumour cannot be assessed	0 No evidence of primary tumour	Is Melanoma <i>in situ</i> (has not spread to neighbouring tissue)	1 - 4 Size and/or extent of the primary tumour	T1 T2 - T4	a - without ulceration and mitoses < 1/mm ² b - with ulceration or mitoses ≥ 1/mm ² a - without ulceration b - with ulceration
N <i>Regional Lymph Nodes</i>	X Regional lymph nodes cannot be evaluated	0 No regional lymph node metastasis	1 - 3 Indicates the number (a range) of regional nodes involved		a - micrometastasis (diagnosed after sentinel lymph node biopsy) b - macrometastasis (clinically detectable nodal metastases confirmed pathologically) c - satellite or in-transit metastasis without nodal metastasis (for N2)	
M <i>Distant Metastasis</i>	X Distant metastasis cannot be assessed	0 No distant metastasis	1 Distant metastasis		a - metastasis to skin, subcutaneous tissues, or distant lymph nodes b - metastasis to lung c - metastasis to all other visceral sites or distant metastasis at any site associated with an elevated serum lactic dehydrogenase (LDH)	
Clark's level *	I Pre-invasive	II Thinly invasive	III - IV Moderately invasive	V Deeply invasive		

* Note, Clark's level is only used for the T1 (melanoma ≤1.0 mm in thickness) category. The various levels of classification are depicted in Figure 2-14.

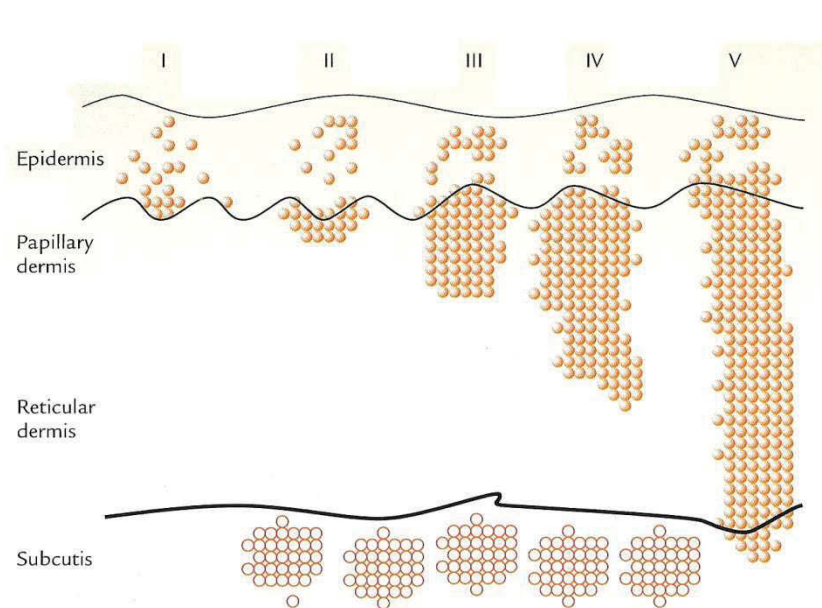


Figure 2-14: The Clark levels of invasion (from Cochran and Crotty).^[54]

a powerful survival predictor in patients with stages I, II and III melanoma, as is mitotic rate for patients with localised melanoma.^[73] For stage III melanomas, the number of metastatic nodes and tumour burden are also powerful survival predictors, as is the anatomic site of distant metastases in stage IV patients. Studies such as those conducted by Balch *et al* clearly show how radically survival rates may vary according to the melanoma TNM and staging category assigned and in turn how important correct melanoma staging will be to the treating physician as well as informing the patient.^[71,74] Table 2-3 provides a summary of the key prognostic factors for melanoma.

Once the staging component of the tumour analysis is completed by the pathologist, a report detailing the results is produced to assist the treating physician(s), along with the results of any further pathology testing, such as those obtained from immunohistochemical assays, to determine the prognosis, type of treatment to be employed, as well as survival

Table 2-3: Key prognostic factors in melanoma.^[37]

		Prognostic factor
Tumour-related	Key factors	TNM Stage Breslow thickness
	Generally considered to be of importance	Ulceration Mitotic rate Regression Clark's level (mainly of importance in thin melanoma) Lymphovascular invasion
	Of uncertain or disputed importance	Tumour infiltrating lymphocytes Growth phase Desmoplastic type
Host-related		Age Gender Site Raised serum LDH* Poor performance status*

* In stage IV disease

indicators. To be of greatest assistance to the treating physician, the pathologic report should contain detail similar to that displayed in Table 2-4.

2.1.9 Treatment

The survival rates of an individual diagnosed with melanoma depends highly on how early the cancer is detected, and therefore accurate diagnosis is critical in assisting with treatment and prolonging life expectancy. Treatment strategies vary according to the staging of the melanoma, and may range from a single procedure, such as performing a simple therapeutic excision, to a complex strategy that can include further surgery as well as chemotherapy and immunotherapy.^[3]

Table 2-4: An example list of the data that may be included in a pathological report on a melanoma.^[31,45,76]

Component	Description
Age	Patient age
Gender	Male / female
Site	Anatomical location of the melanoma
Macroscopic Description	Size of the excision specimen and the melanoma, as well as description of the melanoma (i.e. contour, borders, regression, pigmentation).
Diagnosis	What is the lesion, e.g. malignant melanoma.
Histological subtype	e.g. superficial spreading
Tumour thickness	Maximum tumour Breslow thickness (nearest 0.1 mm)
Ulceration	Present or absent; diameter measurement (mm)
Dermal mitotic rate	Mitotic rate per square mm
Level of invasion	Clark's measurement
Vascular / lymphatic invasion	Present or absent; evidence of tumour infiltrating lymphocytes (TILs)
Neurotropism	Is the melanoma affecting nervous tissue?
Desmoplasia	Signs of fibrotic tissue in the healthy tissue
Satellites	Presence or absence of microsatellite lesions
Regression features	Presence and extent
Predominant cell type	e.g. epithelioid, spindle, naevoid
Associated neavus	Description if present, e.g. dermal naevus
Completeness of excision / excision margin measurements (radicality)	Provides a measure of the distance of the invasive component of the tumour to the edge of the excised tissue in a number of dimensions (lateral and deep margins)
Growth phase	Horizontal, vertical, or both.

Wide local excision (WLE) is the standard treatment for primary melanoma and involves removal of the lesion as well as a margin of surrounding skin and subcutaneous tissue.^[37]

The goal of any excision is to remove not only the melanoma in situ, but also any invasive components, particularly at the periphery. Confirmation of sufficient tissue removal is performed by a pathologist, who undertakes a comprehensive histological examination of the excised tissue. RGP melanoma is generally cured using this approach as its spread is relatively localised to the epidermis and upper layer of the dermis. Local recurrences, satellites and in-transit metastases may also be treated by excision.

The minimum width of the radial excision margin taken is usually based on the maximum Breslow thickness of the primary melanoma and may range from 5 mm to 2 cm.^[37] A flap repair or skin graft may be required following excision due to the amount of tissue removed, and as with any wound, infection, swelling and scarring may result. In cases where the primary melanoma thickness exceeds 1 mm or possesses a Clark level of IV, SNB should be considered before the wider local excision to provide a rapid prognosis to the patient.

Following excision of a melanoma lesion, the risk of recurrence is highest in the first few years following its removal (particularly for thick lesions), with approximately 80% of recurrence occurring in the first three years.^[58] Hence, post-operative follow-up by treating physicians, long-term clinical follow-up (particularly in deep, but non-metastatic cutaneous melanoma patients), as well as regular self-examination, is essential for all patients following melanoma lesion excision, so that local-regional recurrences and nodal recurrence can be detected early.

Whilst most researchers agree that the pathological examination of SNBs provides valuable information for melanoma staging, the accuracy of SNB metastatic disease detection (particularly the rate of false positives) and its prognostic value is a point of concern for some investigators. Thomas suggests that the detection of melanoma deposits in a sentinel

lymph node does not necessarily indicate that an eventual progression into metastatic spread, and that some micrometastases may in fact remain dormant or possibly die.^[77] This in turn means that some patients may be undergoing what may possibly be an unnecessary immediate lymphadenectomy. Not surprisingly, the therapeutic value of SNB removal, along with complete regional node field dissection in patients where the SNB yielded evidence of micrometastatic melanoma, remains an area of considerable debate. However, in terms of the questioned therapeutic value of SNB removal alone, as Scoggins *et al* points out, the therapeutic benefit of other cancer staging modalities (e.g. CTs and MRI) is rarely, if ever, scrutinised.^[78]

The value of elective lymph node dissection (ELND), which is the removal of a lymph node area in the lymphatic drainage region of a tumour, in treating melanoma is still being investigated, particularly for lesions between 1 and 4 mm thick.^[45] While some studies have supported the validity of performing SLNB, some researchers argue that the data is insufficient and wide excision biopsies may be considered the better treatment option.^[79] CLND's therapeutic value is also a topic of debate. The rationale behind CLND is that by removing the regional lymph nodes, one may be eliminating the most likely site of early metastases.^[4] Common side effects of this procedure may include initially bruising, pain, and then potentially lymphedema (accumulation of lymph fluid in the tissue which causes swelling). Hence, the treating physician must weigh the possible survival benefit against the impact such a procedure may have on patient quality of life.

Post-operative irradiation therapy may be of use in a number of specific clinical situations for melanoma treatment, such as in the case where residual disease must be left in situ and further surgery may not be possible to remove it, or in regions of the body such as the head and neck where, due to the lesion's depth, the risk of local recurrence is considered high.^[45] Radiation therapy may also be employed as a palliative treatment

option.^[76] Unfortunately, melanoma cells are particularly resistant to radiation and show the ability to repair themselves following significant radiation damage.^[45]

Chemotherapy, using single-agent drugs such as dacarbazine (DTIC), cisplatin, nitrosoureas and vinca-alkaloids, generally only produce objective response rates of < 20%, but in some cases have been known to achieve long-term disease control and even a cure.^[19] In cases where there is a high statistical risk of relapse, even though all of the detectable melanoma at the time of diagnosis has been removed, adjuvant systemic therapy should be considered (e.g. AJCC stages IIC, IIB and IIC which possess < 50% ten-year survival).^[37] Interferon-alpha2b (IFN- α -2b) is the only drug which has demonstrated any efficacy, with one group claiming strong evidence of improved relapse-free survival time.^[80] Unfortunately the high doses necessary to be of benefit to the patient are also considered toxic (although the effects are rapidly reversible) and require that the patients be treated in an experienced medical oncology facility. Accordingly, patients at intermediate levels of risk (stages IIA, IIB and IIIA which has a 51 - 64% ten-year survival) should be considered only for clinical trials of adjuvant therapy, the benefits of which must be weighed against the impact of any potential side-effects.

Various combinations of multiple drugs have been examined in numerous clinical trials, as well as combining chemotherapy drugs with immunomodulatory or hormonal agents. Research into gene therapy and potential vaccines is ongoing and remains at the investigational stage. Evaluation of new approaches, such as protein kinase inhibitors, or agents that act on apoptotic mechanisms (targeting cellular events critical to tumour formation and progression), some in combination with chemotherapy, are attempting to address melanoma's resistance to conventional chemotherapy treatments.^[19,81]

Unfortunately, for patients with stage IV melanoma, the median survival rate is poor, with an estimated five-year survival of 5 - 10%, although these values will depend on a number of prognostic factors such as the extent of metastasis.^[37] One option, for some patients, is

to undergo further surgical resection. The resistance of melanoma to chemotherapy, radiotherapy and immunotherapy, attributed mainly to its resistance to induced-apoptosis, means that chemotherapeutic treatment is not highly successful.^[22] Hence, for patients with unfavourable prognostic metastases, the Clinical Practice Guidelines for the Management of Melanoma in Australia and New Zealand recommend that patients receive palliative treatments such as surgery, radiotherapy, steroids and attention from palliative care specialists.^[37]

2.1.10 Accuracy and Reliability of Diagnosis

In order to manage melanoma most effectively, it is critical that the disease is diagnosed early (tumour burden is at its least). Identification of the various cytological and architectural characteristics of malignant melanocytic and dysplastic tissue can, as mentioned earlier, be very difficult.^[58] Misdiagnosis of melanoma may be associated with disastrous outcomes such as unnecessary surgery, disfigurement and morbidity in the case of false positives (i.e. diagnosing a naevus as melanoma), delayed treatment and death in the case of false negatives, and expensive medical malpractice lawsuits as a result of either error.^[57]

Unfortunately, disease diagnosis based purely on a morphological analysis of tissues submitted for pathological examination is limited by its subjective nature. Discordance between expert pathologists is not uncommon, as was highlighted in a 1996 study published by Farmer *et al* in which the participating pathologists reached unanimous agreement as to the neoplasm classification (i.e. benign, malignant or indeterminate) of a number of “classic cases” only 30% of the time.^[82,83] However, it should be noted that this study did not employ an AJCC or similar staging system for tumour classification, involved only a small sample set (37 single sections, mounted on slides) and examination of the tissue appears to have been limited to visual techniques.

Although standardised staging systems and additional pathology diagnostic tools and techniques are continuously becoming available, the cost of medical malpractice to health care service providers continues to be of concern. Table 2-5 provides a sample of figures regarding civil litigated and unlitigated claims from an Australian organization specializing in medical legal defence. Whilst the majority of claims appear to be due to diagnosis failure by the consulted physician, there are also a number of claims arising from incorrect pathology reports. A number of American articles examining medical malpractice lawsuits related to surgical pathology have concluded that the most common reason for such cases is the alleged missed diagnosis of melanoma based on a skin biopsy specimen.^[84-86]

Table 2-5: Unlitigated and civil litigated claims - National Medical Defence Organisation (1990 - 2005)* ^[37]

Reason for Claim	Surgeons (8) [†]	Dermatologists (2)	GPs (44)
Total number of claims	7	2	38 [‡]
Failure to diagnose patient observed melanoma (delayed or missed)	3	1	32
Incorrect pathology report	2	1	5
Problems in surgical technique and treatment	2		3
Failure to inform patient of diagnosis			2
Failure to obtain pathology report			1
Delay in referral for definitive specialist treatment			1
* 47 claims in total - 32 litigated civil claims and 15 unlitigated matters † No claims for the period 2000-2005 ‡ Six GPs involved in one claim			

According to Troxel, commonly encountered false negative findings included the misdiagnosing of melanomas as Spitz nevi, dysplastic nevi, spindle cell squamous carcinoma, atypical fibroxanthoma and dermatofibroma.^[84,86] Additional errors introduced into pathology examinations also included the use of punch or shave biopsies (providing inadequate lesion sample and also incomplete excision), incorrectly interpreting melanomas with a lymphatic immune response as a nevus displaying chronic inflammation, as well as lymph node metastasis being misdiagnosed as malignant lymphoma.^[87] Aside from incorrect pathological diagnoses, false negative sentinel lymph node biopsies may also be a result of deficiencies in nuclear medicine or the surgical procedure.^[88] False positive results may be produced by incorrectly labelling as melanoma naevus cells, macrophages, or antigen-presenting interdigitating dendritic cells.^[49]

The use of immunohistochemical staining has assisted considerably with improving the accuracy of some aspects of melanoma diagnosis. In some situations it has been demonstrated that H&E sections cannot be used solely to differentiate all types of neoplastic cells within a lymph node tumour.^[87] Immunohistochemical staining, in conjunction with H&E and multiple level sections, can be of great assistance in not only differentiating neoplastic cells, but also assist in highlighting the presence of melanoma in a sentinel lymph node, even if only a single cell or a small cluster of cells are present.^[89,90] In contrast to the improved detection rates garnered in sentinel lymph node examinations from the use of immunohistochemical staining, there appears to be no diagnostic advantage over H&E when applied to samples from completion regional lymph node dissections.^[56]

In an ideal world, each tissue specimen obtained, be it a skin lesion or lymph node, should be examined by a pathologist with the necessary extensive experience in melanoma diagnosis using the most thorough examination processes available. However, as Scolyer *et al* point out, examining a tissue sample such as a lymph node in its entirety is impractical and prohibitively expensive, meaning that medical service providers must devise

histopathological protocols which balance cost, labour and time involved along with clinical value and accuracy.^[49]

It is clear from the above information that ongoing research into rapid and accurate melanoma detection techniques is desirable to assist pathologists in making their diagnoses, which will also enable treating physicians to quickly and efficiently deal with the ever increasing numbers of patients presenting with skin cancer in Australia. This in turn assists in reducing the financial burden on the health system as accurate, quick and sensitive diagnostic methods mean that the correct treatments may be sought in the most cost efficient manner and any potentially expensive litigation avoided.

2.1.11 Melanoma Imaging

Of considerable interest is the application of chemical imaging technology to pathology cases which are difficult to conclusively diagnose using standard histopathology techniques, such as samples that, whilst exhibiting a benign cellular morphology, may in fact possess chemical heterogeneity indicative of a particular cancer type or stage. Due to the diagnostic difficulties associated with the histological analysis of neoplasms for melanoma, in addition to other skin cancers, it is not surprising that researchers have turned to a variety of chemical imaging techniques.

Imaging technology also offers the possibility of automated sample analysis and a possible screening tool to assist in reducing pathology workloads. A review of some of the melanoma imaging techniques explored to date in melanoma and skin cancer studies is given below, with the first section reviewing a wide variety of imaging methods, and the second focusing purely on the technique of Fourier Transform Infrared Chemical Imaging (FTIR CI). Although there has been a considerable body of work investigating the use of *in vivo* detection methods, the literature reviewed below is for studies involving mainly tissue

sections which have been removed during biopsy for pathological analysis, as these are the same sample types that this research project is examining.

Non-FTIR CI Techniques

The employment of computer-aided instrumentation for the diagnosis of primary melanoma and other forms of skin cancer has led to the development of a number of techniques for use by clinicians and pathologists. Techniques such as epiluminescence microscopy image analysis (dermoscopy), *in vivo* confocal microscopy, Raman and near-infrared spectroscopy, fluorescence imaging/spectroscopy, positron emission tomography, computed tomography, magnetic resonance imaging, high frequency ultrasound, optical coherence tomography, multiphoton laser scanning microscopy and terahertz imaging have all been examined as possible computer-aided diagnosis tools to assist in pigmented skin lesion examination and tumour detection *in vivo* with varying levels of success.^[91-95] Whilst none of these methods has garnered sufficient data to demonstrate accuracy and reliability sufficient to replace the reference standard of biopsy followed by histopathologic assessment, some of these techniques are exhibiting great potential as supplemental clinical diagnostic methods, particularly for use by non-expert primary care physicians.^[96] However, as Binder and Menzies point out, the ability of any automated diagnostic system will always be restricted by the rates of histological diagnosis disagreement for early and invasive *in situ* melanomas, as it is by fundamental training with such 'histologically classified' reference samples that such systems are devised.^[91]

The application of Raman spectroscopy for melanoma and skin cancer diagnosis for both *in vitro* and *in vivo* tissue samples has been assessed in a number of studies over the past decade with some promising results. An optical method, Raman spectroscopy irradiates a sample with laser light which produces inelastic light scattering, the frequency shifts of which can then be analysed and recorded as a Raman spectrum. Raman spectroscopy is used to collect information on the vibrational and rotational information specific to the

chemical bonds present in molecules and is considered a complimentary technique to mid-infrared spectroscopy. Earlier studies established that Raman spectroscopy could be used to distinguish malignant from benign tissue, as well as melanoma from other skin tumours that are sometimes clinically confused with melanoma (e.g. pigmented nevi, BCC and seborrheic keratoses).^[97-100]

A study published by Nijssen in 2002 demonstrated that Raman imaging possesses high accuracy in discriminating between BCC and non-cancerous tissue in excised skin tissue sections.^[101] The spectroscopic and structural features of human skin melanocytes and keratinocytes, both from cultivated and thin skin biopsy sections, have been examined by Short *et al* to seek an understanding of the distribution of the numerous chemical components within a cell (i.e. DNA, RNA, lipids, melanin and proteins) and how this distribution varies between different cells. Raman imaging of cultivated human skin melanocytes and keratinocytes, along with skin biopsy thin sections, was performed by Short *et al* to examine the distribution of different molecular components in normal and malignant cells.^[102] Automation of the detection and imaging process of BCC in excised tissue sections (compared to healthy tissue) has also been explored by Larraona-Puy, providing excellent correlation with the corresponding H&E sections and good sensitivity (90±9%) and specificity values (85±9%).^[103]

Imaging employing ion microscopy is a technique which enables the collection of multiple ion spatial distributions across the sample's surface. At its most simple level, ion microscopy imaging employs an ionising device, which when coupled to a mass spectrometer and computerised instrumentation, is capable of separating the ions ejected from the sample following ionisation (based on their mass to charge ratio (m/z)) and simultaneously recording the topological information of their origin. An image is then constructed from the ion details recorded at each spatial location targeted for ionisation. Fartman *et al* in 2004 investigated the application of time-of-flight secondary ion mass

spectrometry (TOF-SIMS) and non-resonant laser secondary neutral mass spectrometry (NR-Laser-SNMS) to examine human melanoma cells to obtain both elemental and molecular images.^[104] The focus of this research was to gain an understanding of the intra- and intercellular boron distribution in tissue sections for the development purposes of a targeted tumour treatment (boron neutron capture therapy) with the technique displaying high sensitivity and subcellular resolution.

An ion microscopy imaging study published by Guerquin-Kern *et al* also in 2004, employed a newly-developed SIMS microprobe to examine melanoma cell colonies as well as mouse tumour tissue sections.^[105] Using the ion images produced, the technique confirmed the high affinity that *N*-(2-diethylaminoethyl)-4-iodobenzamide (I-BZA), a melanoma-targeting molecule agent used during scintigraphic exploration, has for melanin, and produced images capable of showing the spatial distribution of various ions within a cell as well as across a tissue section. A similar study, again demonstrating the tissue and cellular distribution of I-BZA using SIMS imaging, but this time more focused on the biological mechanisms involved in its cellular incorporation, also demonstrated the effectiveness of ion microscopy in the development and assessment phases of radiopharmaceutical research.^[106]

In terms of melanoma investigation and histopathological application, the techniques known as matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) and imaging laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) have also been examined. MALDI employs a laser to desorb and ionise molecules in a tissue sample which has been cocrystallised with a suitable matrix (usually a small aromatic molecule), enabling the collection of location-specific information on the proteins present in the tissue. Hardesty and Caprioli demonstrated the usefulness of MALDI ionising mass spectrometry as a tumour imaging tool by obtaining various images based on tumour-specific proteins to highlight the presence of metastatic melanoma cellular regions within human lymph node tissue.^[107] Imaging LA-ICP-MS utilises a laser beam of 10 - 200 µm diameter

which is fired onto the surface of the sample, vapourising part of the matrix. This vapour, along with ejected particles, is then carried to the ionisation source where it is rapidly desolvated, atomised and ionised and readied for separation by a quadrupole mass spectrometer. An imaging LA-ICP-MS study published by Hare *et al* in 2009 assessed the distribution of eight isotopes in the tissue: ^{31}P , ^{66}Zn , ^{13}C , $^{32,34}\text{S}$, $^{56,57}\text{Fe}$ and ^{63}Cu in a number of human lymph node thin sections which had been partially infiltrated by melanoma cells.^[108] By comparing the images obtained with corresponding H&E stained tissue, it was found that a decreased relative phosphorous concentration correlated with tumour presence and that tumour boundaries could be visualised using the $^{31}\text{P}^+/^{66}\text{Zn}^+$ ratio.

Fluorescence imaging is a technique that may either utilise the natural fluorescent properties of a sample or involve the addition of a fluorescent compound to a tissue sample in order to enable a fluorescent image to be captured from a tissue section. Valesky *et al* demonstrated the usefulness of fluorescence imaging for studying the effects of inhibiting expression of basic fibroblast growth factor and fibroblast growth factor receptor-1 in melanoma cells.^[109] The method employed in Valesky *et al*'s study involved treating excised primary melanoma tissue sections with either rhodamine stain or cyanine fluorochrome-labelled antibodies, and then imaging them using a combination of excitation and emission filters. Smith *et al* in 2003 also published a study which utilised fluorescent hybridisation signals to produce images of naevus tissue sections to examine the expression of various biochemical entities involved in the transcription factors STAT1 and STAT3 (proteins which control the rate at which DNA is copied from genes in a cell).^[110] The fluorescence images, taken at both the cellular level and across the entire nevus tissue surface, were thereby able to provide information on the cellular activity prior to and following treatment by low-doses of IFN- α in atypical naevi.

Aside from examining cellular processes in relation to development of melanoma-targeting treatments, fluorescence imaging has also been trialled more recently as a melanoma

demarcation tool in thick fresh skin excisions by Tannous *et al.*^[111] In this study, reflectance and fluorescence polarization imaging was employed to examine clinically suspicious pigmented lesions which had been excised for histopathological examination. To enable fluorescence imaging of the tissue, the samples were treated with either one of two exogenous chromophores, methylene blue or tetracycline, and then excited (using a Xenon arc lamp with bandpass filters) and observed over a variety of wavelengths. The technique successfully delineated the tumour margins of the non-melanoma skin cancers, and demonstrated potential for use as a rapid, intraoperative detection technique for the demarcation of dysplastic nevi and melanoma within surgically excised tissues.

FTIR CI Techniques

FTIR CI offers an extremely powerful analytical tool for the biologist in that, with the exception of a few homonuclear molecules, all molecular species absorb infrared radiation, and consequently it is possible to identify the various organic compounds of which many biological samples are comprised, as well as their spatial location in the sample. The motivation behind such research is the concept that all changes in tissue composition which are a result of disease are accompanied or preceded by biochemical changes, and that the chemical species involved in these biochemical changes may potentially be detected and identified using infrared analysis methods. Consequently, the innumerable biochemical components which comprise a biological tissue sample may be employed as a means by which the differing cellular components, such as varying cancerous cell types, are able to be identified and classified using FTIR chemical data. The information thus obtained may then be used to assist the pathologist in reaching his or her diagnosis, and subsequently provide the patient with a more accurate prognosis.

Existing research has shown that FTIR chemical imaging is capable of detecting subtle biochemical changes in biological samples, and has been employed for the discrimination and classification of a variety of cellular states in tissues and cells found in the breast,^{[112-}

^{117]} lung,^[118] oral cavity,^[119-122] lymph nodes,^[123-126] cervix,^[127-129] prostate,^[130-136] brain,^[137-142] esophagus,^[143] colon,^[144-148] liver,^[149,150] and even teeth.^[151] Based on the results achieved in such studies as those listed above, the application of infrared hyperspectral imaging analysis of biological tissues for evidence of melanoma possesses the potential to be an excellent pathological diagnostic tool and hopefully assist in improving the histological diagnostic concordance rate.

Prior to the development of imaging technology, infrared analysis of skin tissue by Wong *et al* had indicated that distinct spectroscopic patterns could be observed between BCC and normal skin.^[152] Further support for the use of infrared spectroscopy in melanoma research came from studies conducted by groups such as Skrebova *et al*, Mordechai *et al*, Hammody *et al* and Diem *et al*, who demonstrated such potential advantages as its ability to discriminate between various forms of skin cancer and to analyse the biochemistry of various cellular processes.^[127,153-156]

Studies on various skin tumour types have been conducted by McIntosh *et al* using single point spectra and mapping of biopsied tissue.^[157,158] In one study, the distinction of basal cell carcinoma, squamous cell carcinoma and melanocytic lesions was achieved using linear discriminant analyses, with spectral separation of abnormal versus normal tissues being achieved with high sensitivity and specificity. An area of difficulty encountered was the differentiation of the various tumour types from normal epidermis, and highlighted the need for the removal of subjectivity in spectral interpretation. Simple superimposition of normalised spectra of the tumour and epidermal tissue was found to lead to erroneous interpretation of the spectra obtained, and hence emphasised the need to employ more sophisticated data analysis techniques.^[159]

Lasch and Naumann have conducted studies on biopsy tissue thin sections such as that from human melanoma primary lesions.^[160,161] In one study, Lasch and Naumann performed spectroscopic mapping of freeze-dried, thin tissue sections mounted on calcium fluoride

windows, obtaining up to 15,000 spectra per mapping experiment. The images obtained were subsequently examined using functional group mapping and a combination of chemometric techniques such as cluster analysis, principal component analysis and artificial neural networks. It was found that the functional group mapping was unable to reliably differentiate between different tissue regions within the sample. Nevertheless, the employment of the more sophisticated pattern recognition techniques produced images with good contrast that confirmed the tissue changes observed by standard histopathological analysis of the same sample. This research also demonstrated the potential ability for FTIR CI to increase image contrast, particularly between regions of normal and cancerous cell growth.

The examination of archival tissue is of great significance in cancer studies, as often retrospective analysis of tissue is required.^[162] Archival tissue stores are also an excellent source of case study tissue of known histopathological grading, and therefore more recent research has been examining the impact of retaining the paraffin in tissue sections to remove the need for an additional sample treatment step (and possible further chemical alteration). A study published by Tfyali *et al* in 2005 demonstrated that good discrimination could be achieved between naevus and primary melanoma tissue sections which remained embedded in paraffin using spectral regions known to be free of paraffin contribution.^[163] More recent FTIR CI studies conducted by Ly *et al* have also examined non-melanoma skin cancer (BCC, SCC and Bowen's disease) sections without the prior removal of paraffin.^[148,164] In these studies, the paraffin component of the tissue section was removed from the collected data using a technique known as extended multiplicative signal correction. The research also examined the use of a variety of chemometric techniques, including PCA, LDA and genetic algorithms for tissue differentiation, with the results indicating that paraffin-embedded tissue may not require any chemical pre-treatment prior to imaging.

In a study by Chew *et al*/ human leukocyte antigen (HLA) class I expression was assessed in metastatic tumour sections using FTIR CI as an alternative to immunohistochemical staining.^[165] This research discovered that FTIR CI was capable of differentiating HLA-class-I-positive and -negative areas without the need for antibody attachment. FTIR CI has also been employed in skin cancer research by Acerbo and Miller to assist in gathering information regarding the role of boric acid (BA) in melanoma cell growth and proliferation.^[166]

An alternative approach to melanoma research utilising infrared imaging has been proposed using engineered tissue.^[167] In this research, which followed on from an earlier study by Bhargava and Kong^[168], an engineered tissue model of melanoma was constructed so that the authors could examine the process of tumour progression in this particular form of cancer in a controlled environment. The study was comprised of two stages: the first to characterise the biochemical properties and spectral variation of the differing skin layers, and the second to examine the simulated tumour formation and growth after the introduction of malignant melanocytes to the tissue material. Of particular interest to this research group was to investigate the role of stromal cells in tumour formation and progression. Stromal cells are a type of connective tissue cell whose role it is to release growth factors and promote cell division in organ tissue. In the case of human skin, stromal cells are found at the base of the epidermis, and it is their interaction with melanocytes (which are themselves located at the epidermal-dermal junction) which is of particular interest. By using comparative spectral profiles of the tissue of the tumour mass, stroma (the supportive tissue formed from a number of stromal cells) located close to the tumour (10 - 100 μm) and from further afield (at least 500 μm away), the authors were able to demonstrate that the stroma underwent biochemical remodeling within 50-100 μm of the tumour mass. This study clearly exhibited the potential role FTIR offers in the study of stromal-epithelial interactions in tumour growth, as well as the valuable research and clinical information that engineered tissue studies combined with FTIR-imaging offers.

2.1.12 Data Analysis and Classification Automation

Due to the natural complexity of tissue chemistry and the fact that the infrared data obtained from chemical imaging techniques of whole tissue sections is comprised of numerous infrared absorbing species, it is difficult to assign changes in cellular structure to any one particular frequency (Table 2-6). Differences in ratios between peak areas have proven useful in some instances,^[126] however a more sophisticated and more objective analysis technique is desirable.

The combination of unsupervised cluster analysis techniques with supervised pattern recognition methods not only permits the exploration and objective grouping of the different tissue structure spectral features within a sample, but also the comparison of different tissue samples which may possess multiple pathologies (e.g. differing neoplastic classifications). Whilst methods such as HCA are truly objective imaging techniques in that no prior knowledge of the sample composition is required, the computational requirements for the calculations are high, particularly when one considers that each image will be comprised of thousands of pixels, each with its own spectrum of numerous data points. In addition to this, the number of clusters chosen for display is usually obtained after comparison of the clustered image with that of an H&E stained section. Further comparison of the spectral characteristic of each cluster must then be performed to assess what each cluster represents in terms of the tissue composition.

Subsequent automation of the identification of tumour-bearing regions of tissue samples will require the employment of pattern recognition techniques. Pattern recognition is based on the same fundamental principles of pathological tissue analysis, the so-called "gold standard". Put simply, a sample of unknown tissue quality is examined in comparison with a library of known cell or tissue classification. In terms of spectral imaging, computerised pattern recognition techniques process the test sample image by comparing the spectrum from each pixel against a reference spectral data set of known histopathological diagnosis. What varies

Table 2-6: An example of the large number of IR spectral features that are assigned to skin, obtained from Kong *et al.*^[167]

Mode	Origin	Literature (cm ⁻¹)	Human skin (cm ⁻¹)	
			Epidermis	Dermis
Amide A (N-H stretching)	Peptide, protein	3300	3298	3298
Amide B (N-H stretching)	Peptide, protein	3080	3078	3078
C-H stretching of alkenes	Phospholipid, glycolipid, fatty acid, ceramide, triglyceride	3010	3010	3010
Asymmetric CH ₃ stretching	Phospholipid, glycolipid, fatty acid, ceramide, triglyceride	2957	2960	2960
Asymmetric CH ₂ stretching	Phospholipid, glycolipid, fatty acid, ceramide, triglyceride	2920	2920	2920
Symmetric CH ₃ stretching	Phospholipid, glycolipid, fatty acid, ceramide, triglyceride	2872	2868	2868
Symmetric CH ₂ stretching	Phospholipid, glycolipid, fatty acid, ceramide, triglyceride	2851	2850	2850
CO stretch	Phospholipid	1738	1740	1740
Amide I (80% CO stretch)	Peptide, protein, ceramide	1655	1654	1654
Amide II (60% N-H in plane bend, 40% C-N stretch)	Peptide, protein, ceramide	1545	1548	1548
CH ₂ scissoring	Phospholipid, glycolipid, fatty acid, triglyceride	1468	1468	1468
CO stretch of COO ⁻	Fatty acid	~1395	1400	1400
CH ₃ symmetric bend	Phospholipid, fatty acid, triglyceride	1378	1384	1384
CH ₂ wagging	Phospholipid, fatty acid, triglyceride	1343-1370	1344	1338
Amide III (40% C-N stretch, 30% N-H in plane bend, 20% methyl-C stretch)	Collagen	1283	1284	1284
Asymmetric PO ₂ ⁻ stretch	Phosphate salt, nucleic acid, DNA, RNA	~1245	1242	1240
Amide III (40% C-N stretch, 30% N-H in plane bend, 20% methyl-C stretch)	Collagen	1236	1236	1234
Asymmetric PO ₂ ⁻ stretch	Phosphate salt, nucleic acid, DNA, RNA	1223	1224	1226
Amide III (40% C-N stretch, 30% N-H in plane bend, 20% methyl-C stretch)	Collagen	1204	1204	1204
Ester C-O asymmetric stretch	Ester	1170	1170	1168
Symmetric PO ₂ ⁻ stretch	Phosphate salt, nucleic acid, DNA, RNA	~1080	1082	1082
C-OP stretch	Phosphate ester	1047	1056	1058

from one pattern recognition method to the next will be the mathematical ‘matching’ process, or algorithm, used. In the analysis of lymphatic tissue using FTIR CI, the suitability of the pattern recognition method is then simply tested by comparing the results of the automated method on a number of ‘test’ tissue samples which have also been analysed using current standard diagnostic techniques.

An early study conducted by Lasch *et al* demonstrated the effectiveness of combining unsupervised and supervised classification methods by employing PCA to define the training and validation data sets for an ANN classifier of human melanoma thin sections.^[161] A recent study conducted by Tosi *et al* has also successfully employed HCA combined with PCA to examine the spectral changes of melanocytes within the infrared images of deparaffinised samples of ordinary dermal and Reed nevi, dysplastic and malignant skin lesions, enabling good discrimination between the various lesion types.^[174]

A similar hyphenated chemometric approach for the automated assessment of disease state in human breast cells was performed by Zhang *et al* using a combination of *K*-means clustering and ANN.^[115] Ly *et al* examined the application of *K*-means clustering, as well as data reduction using principal components analysis, to assist in the development of an automated LDA discrimination method for BCC, SCC and Bowen’s disease tissue samples.^[164] Bird *et al* demonstrated the effectiveness of performing hierarchical clustering analysis to select suitable characteristic spectral features which could then be used for the building of a diagnostic algorithm (utilizing artificial neural networks) to differentiate benign and malignant tissues within axillary lymph nodes.^[175] The combination of *K*-means clustering for reference spectra selection followed by image classification using SIMCA has also been explored for the identification of primary tumours (i.e. the origin) of brain metastases with encouraging results.^[139] It is clearly apparent from these examples that the combination of unsupervised and supervised classification methods merits further investigation when attempting any FTIR chemical imaging.

Biological tissues are by their very nature highly complex, and comprised of many different cell types, the interaction between which is mediated by various chemical pathways characterised by numerous chemical species. Infrared spectroscopy researchers such as Diem *et al* ^[176] stress the necessity of understanding which spectral features are characteristic of particular cells, and for the spectroscopist to realise that differentiation, maturation and natural stages in the cell cycle may also contribute to spectral variation, the awareness of which is critical for accurate spectral interpretation. Spectroscopic variation between samples from the same tissue donor, as well as natural cell abnormalities present in healthy tissue, are yet further confounding variables which must be anticipated by the spectroscopist. Thus, minimizing variation and contamination due to sample preparation is of utmost importance.

The ideal for routine pathological analysis will involve the construction of an automated analysis scheme, relying on human interaction only for the initiation of program sequences to produce a completely objective classification result for comparison with the diagnosis obtained by the pathologist using standard histological examination techniques.

Based on the discussion provided above, the perceived advantages and disadvantages of employing infrared chemical imaging for melanoma diagnosis in both skin and lymphatic tissue are as follows:

Advantages of using FTIR CI for melanoma diagnosis:

- Non-destructive to mounted tissue sections
- Nil requirement for human interpretation of results (ideally)
- May be developed to examine numerous types of different tissues
- One preparation method and minimal chemicals required
- Samples are stable and easily archived
- Method could potentially be validated and optimised

- Archival tissue may be examined
- Automation potential (time-saving and less labour-intensive)

Disadvantages of using FTIR CI for melanoma diagnosis:

- Outcome could highly depend on tissue preparation method
- Requires access to expensive instrumentation
- Requires database establishment and access to chemometric software
- Current instrumentation and image capture capability slow (particularly if high spatial and spectral resolution images are required)
- A large library of tissue samples may be required due to the high variability observed within the diseased tissue and between donors

To date there have been minimal to no published examples of routine employment of FTIR CI for the classification of human melanoma lesion and lymph node tissue in a clinical pathology setting. A number of excellent articles published by Bhargava *et al* and Fernandez *et al* have examined the acquisition, classification and validation issues integral to the establishment of practical protocols for automated histological assessment.^[130,132,177,178] Fundamentally, infrared imaging must offer a comparable level of quality, efficiency and reliability when compared to the “gold standard” of trained human pathological examination of stained tissue sections. It is the first steps towards this goal that the following report attempts to address.

2.1.13 Project Research Goals

The purpose of this research was to apply this technology to the study of melanoma in lymph nodes and skin lesions using paraffin-embedded archival tissue, and to determine if infrared hyperspectral imaging (using a combination of hierarchical clustering analysis and the spectral angle measure classification algorithm) may be of assistance to pathologists in cancer detection and staging.

Besides offering an automated classification method to distinguish healthy from malignant tissue, it is hoped that FTIR chemical imaging may be used to assist pathologists in diagnosing tissue samples that show slightly ambiguous morphology, as it is known that biochemical changes often precede visible morphological changes in disease-affected tissue. Hence, this preliminary research attempted to address the following:

- Step 1** To determine if FTIR CI can distinguish naevus from primary melanoma lesions, as well as healthy lymph node tissue from melanoma metastatic material, in clearly defined melanoma cases; and
- Step 2** Identify a possible hyperspectral classification algorithm with the potential future capability of performing automated (and therefore objective), computerised recognition of melanoma using FTIR chemical images of excised skin lesions and lymph nodes.

Step 1 was performed using an unsupervised multivariate chemometric technique. Step 2 then examined the employment of automated pattern recognition approaches to determine the most appropriate method of image classification. The final computerised images were then compared against corresponding H&E stained slides of a serial section of the same tissue specimen.

Although there has been considerable research conducted on a variety of cancer types in a large number of tissues, the examination of melanoma in lymph node tissue using FTIR chemical imaging has yet to be comprehensively addressed. Whilst the application of infrared chemical imaging to skin lesions is not new, the employment of the spectral angle measure as an image classification algorithm for this type of tissue is, with a growing body of literature providing evidence of its usefulness for biological material study^[179-183] and even tumorous tissue discrimination^[184]. Also exploratory was the attempt to develop a classification protocol suitable for both skin and lymph node tissue types, and the combined investigation into the spectral features of melanoma in both skin and lymphatic tissue.

2.2 EXPERIMENTAL METHOD

2.2.1 Ethics Approval

The collection of human skin and lymph node tissue was performed during routine surgical procedures and archived at the Department of Anatomical Pathology based at the Royal Prince Alfred Hospital (RPAH), Camperdown, Australia. Ethical clearance for access to and sampling of archival tissue samples was provided by the Ethics Review Committee of the Central Sydney Area Health Service, RPAH Zone (protocol number X04-0183). External ratification of this human ethics protocol was provided by the University of Technology, Sydney (UTS) Human Research Ethics Committee (approval number UTS HREC 2004-138A).

Archival tissue was selected by representatives of the Sydney Melanoma Unit based on the presence of histopathological features required for this study. Tissue blocks were sectioned on the premises of the RPAH. Nil identifiers were recorded with the slides, aside from an archive reference number which could only be cross-referenced against the Anatomical Pathology department database by RPAH staff. As a consequence, the patients from whom the tissues originated remained anonymous to those conducting the imaging at UTS.

2.2.2 Sample Selection and Preparation

Human lymph node and skin tissue sections (5 - 6 μm thick) were obtained from paraffin-embedded tissue blocks and mounted on infrared reflective glass microscope slides (Low-e Microscope Slides, Kevley Technologies). In the early stages of the study, tissues were deparaffinised prior to release to the researchers at UTS. However, additional sections with the paraffin still embedded in the tissue were obtained at a later date.

A total of 22 lymph nodes, 8 primary melanoma and 10 naevus lesions were obtained (see Table 2-7 for a break-down of the sample details).

Table 2-7: Tissue samples employed in this study.

Tissue	Number of individual nodes/lesions	Number of source tissue blocks	Number initially paraffin embedded
Primary Melanoma Lesion	8	5	8
Naevus Lesion	10	4	10
Lymph Node with melanoma metastasis	13	9	8
Normal Lymph Node	9	5	9

The histological status of each sample had been determined prior to block selection by a pathologist specialising in melanoma diagnosis. For each tissue section obtained, a corresponding serial section was also cut, mounted on a standard glass microscope slide, de-paraffinised and stained with haematoxylin-eosin (H&E) for later comparison with the chemical images obtained.

Due to concerns about the effect that paraffin retained in the tissue may have on the chemical information contained within the tissue, one component of this study examined the effects of paraffin-embedding and its subsequent removal on the FTIR chemical image classification results. Once the images for the samples received with paraffin were obtained, the tissue samples were then deparaffinised (using an initial wash with xylol followed by a series of progressively more diluted alcohol aqueous solutions ranging from 100 - 70% ethanol concentration) and re-imaged. Confirmation of paraffin removal was performed by obtaining infrared spectra from the slide surface in regions devoid of tissue, as well as at a number of points containing tissue and checking that the characteristic strong peaks of paraffin at 1450 - 1465, 2840 - 2850 and 2910 - 2915 cm^{-1} could not be observed in the tissue spectra.

All of the tissue slides were stored in a dessicator in a temperature-controlled laboratory prior to and following their use for the duration of the study.

2.2.3 Instrument and Image Collection Parameters

The samples were examined using a Digilab Stingray IR Imaging System comprised of an FTS 7000 FTIR spectrometer, UMA 600 IR microscope (S/N 010-0232-0092), Lancer 64 x 64 FPA detector (S/N 02-2134), with instrument control and data collection performed using the Digilab Win-IR Pro (version 3.4.0, Digilab, Massachusetts, USA) and Resolutions Pro (version 5.1.0.829, Varian, Inc.) software. A background image for each sample was collected prior to measurement using a blank infrared reflective glass microscope slide. All images were collected using a nominated spectral range of 900 - 4000 cm^{-1} with a spectral resolution of 4 cm^{-1} . 32 scans were co-added to form each spectrum and an undersampling ratio value of 2 was employed.

Sample image sizes varied according to the type of tissue being examined. For example, naevus and primary melanoma tissue tended to only require imaging of the epidermis and upper dermal layers of the skin (a relatively small area), whereas the lymph nodes varied considerably in size (millimetres to over a square centimetre in area). The majority of samples were imaged at a maximum 16 x 16 tiled images (256 tiles in total), with a value of 4 for pixel aggregation (to assist in reducing file sizes), creating a 128 x 128 (approximately 1.4 x 1.4 mm) image.

Unfortunately, limitations of the computer memory required that larger physical dimension files (i.e. file sizes greater than 1 GB of data) be collected in a multi-file format. In this process, each tile is saved as an individual file and the final image “stitched” together in the Win-IR software program.

2.2.4 Data Processing

Pre-processing of image data

All data pre-processing and unsupervised classification was performed using the multivariate imaging software program CytoSpec (version 1.4.02, build 00327b, CytoSpec Inc., New York, USA). Although smaller image files could be directly imported into CytoSpec from Win-IR, the larger multi-file images required conversion into a single ASCII file, which was performed using ENVI (version 4.7, ITT Visual Information Solutions, Boulder, USA). Prior to importing the infrared images into CytoSpec, the files were first imported into Microsoft Office Excel 2007 (Microsoft Corporation, California, USA), where random noise spikes could be removed from the data (using an averaging method). Wavenumber labelling was then performed to obtain a file which conformed to the importing requirements of CytoSpec.

Prior to chemometric analysis, the images were subjected to a number of data pre-processing steps in CytoSpec consisting of spatial subsetting (to reduce the file size and remove void space), quality testing (thickness testing to remove areas of bare slide), derivatisation (second derivative with fifteen smoothing points), vector normalisation and spectral truncation (cut to 1000 - 3100 cm^{-1}). The aforementioned measurement and data-processing parameters were selected based upon information obtained from literature references examining melanoma, skin and lymphatic tissue and employing similar infrared instrumentation, as well as by performing a number of smaller preliminary sample classification studies.

Image classification

Data processing was performed in two stages:

Stage 1: Unsupervised classification and selection of reference spectra

This first stage involved identification of suitable spectra representative of the known cell/tissue types present in the samples by means of an unsupervised classification method. Hierarchical cluster analysis (HCA) was performed on each sample using D-values and Ward's algorithm over two spectral regions: 1000 - 1750 cm^{-1} and 2800 - 3100 cm^{-1} . HCA groups spectra based on spectral features alone, and does not require any *a priori* information to be provided by the spectroscopist. This chemometric technique analyses spectral data for similarities, such that spectra with n wavelengths are represented as single points in n -dimensional space; the distance in this space between each pair of spectra is then calculated. Numerous small groups of 'similar' spectra are formed, with the algorithm gradually merging the groups based on similarity until only one group, or cluster, containing all of the spectra remains. A chemical image can then be 'coloured' to show the regions of the tissue sample determined as similar or different according to the clustering performed. Hence, the demonstration of two clusters - the simplest sorting of the component spectra into two groups - would be given as only two colour tones in the image.

The number of clusters employed is chosen by the spectroscopist, as well as the method of distance calculation. Comparison of the cluster images with those obtained using standard histological staining protocols may assist in the assessment of the clustering method chosen. The final clustered images are compared to the corresponding H&E stained tissue sections to assess the quality of tissue type differentiation and to select representative, or 'reference' spectra, to be used in the second stage of data analysis (supervised classification).

The reference data to be used for the subsequent supervised classification stage was obtained using a variety of methods, such as obtaining the average spectrum of the pixels selected for each cluster region selected, or manually selecting pixels from an imaged sample based on the corresponding H&E slide and the identified clusters, and where possible not including pixels near cluster borders.

Stage 2: Supervised classification

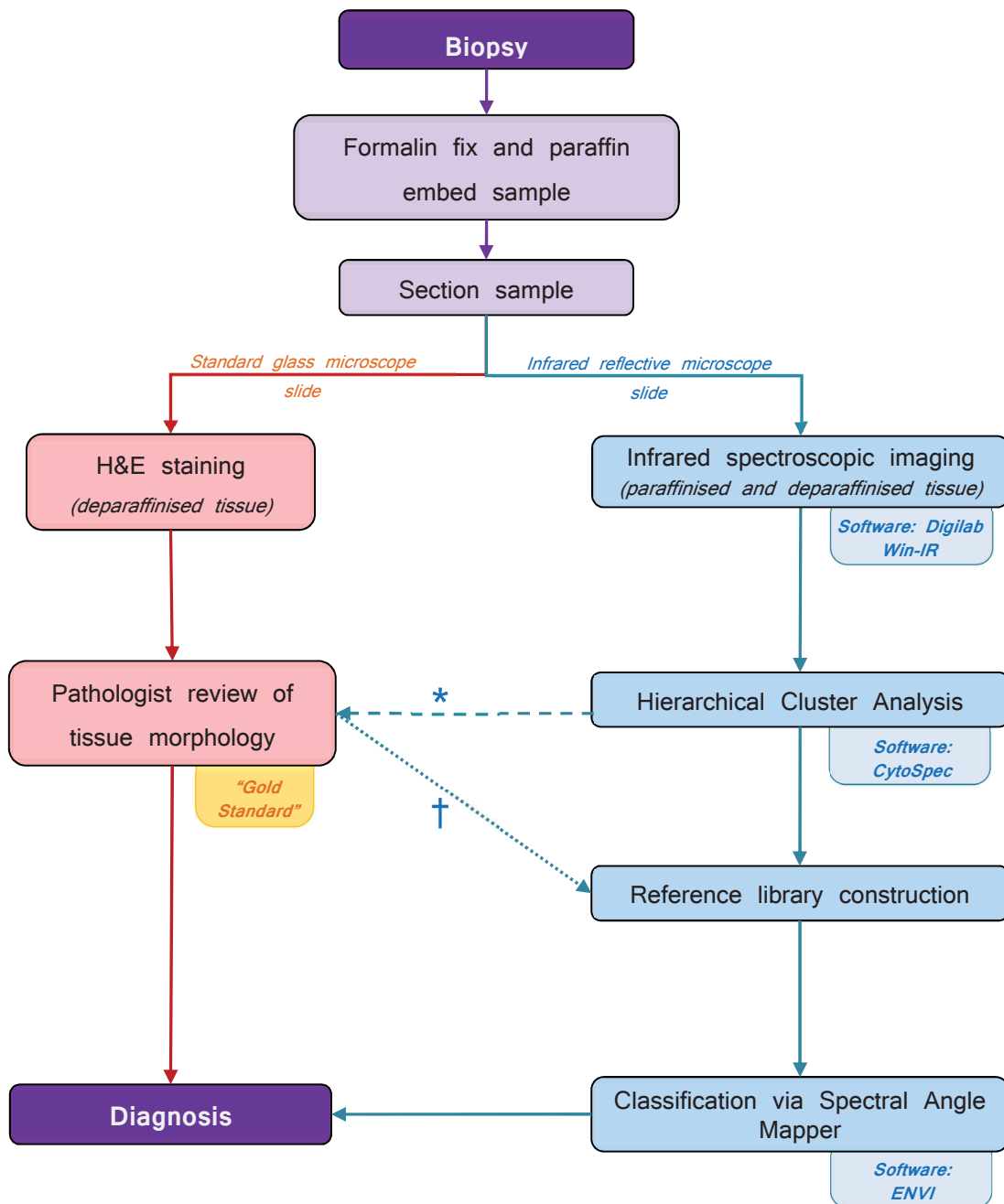
Prior to performing any supervised classification, the images to be examined were subjected to the same data pre-processing steps in CytoSpec. In this second stage of data processing, the selected reference spectra were imported into ENVI and the images classified using the Spectral Angle Mapper (SAM) algorithm. When applying SAM to a sample, the user is prompted to select the maximum angle value at which two spectra will be considered a match (effectively a 'cut off' value or tolerance level). Therefore, the maximum angle selected must balance the likelihood of false-positive (too large an angle of tolerance) and false-negative values (too small an angle of tolerance). In terms of imaging, the result is displayed as a false-colour map, with the user selecting the colour to be used to depict the reference spectra groups (e.g. red for cancer, green for healthy tissue, etc.).

As the computer is only comparing each pixel against a library of spectra (which is often comprised of fewer spectra than in the sample itself), the processing time required to classify each sample is significantly less than in the HCA technique. The drawback with this imaging method is that in cases where the spectrum at a pixel shows qualitative properties from a mixture of spectra (e.g. both healthy and abnormal tissue), the mapper is only able to depict one value for that pixel, increasing the possibility of a false-positive or negative outcome, and hence the classifications obtained are significantly affected by the quality of the reference spectral library employed. However, construction of a reference library of spectra from a wide variety of tissues of known composition will alleviate this problem somewhat.

After classification, the false-colour tissue images were then compared to their corresponding H&E slides and the accuracy of the various reference spectra selection methods and classification method parameters assessed. Based on the results obtained, the optimum spectral reference library construction method and pattern-recognition parameters were then defined.

Any additional chemometric techniques employed for the above tissue image analysis (e.g. the need for paraffin removal digitally, additional classification techniques attempted, etc.) will be further expanded upon, where required, in the results section of this thesis.

A summary of the analysis approach taken in this study is given in Figure 2-15.



* The number of clusters used will be determined after comparison of the HCA images with the stained comparison with the H&E stained corresponding sections.

† The spectra employed to build the reference spectra library will be collected in a variety of ways to assess the impact of spectra from boundary regions.

Figure 2-15: Flow chart representing the analysis approach taken for the infrared imaging of tissues for melanoma diagnosis (adapted from Bird *et al*).^[175]

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CHAPTER 3:

MELANOMA STUDY PART 2

(RESULTS AND DISCUSSION)

3.1 RESULTS

3.1.1 Tissue Sections - Visible Features

Although a pathologist had already diagnosed the tissue status of each archival sample provided in this study, assistance was required (for those samples listed as diseased) to identify the regions of the tissue sections exhibiting the cellular / structural features indicative of melanoma diagnosis (cellular and nuclear pleomorphism, nuclear hyperchromatism and abnormally increased mitotic activity).^[1,2] While the macroscopic and histopathologic features of an excision specimen are used for diagnosis, it must be noted that none of the individual features alone (e.g. cytologic parameters, tissue architectural features, etc.) can be considered diagnostic as they can often be observed in melanomas as well as benign naevi.^[3]

To address the need for tissue pathology identification, instruction from a pathologist was given in a variety of forms including the marking of corresponding H&E stained sections to indicate the cancerous regions (Figure 3-1), as well as visual demonstration using a light microscope. This in turn would permit the correct targeting of areas suitable for spectral library construction as well as determine if the images were correctly classifying for the test samples.



Figure 3-1: An example of an H&E-stained serial section of a lymph node provided by the Sydney Melanoma Unit. The black markings were made by a pathologist on the slide to indicate the tumour and 'normal' lymph node (LN) regions.

Examples of the various tissue states analysed in this study have been given in Figure 3-2 to Figure 3-6. As may be observed in the images, the distinct tissue and cellular differences characteristic of the primary melanoma and metastatic disease states are not so easily identifiable to the untrained eye. Also, the images clearly demonstrate the variety of cellular types (e.g. melanocyte, naevus, melanoma and Langerhan cells in the skin tissue sections; reticular, melanoma and lymphocyte cells in the lymph nodes) and structures that may be found within each tissue section (e.g. blood vessels, epidermis, dermis, connective tissue in the skin sections; capsule, cortex, medulla and hilum in the lymph nodes).

In the cases of the tissue samples exhibiting both normal and malignant pathology, the latter samples exhibited clear regions of cellular structure consistent with the diseased state, making the tumour regions relatively straight-forward to identify. In addition to this, it should be noted that the cancerous regions may be interspersed with cells exhibiting normal morphology, particularly at the border regions of a growing cancerous mass, and hence the concept of identifying and selecting “pure” melanoma regions for spectral sampling may in fact be unachievable in reality. Consequently, multiple regions within each sample and across multiple samples were targeted for spectral library construction in an effort to minimise the impact of potentially ‘mixed’ spectral data.

3.1.2 Image Collection

A standard infrared image tile obtained using the Stingray instrument has dimensions approximately 350 x 350 μm . If one utilises the EFOV option, these dimensions increase to approximately 700 x 700 μm . Each single image tile possesses 64 x 64 pixels, producing a theoretical pixel size of 5.5 x 5.5 μm in standard view and 10.9 x 10.9 μm for the EFOV. It must be noted that these values are nominal as each array element does not perfectly capture such an area due to manufacturing and physical (i.e. diffraction) limitations.

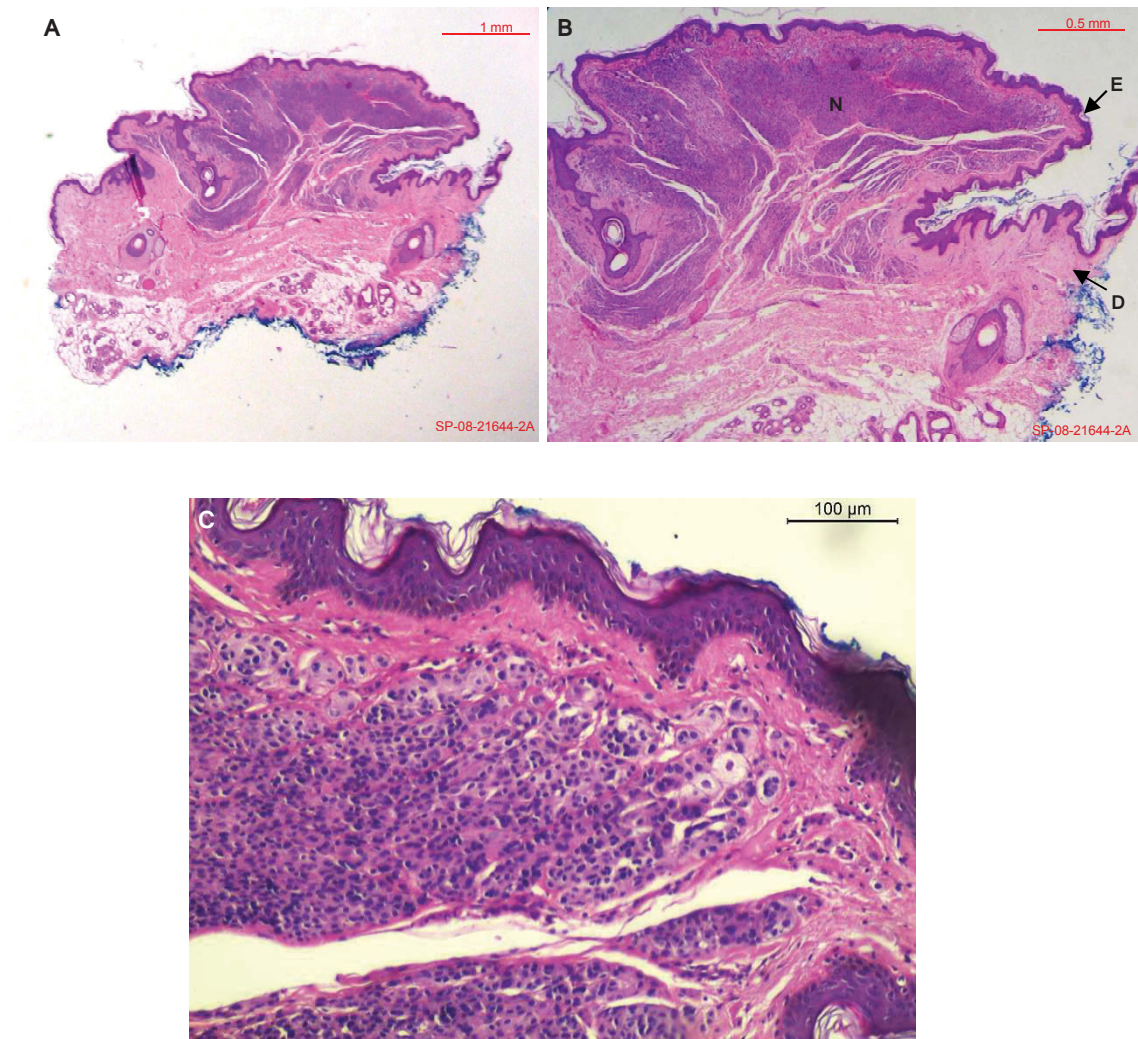


Figure 3-2: (A) An example H&E stained section of naevus human skin tissue at x1.6 objective magnification. (B) A magnified region of the naevus tissue displaying the dense accumulation of pigmented melanin cells at x4.0 objective magnification. The epidermis E, dermis D and the naevus growth N are all clearly visible. (C) A closer view of the naevus growth displaying the dense accumulation of pigmented melanin cells at x10 objective magnification.

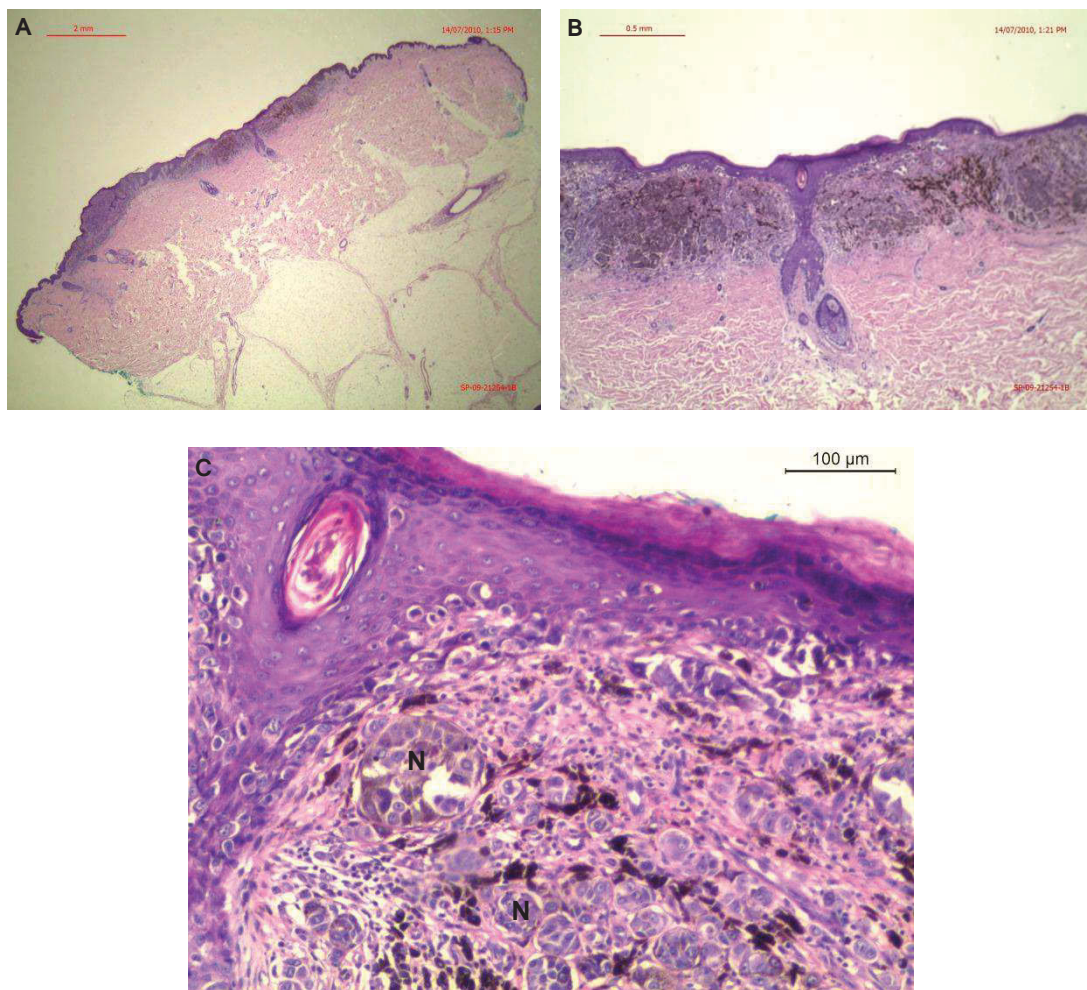


Figure 3-3: (A) An example H&E stained section of a human skin tissue section exhibiting primary melanoma at x5 objective magnification. (B) A closer view of a region of the same tissue sample displaying melanoma cell growth. (C) A magnified (x100 objective) region of the same tissue sample. Note the nests of atypical melanocytes **N** occupying all levels of the epidermis which are considered typical of superficial spreading melanoma.

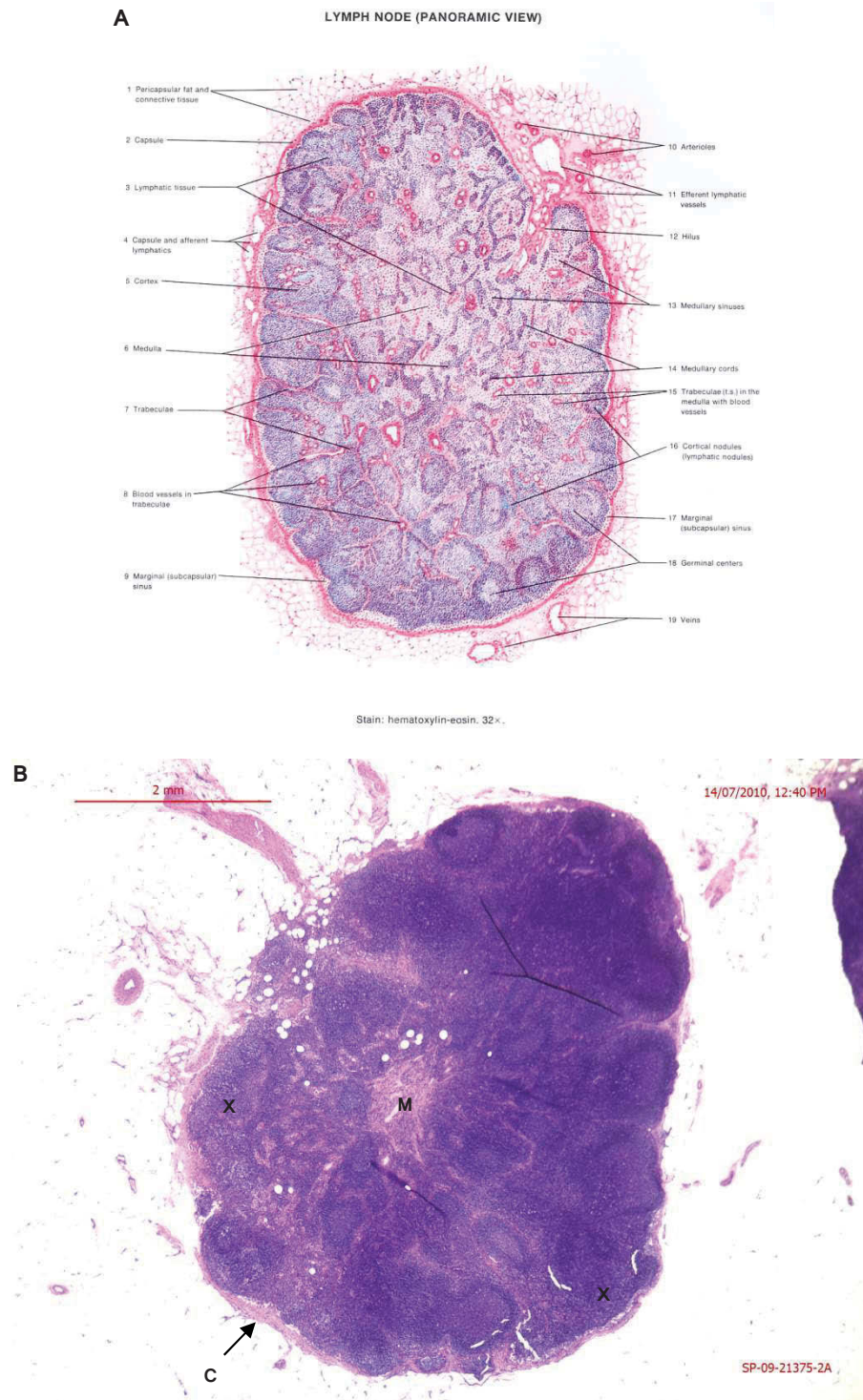


Figure 3-4: (A) An example of a normal lymph node from Fiore.^[4] (B) A x5 objective magnification of an H&E stained section of a normal human lymph node section used in this study. The capsule C, cortex X and the medulla M are all clearly visible.

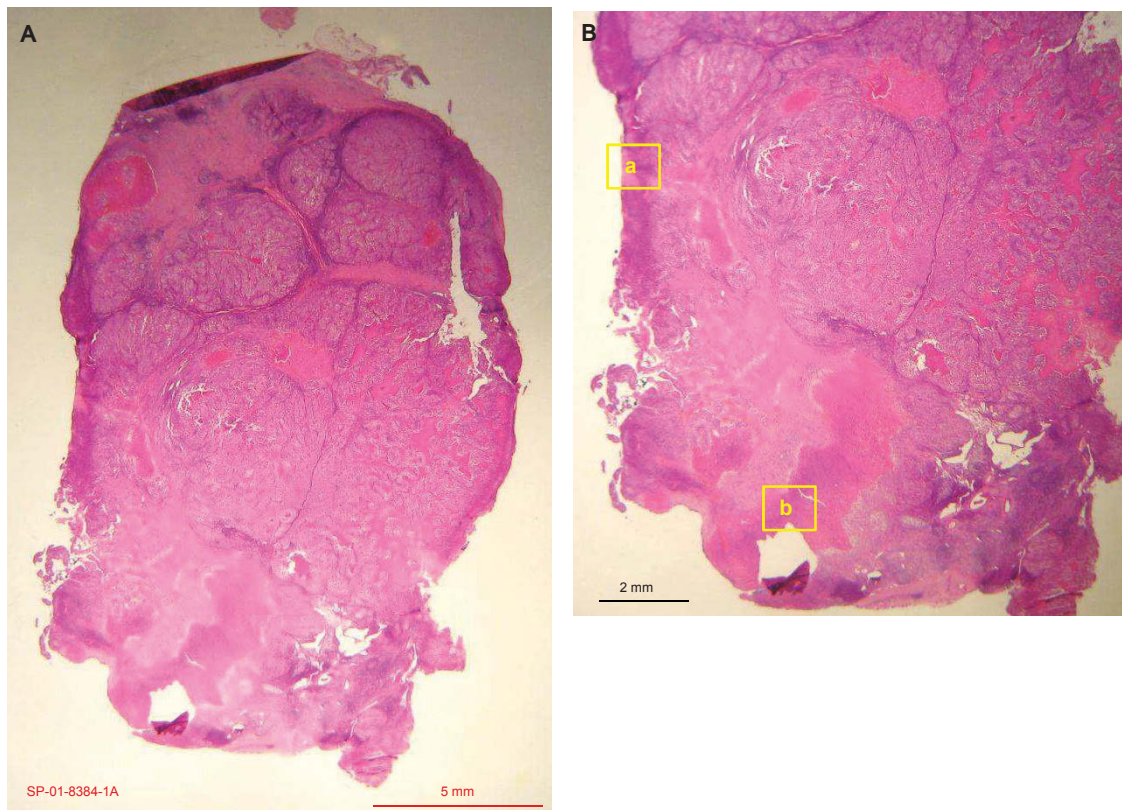


Figure 3-5: (A) A human lymph node at x0.63 objective magnification, but in this case the sample is exhibiting metastatic involvement. (B) Magnified region of the metastatic lymph node (x1.0 objective magnification) from (A) shows distinct differences from the normal lymph node tissue section observed in Figure 3-4. The marked rectangles correspond with further magnification of the selected tissue regions displayed in Figure 3-6.

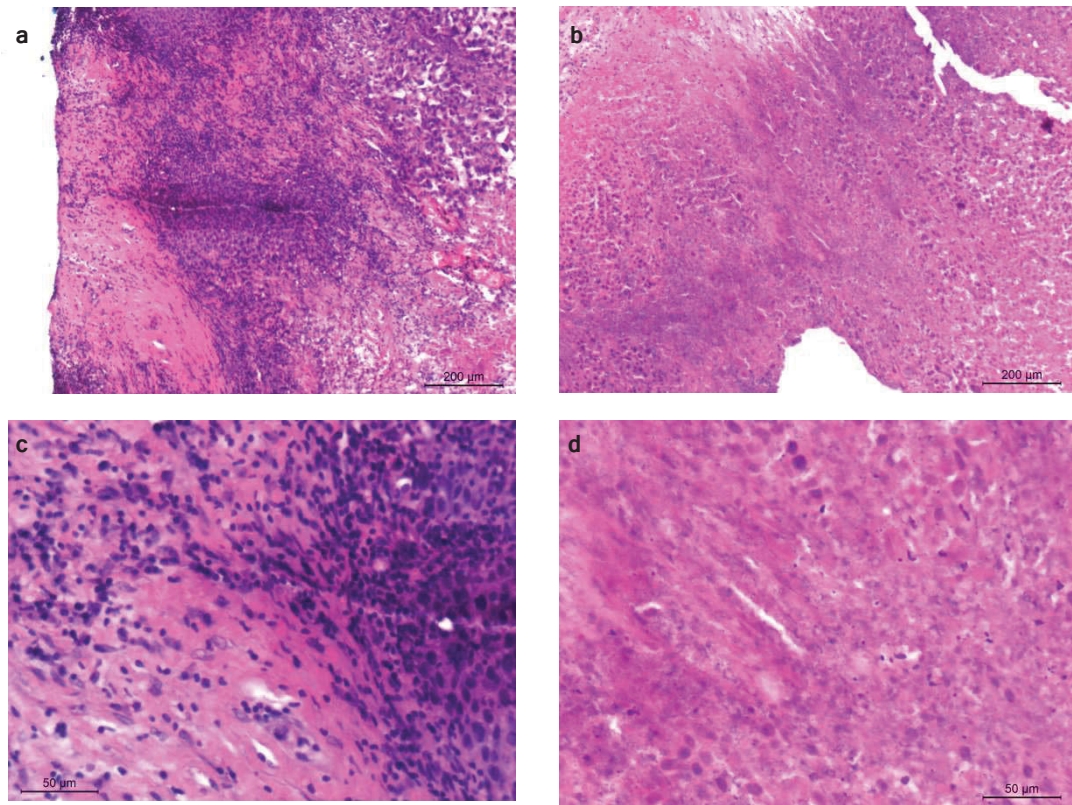


Figure 3-6: Magnified regions (x5 objective magnification) **(a)** and **(b)** marked in Figure 3-5B showing the variety of cell types and shapes observed in the metastatic lymph node. **(c)** & **(d)** are x20 objective magnification images of regions **(a)** and **(b)** respectively. The cells in **(d)** are not clearly distinct entities and in particular exhibit irregular shapes and lack a clearly staining nucleus, particularly when contrasted to the tissue shown in **(c)**.

Due to the limitations of the operating system on the computer interfaced to the infrared spectrometer, large area images needed to be collected in either the expanded field of view (EFOV), or in multi-file mosaic file format, or a combination of the two. In addition to this, the background image collected could not exceed a file size of one gigabyte due to operating system memory restrictions, so background correction was usually performed using a repeated image tile or a small mosaic of tiles, rather than a single image covering the entire sample area. This strategy meant that spectra in the sample image were not ratioed to background spectra from the identical microscope stage position. This could introduce artefacts if (for example) the stage was not perfectly horizontal, but this problem was unavoidable for images of this size. It was hoped that such artefacts would be removed by spectral preprocessing.

As a result of some of the tissue samples being so large in area, giving file sizes (at the settings chosen) too large for the instrument computer to handle, spatial sub-sampling on the images was performed. While the use of an expanded field of view and pixel aggregation for image collection was employed to assist in imaging larger areas of some of the tissues, it was not desirable for all samples due to the potential loss of spatial resolution. Hence, the instrument parameters employed for imaging needed to balance spectral resolution, spatial resolution and the resulting data file size.

For this study the majority of images were collected as multi-file mosaics. While imaging of areas of several square centimetres is possible using this approach, the resulting compiled image would take hours to days to collect and would be too large for subsequent chemometric analysis. In order to minimise file size but maximise the image area, pixel aggregation was employed when collecting the infrared images. This process involves averaging neighbouring pixels (for example in a 2 x 2 or 4 x 4 square pattern, etc.) to give larger pixels. The larger the pixel aggregation value, the greater the potential loss of spatial resolution and spectral information, particularly in cases where the chemical

composition of a sample varies on a microscopic level (e.g. cellular). However, if a sample possesses large regions of chemically homogenous materials then one would only expect pixel aggregation to impact where different materials border each other. Again, computational restrictions meant that pixel aggregation became a necessity if large image areas were to be collected.

Hence, images were capped at a maximum of 256 component tiles (e.g. a 16 x 16 tile image) using a pixel aggregation value of 4 (producing a 128 x 128 pixel image with dimensions of approximately 5.4 x 5.4 mm, or 10.8 mm x 10.8 mm in EFOV). In total, approximately 165 images from the 46 tissue samples (23 slides) were obtained, with a number of the tissue sections requiring numerous sub-regions to be imaged due to the sample's large surface area.

3.1.3 Data Preprocessing

The first preprocessing step was to reduce the file size by spectral subsetting. This involved initially truncating the spectral range to 1000 - 3100 cm^{-1} wavenumbers. It was believed that retaining this region would reduce file size without compromising the amount of information retained. This truncation was performed in CytoSpec, which, unlike ENVI, permits the retention of only one subset region from the spectrum (therefore the region between the amide I and C-H stretching bands, roughly the 1750 to 2800 cm^{-1} region, which contains no useful information in the biological tissue samples studied here, was not removed during initial data preprocessing).

To remove unwanted pixels (representative of the glass slide) that were captured in some sample images, the 'sample thickness' criterion of the spectral quality test in CytoSpec was employed. This tool obtains the integrated absorbance over a user-specified spectral range and checks the value against a nominated threshold value. In some images the sample

thickness tool in isolation was not totally effective in removing noisy spectra/pixels and so a signal-to-noise ratio test was then used to remove further unwanted pixels.

Initial data analysis and published observations^[5-8] indicated that taking the second derivative, followed by vector normalisation, could produce reasonable classification results. It was observed during the preliminary studies that a high smoothing value appeared to reduce the 'tiling effect' (Figure 3-7) which appeared in some samples following image classification, a result observed by Wood and McNaughton also.^[5] However, increasing the smoothing value too greatly may also present a high risk in the form of data loss (through removal of potentially diagnostic inflection points) and poor subsequent clustering, so an optimal value that balances these considerations must be decided upon prior to *en masse* sample analysis.^[5] Based on the preliminary classification studies, a value of 15 smoothing points was selected for this research.

3.1.4 Spectral Reference Library Construction

Each tissue section nominated for the reference (or 'training') library data set was closely examined under a standard light microscope and the target regions of tissue identified. Three approaches for selecting reference library spectra were trialled on the training images. The first approach taken to construct a spectral reference library involved subjecting the pre-processed training set tissue section images to unsupervised classification in the form of hierarchical cluster analysis (HCA). The number of clusters used for each image was determined by comparing the clustered images to the corresponding H&E stained sections.^{[6,9-}

^{12]} For each cluster of interest, the average spectrum was extracted.

A second approach employed for spectral library construction involved manually sampling spectra from the training sample images. Bhargava strongly advises that, when extracting reference library spectra, the pixels selected should be chosen independent of validation algorithms and that boundary pixels should be excluded in both training and validation

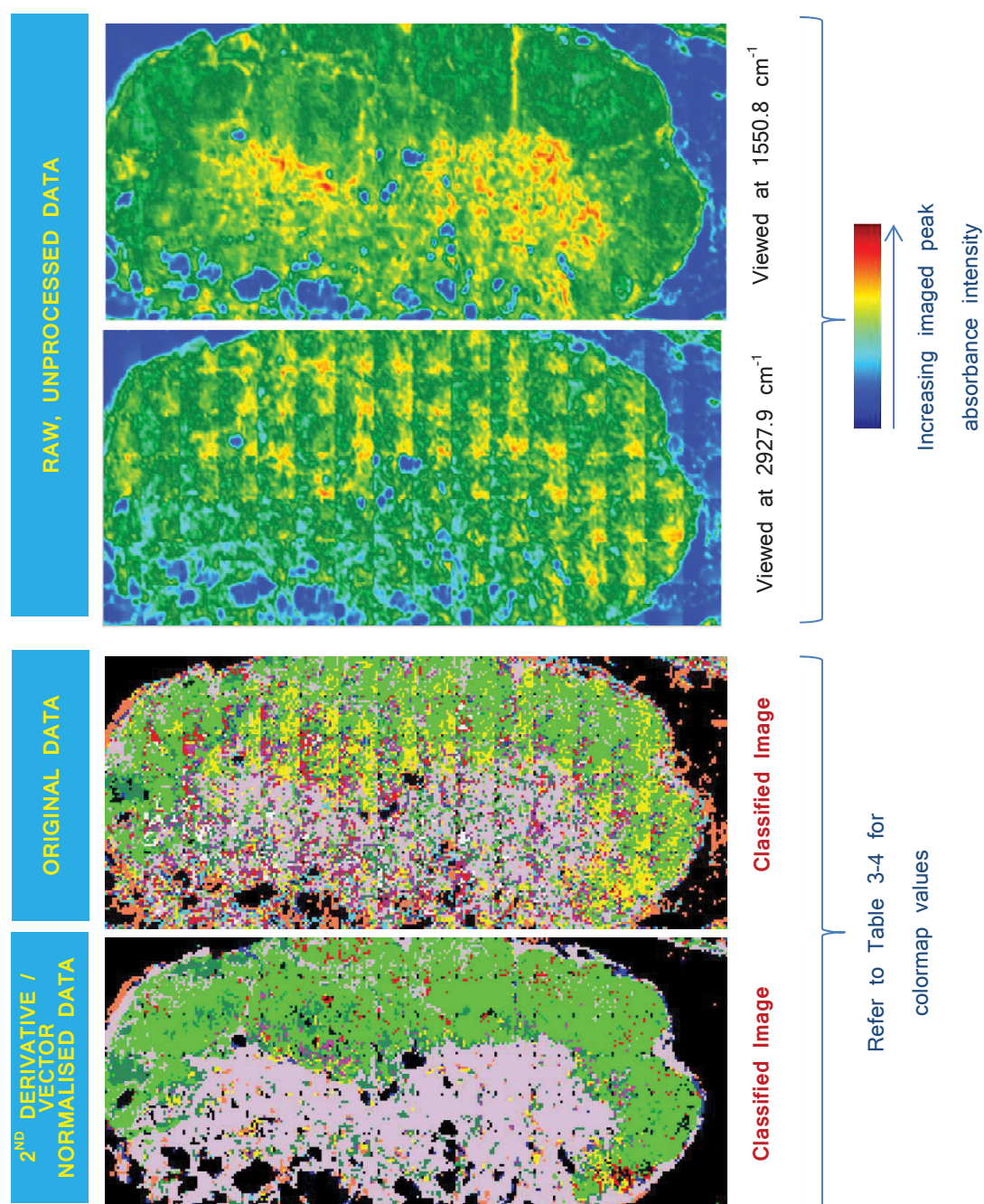


Figure 3-7: An example of the 'tiling effect' observed in a lymph node sample and the degree to which its appearance varies when the image is viewed at different wavenumber values. This effect may be even more visible following image classification if effective data preprocessing is not employed. The source of the effect is attributed to a number of causes which include subtle stage calibration issues, uneven illumination of the sample, as well as background collection limitations (using a 4-tile background for a 16-tile image) and instrumental variations over the long sample collection times.

stages.^[13,14] Spectral extraction was performed by selecting a region of interest (ROI) from the tissue image and then calculating the average spectrum for the ROI. To assist in ROI determination, cluster regions were subsampled from the HCA image (i.e. an ROI was taken from within the cluster region, avoiding border regions where possible). In cases where the HCA cluster regions did not adequately reflect the structures expected to be observed in the sample, the ROIs were chosen based on the corresponding H&E stained section. A graphical representation of the two aforementioned spectral library construction methods has been provided in Figure 3-8.

The final spectral library construction approach involved employing a form of spectral unmixing, a multivariate data analysis technique that may prove advantageous when pixels/spectra represent mixtures with varying abundances of component spectra, much as would be expected in the images collected in this study. There are numerous spectral unmixing algorithms available such as the pixel purity index (explored further in Chapter 5), but for the purposes of this melanoma study, the Vertex Component Analysis (VCA) algorithm was selected. An example of the analysis of a training sample image has been given in Figure 3-10.

In summary, the three methods performed to construct the spectral reference libraries were:

Method 1: Perform HCA on selected training samples which have undergone preprocessing (derivatisation and normalisation) using CytoSpec. The clustered images are then compared to the H&E sections and the average representative spectra for the clusters correlating with the various structural features of interest in the tissue extracted.

Method 2: Perform HCA on selected training samples which have undergone preprocessing (derivatisation and normalisation) using CytoSpec. The clustered images are then compared to the H&E sections and the regions of interest (ROIs) manually selected. The average spectra from these ROIs are then used in the library construction.

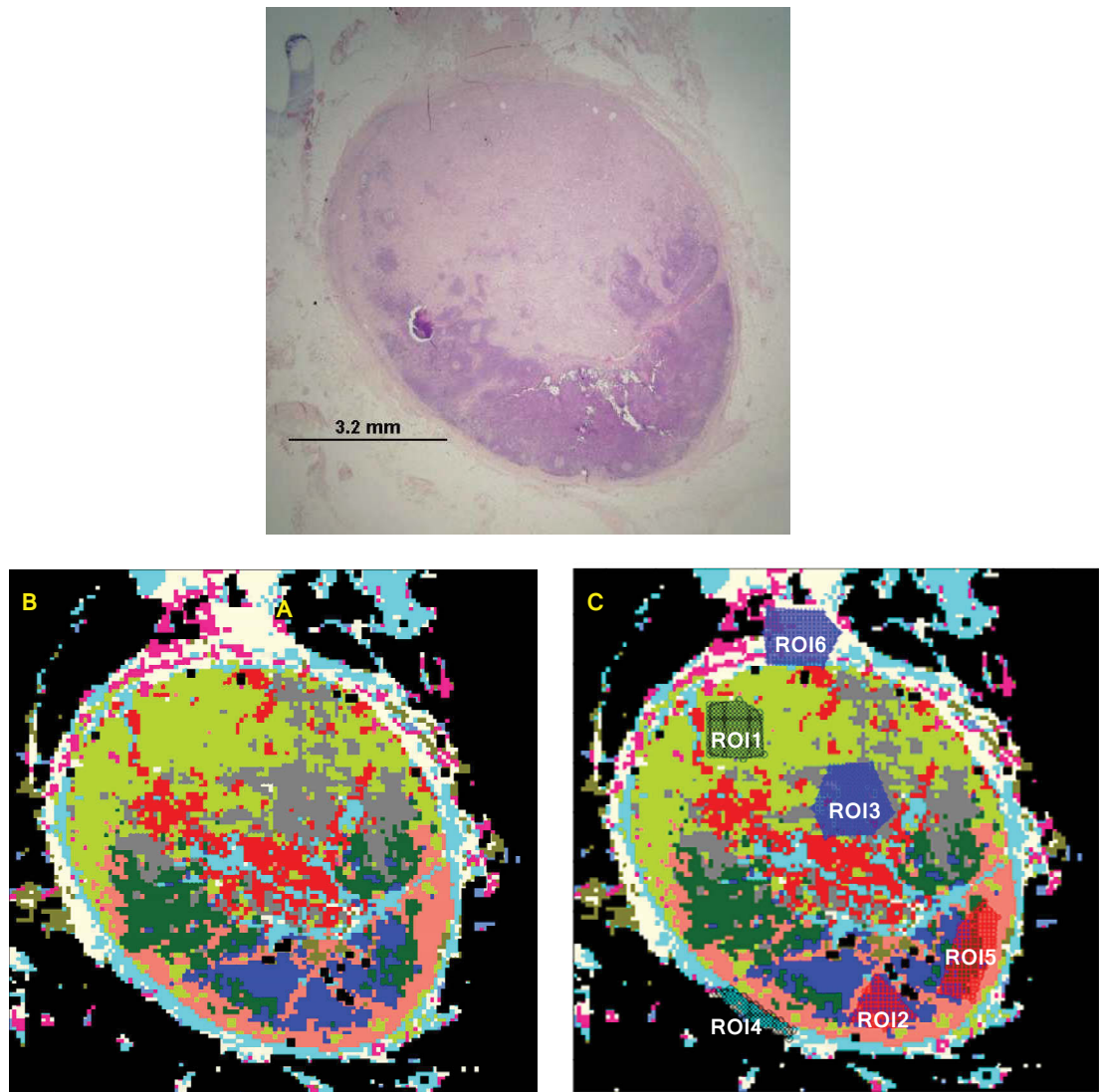


Figure 3-8: (A) An example of one of the training samples used in the spectral reference library construction - an H&E-stained section of a lymph node exhibiting metastatic melanoma. (B) The HCA clustering image showing the first 13 clusters, some of which were then selected for inclusion in the first library construction method. (C) Examples of the ROI selection method conducted in CytoSpec for the collection of average spectra for the second library construction method explored. The average spectra collected during this stage have been presented in Figure 3-9.

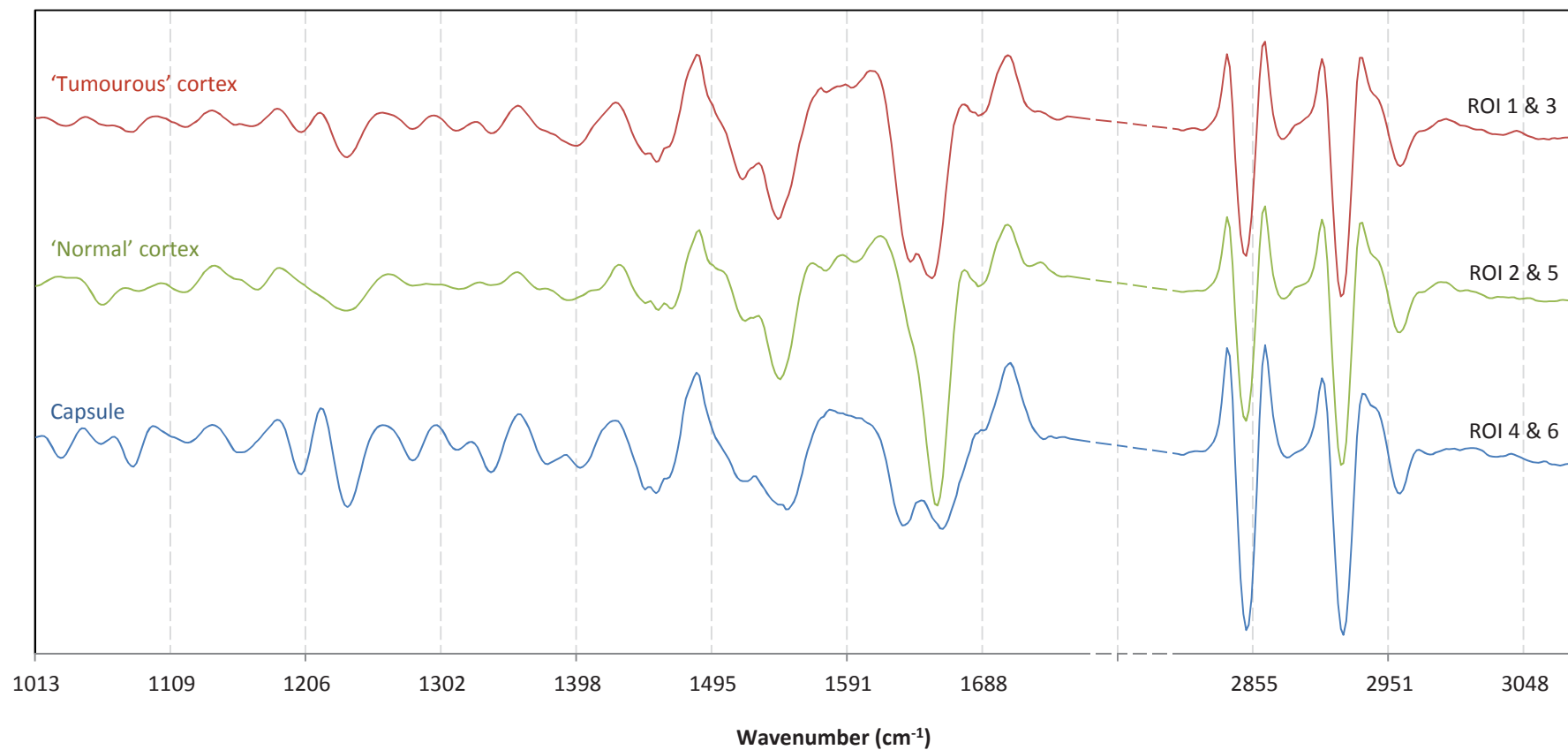


Figure 3-9: Comparison of the average second derivative spectra (offset from each other to assist in ease of viewing) for the three “tissue types” sampled using the ROI regions marked in Figure 3-8C. Two ROIs (i.e. non-contiguous regions) were obtained for each of the three tissue types based on a comparison with the H&E-stained slides in this case rather than relying solely on the HCA cluster regions.

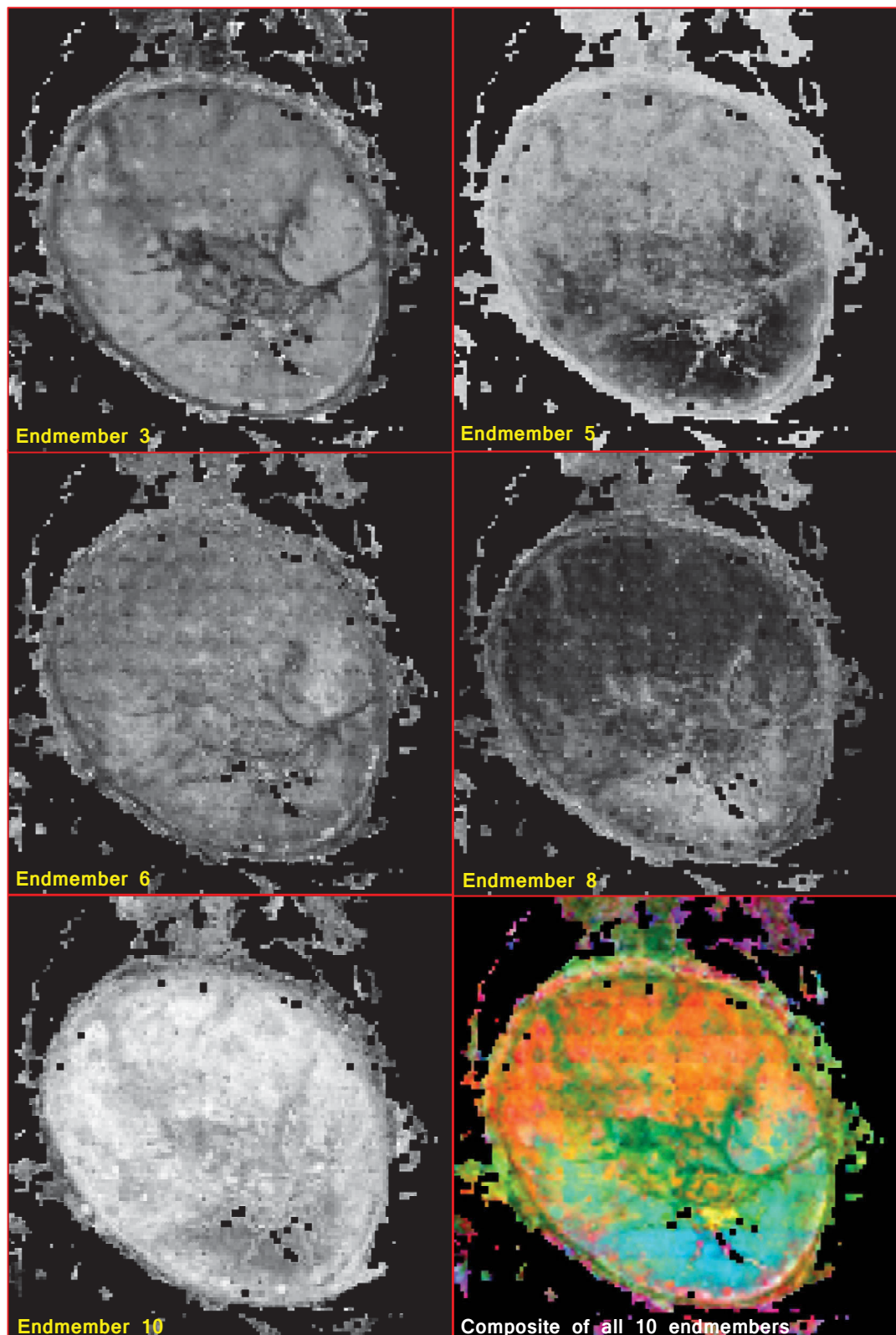


Figure 3-10: Example of the training images obtained using VCA from a deparaffinised lymph node tissue section exhibiting metastatic melanoma growth (pre-processed data block).

Method 3: Perform VCA on selected training samples using original, non-derivatised/non-normalised data as well as those which have undergone complete preprocessing (derivatisation and normalisation) using CytoSpec. Again, the resulting component images are compared to their corresponding H&E sections and the representative component (endmember) spectra extracted.

For each library, spectra were collected from paraffin-embedded and deparaffinised tissue sample images. This meant that, in effect, four libraries were obtained for each library construction method: original spectra from paraffin-embedded tissue, pre-processed (derivative) spectra from paraffin-embedded tissue, original spectra from deparaffinised tissue and pre-processed spectra from deparaffinised tissue. A summary of the library construction methods used has been given in Table 3-1.

Table 3-1: Summary of the spectral reference library construction approaches. ‘Original’ refers to the unprocessed data and ‘pre-processed’ refers the spectra subjected to the pre-processing described in Section 3.1.3.

Method	Spectra Selection Method	Output Spectra
1	HCA, cluster averages	Original and pre-processed
2	Region of interest (ROI) spectra	Original and pre-processed
3	VCA, endmember spectra	Original and pre-processed

A component of this study was to examine the effectiveness of each classification method given a library constructed only from training tissue of the same type (i.e. skin or lymph node) or comprised of both the skin and lymph node tissues. Hence, for each library construction method, effectively three reference spectral libraries were produced (a combined

library, a naevus/primary melanoma tissue library, and a normal lymph node/metastatic lymph node library) with each constructed using either unprocessed or pre-processed data.

For use in ENVI, the spectral libraries were constructed by extracting the spectra from CytoSpec and importing them into Microsoft Excel in a text format. Each column of data (representing the spectrum) was then labelled and a colour assigned. This file was then saved with a header ready for importation into ENVI.

3.1.5 Spectral Similarity Investigation

Constructing a representative spectral library is one of the key steps in any hyperspectral image classification process. However, in this case, while guidance was obtained from a trained pathologist in regards to identifying regions within a tissue sample exhibiting various pathological states, the selection of image regions for representative spectra was performed by an individual with no formal pathology training. Employing a trained pathologist to perform spectra selection would have been ideal, but it would have been costly in terms of the time requirements placed on the participating pathologist. Hence, for this infrared imaging study it was decided that spectral selection performed by the author, under the guidance of information provided by a certified pathologist, would suffice for the preliminary investigation into the suitability of the SAM algorithm classification approach being attempted.

Prior to performing test image classifications, an examination of the library reference spectra was performed to gain some understanding into the variation between spectra obtained from the different tissue regions collected from the training images. A cursory visual inspection of the library spectra does not reveal much in the way of visible differences between the various spectra collected (see Figure 3-11), and therefore a chemometric analysis of the spectra from the various libraries was proposed. Two classification techniques were chosen to assess the similarity of the spectra from the various tissue regions, and from the two main tissue types (i.e. skin or lymph node). A number of classification methods were

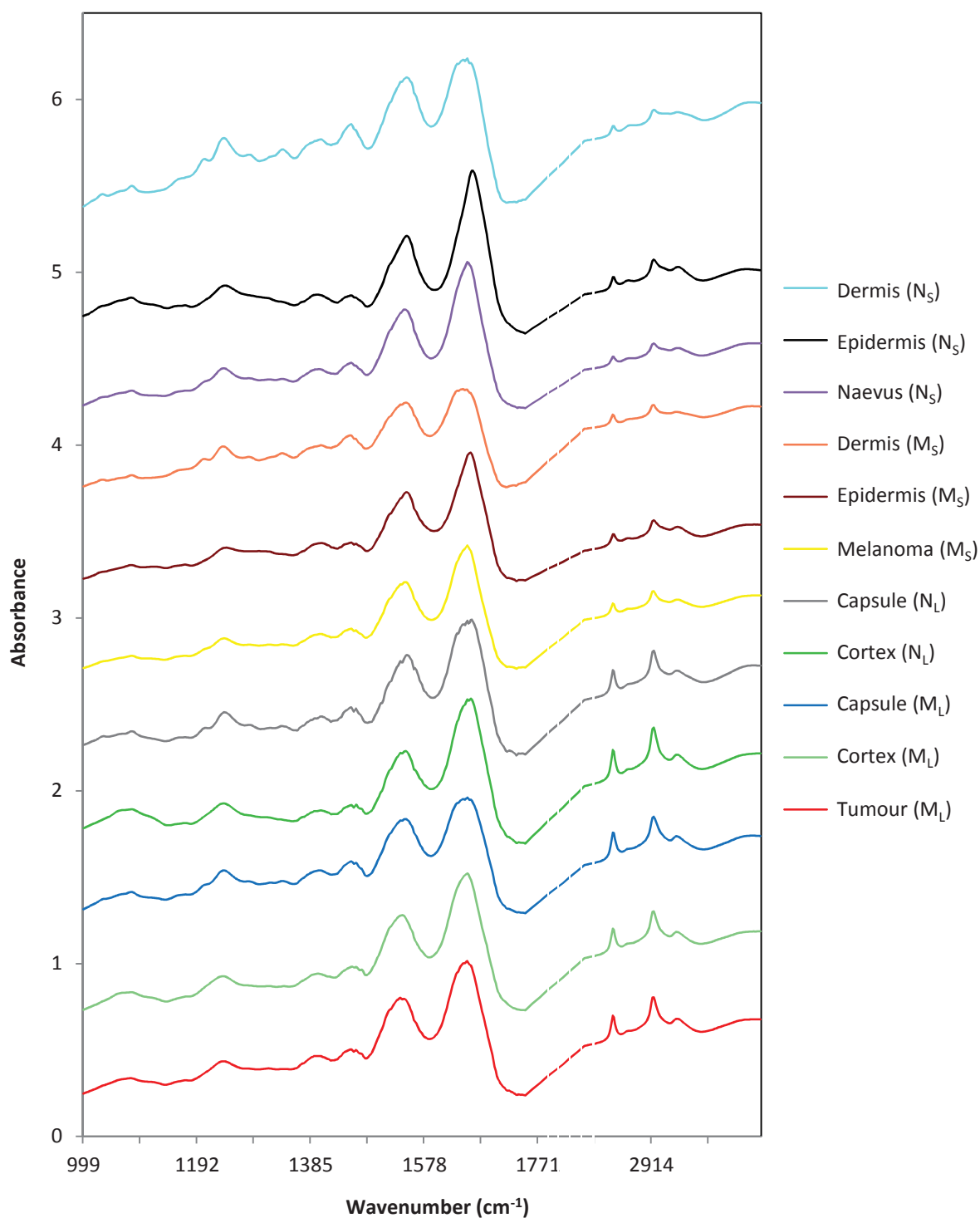


Figure 3-11: Average spectra (offset for convenience of display by 0.5 absorbance units) from the different tissue groups targeted in this study (spectral reference library construction Method 1). Note the high similarity in the appearance of the spectra between the various tissue regions sampled.

considered, as well as those explored in previously published infrared spectroscopy cancer diagnosis studies.^[9,10,15-17] What was preferred was a technique that could be used to produce easily interpretable data with the option of a simple graphical representation of the similarity for all of the library spectra.

Although HCA would produce a simple means of understanding how the spectra would group, it was found that the dendrograms produced were too cluttered to represent the spectral relationships, and the determination of membership (of spectra) involved having to select a predetermined number of clusters. Eventually two approaches familiar to the author were employed: SIMCA and PCA/LDA. Each of these methods produces a substantial amount of data which may then be used to assist in determining not only how similar or different the spectra are, but also the spectral regions or individual features (peaks) that may be responsible for the greatest spectral discrimination.

In this application, SIMCA and PCA/LDA were employed primarily to assist in answering the question of whether there is sufficient spectral variation for any form of spectral classification (i.e. in a more 'supervised' manner). In addition to this, the impact of spectral truncation was also assessed in two stages: by removing the C-H stretch region (reducing the region to approximately 1000 - 1750 cm^{-1}) first, and then truncating again by removing the amide I peak (1000 - 1585 cm^{-1} region retained) for each classification method employed.

An examination of the spectral similarity of the various library spectra and their suitability as library reference spectra was performed using PCA/LDA and SIMCA as implemented in the Pirouette and SPSS software packages. Performing this analysis permitted an investigation into whether there are any strong distinguishing spectral features characteristic of primary melanoma, metastatic melanoma and naevus tissues and how strongly the tissue type may affect image classification. This investigative step also provided insight into how well the library spectra collected reflected the different disease tissue states and assisted in possibly identifying any problems in the image classification methods employed.

Initially the library spectra were simply grouped into their source tissue types: lymph nodes displaying metastatic melanoma, normal lymph nodes, skin tissue exhibiting primary melanoma, and naevus tissue specimens. This meant that tissue structures common to the different source tissue types, such as epidermal tissue in the naevus and primary melanoma, would be labelled as having separate source tissue types. Classification of this type was considered the most crude but provided a quick means of examining spectral relationships such as between the two main tissue types (i.e. skin or lymph node) as well as the possible impact paraffin retention/removal might have on sample discrimination.

The simplest means by which to assess the discrimination capability of the reference library spectra was to analyse the percentage of correctly classified samples for each of the library collection methods. The results of this classification for both the SIMCA and PCA/LDA have been given in Table 3-2 and Table 3-3 respectively.

Table 3-2: SIMCA analysis (% correctly classified) of the library reference spectra grouped into the four source tissue types. First truncation refers to the removal of the C-H stretching region of the spectrum. Second truncation describes the subsequent removal of the Amide I band.

Library Method	Paraffin Present?	Preprocessing Method	Full Spectrum	First Truncation	Second Truncation
1 (HCA)	No	2 nd Derivative	92.3	94.6	94.2
		Original	91.5	94.7	98.2
	Yes	2 nd Derivative	90.2	98.2	97.5
		Original	89.2	96.9	96.3
2 (ROI)	No	2 nd Derivative	93.0	93.9	99.1
		Original	91.0	95.2	91.9
	Yes	2 nd Derivative	85.9	84.5	94.0
		Original	86.1	92.3	90.8
3 (VCA)	No	2 nd Derivative	72.7	73.1	68.8
		Original	58.1	61.8	61.5
	Yes	2 nd Derivative	71.2	80.2	72.7
		Original	64.5	68.1	63.1

Table 3-3: PCA/LDA analysis of the library reference spectra grouped into the four sample type categories of lymph nodes exhibiting metastatic melanoma involvement, normal lymph node, primary melanoma and naevus tissue. The two values represent the percentage of original grouped cases correctly classified followed by the percentage of cross-validated grouped cases correctly classified in brackets.

Library Method	Paraffin Present?	Preprocessing Method	Full Spectrum	First Truncation	Second Truncation
1 (HCA)	No	2 nd Derivative	78.6 (72.5)	74.8 (69.5)	81.7 (75.6)
		Original	82.4 (78.6)	83.2 (74.0)	83.2 (79.4)
	Yes	2 nd Derivative	71.1 (64.4)	81.2 (77.9)	82.6 (65.8)
		Original	67.8 (63.1)	79.9 (74.5)	87.2 (84.6)
2 (ROI)	No	2 nd Derivative	77.9 (69.5)	72.6 (65.3)	82.1 (70.5)
		Original	76.0 (64.4)	79.8 (73.1)	83.7 (75.0)
	Yes	2 nd Derivative	73.4 (64.2)	82.6 (71.6)	78.9 (69.7)
		Original	64.6 (56.9)	77.7 (66.2)	79.2 (70.8)
3 (VCA)	No	2 nd Derivative	45.6 (27.8)	46.7 (28.9)	54.4 (33.3)
		Original	58.7 (48.0)	58.7 (42.7)	60.0 (45.3)
	Yes	2 nd Derivative	54.8 (43.0)	62.4 (41.9)	61.3 (47.3)
		Original	67.1 (56.6)	65.8 (61.8)	76.3 (60.5)

Examples of the type of data that may be extracted from the SIMCA and PCA/LDA data analyses have been given in Figure 3-12 to Figure 3-15 also.

It is clear from the results obtained that the spectral reference library which suffers from the poorest discrimination capability is that of Method 3 (VCA collection method). This is not surprising considering that in a number of the training images clear endmembers representative of the known differing tissue components in the tissue could not be visualised.

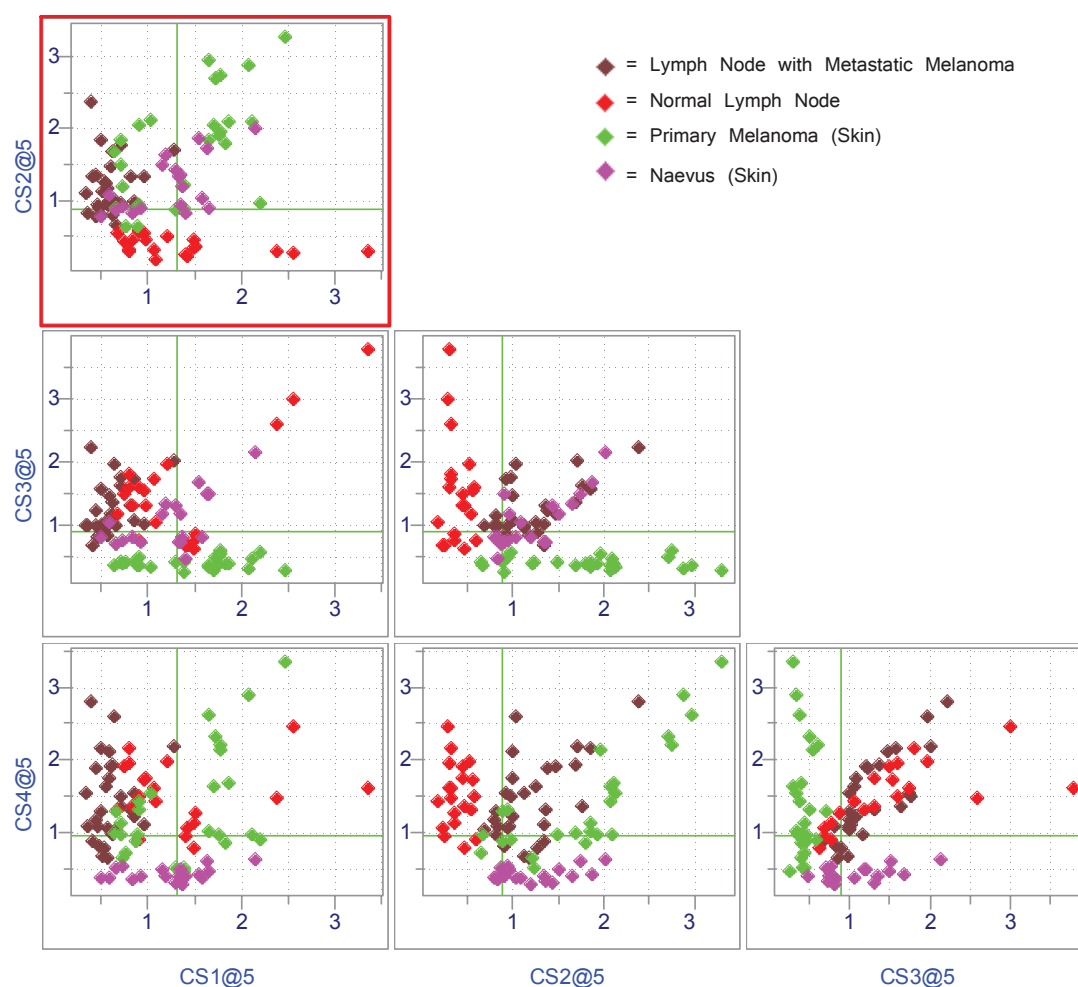


Figure 3-12: Multiplot of class distance for reference library Method 2 (ROI) utilising deparaffinised tissue and the second derivative ($1012 - 1585 \text{ cm}^{-1}$ spectral region). Samples in the top left and bottom right regions indicate clear membership to the x and y axis classes. Samples in the bottom left region indicate samples which could belong to either class and those in the top right region belonging to neither.

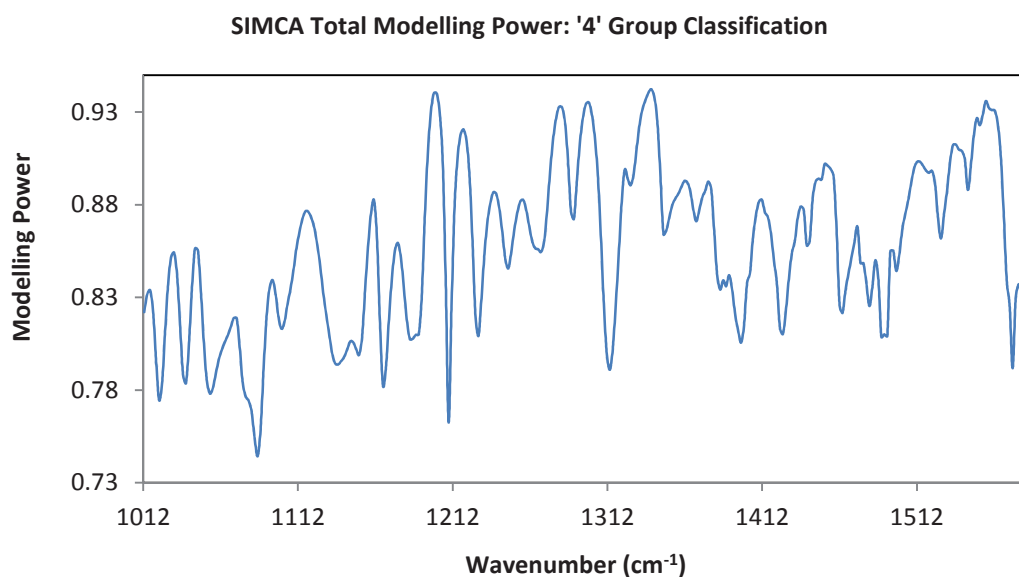


Figure 3-13: Total Modelling Power plot for reference library Method 2 (ROI) utilising deparaffinised tissue and the second derivative (1012 - 1585 cm^{-1} spectral region). It is clear that there is no one specific region of the spectrum which is of greatest importance for any class in the spectral data set examined.

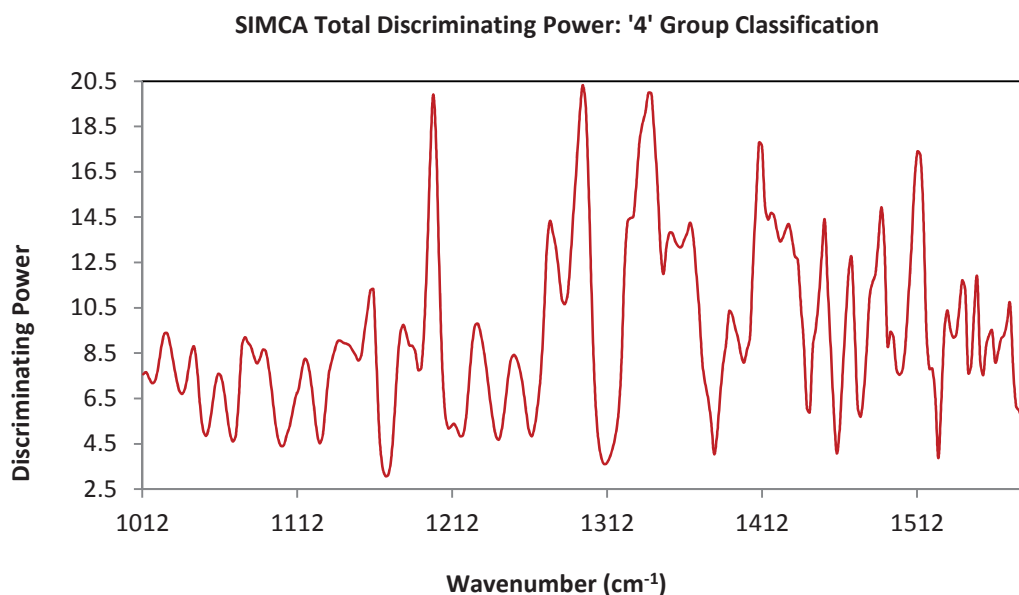


Figure 3-14: Total Discriminating Power plot for reference library Method 2 (ROI) utilising deparaffinised tissue and the second derivative (1012 - 1585 cm^{-1} spectral region).

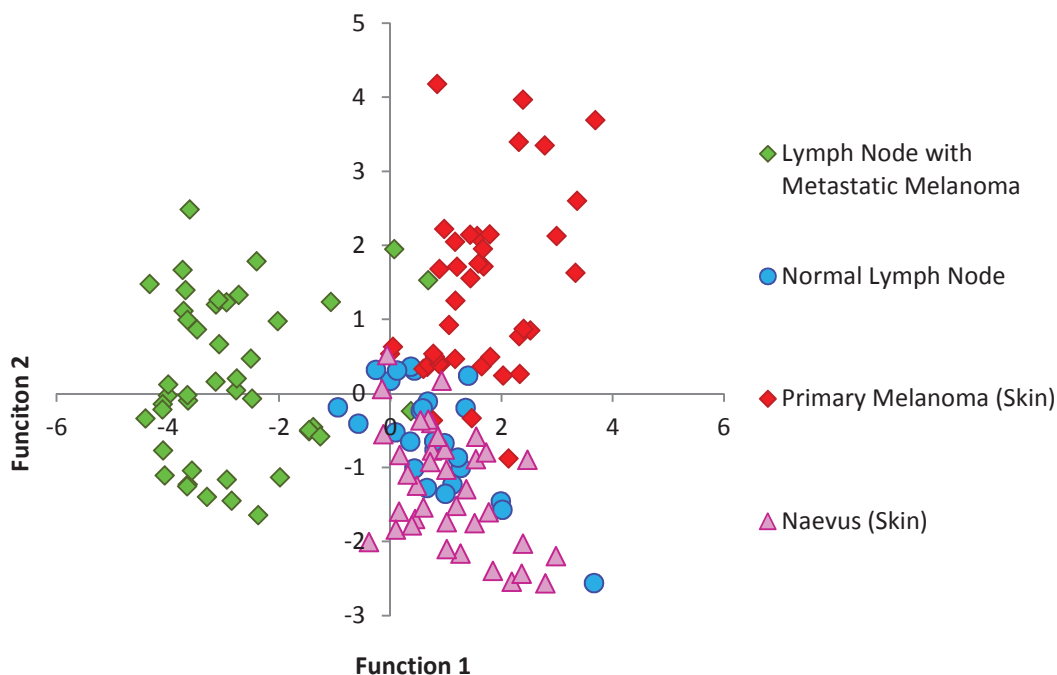


Figure 3-15: PCA/LDA discriminant score function plot for reference library Method 1 (HCA) utilising paraffin-embedded tissue and the original spectra using the $1000 - 1585 \text{ cm}^{-1}$ spectral region. The degree of overlap between the training sample spectra is considerable and strongly indicates a high degree of similarity between the tissue spectra.

A second spectral library examination was then conducted on the reference spectra after the individual spectra were more specifically divided into twelve categories (see Table 3-4). This approach permitted the study of the reference spectra for a range of different cellular structures. The results of this second classification approach employing SIMCA and PCA/LDA have been given in Table 3-5 and Table 3-6. Graphical representations of the variance observed in the data obtained following the PCA-LDA analysis have been given in Figure 3-16 and Figure 3-17.

Table 3-4: The twelve sub-classifications used for the more specifically labelled tissue regions within the training samples.

Main Tissue Type	Sub-classifications	ENVI Colour
Lymph Node exhibiting metastatic melanoma involvement (M_L)	Tumour	Red 
	Capsule	Sea green 
	Cortex	Blue 
	Unknown*	Magenta 
Normal lymph node (N_L)	Capsule	Green 
	Cortex	Thistle 
Skin tissue with a primary melanoma component (M_S)	Melanoma	Yellow 
	Epidermis	Maroon 
	Dermis	Coral 
Skin tissue with a naevus component (N_S)	Naevus	Purple 
	Epidermis	White 
	Dermis	Cyan 

* The 'unknown' tissue describes regions within the lymph nodes classified as possessing metastases that were composed of cells that did not appear similar to those identified in other metastatic melanoma lymph nodes. Their abnormal appearance did not permit their classification into any of the remaining nominated tissue regions.

Table 3-5: SIMCA (% correctly classified) analysis of the library reference spectra grouped into the twelve tissue sample type categories.

Library Method	Paraffin Present?	Preprocessing Method	Full Spectrum	First Truncation	Second Truncation
1 (HCA)	No	2 nd Derivative	65.5	65.9	73.2
		Original	59.4	66.1	59.1
	Yes	2 nd Derivative	77.8	83.1	83.4
		Original	76.6	68.5	66.0
2 (ROI)	No	2 nd Derivative	93.3	90.5	92.5
		Original	80.5	83.8	85.0
	Yes	2 nd Derivative	67.9	67.3	63.3
		Original	78.7	78.5	78.3
3 (VCA)	No	2 nd Derivative	70.7	69.9	69.0
		Original	61.8	61.3	57.7
	Yes	2 nd Derivative	70.5	82.6	77.2
		Original	60.5	62.3	57.9

Table 3-6: PCA/LDA analysis of the library reference spectra grouped into the twelve tissue sample type categories. The two values represent the percentage of original grouped cases correctly classified followed by the percentage of cross-validated grouped cases correctly classified in brackets.

Library Method	Paraffin Present?	Preprocessing Method	Full Spectrum	First Truncation	Second Truncation
1 (HCA)	No	2 nd Derivative	67.2 (49.6)	69.5 (56.5)	74.8 (62.6)
		Original	62.6 (55.0)	73.3 (60.3)	77.1 (63.4)
	Yes	2 nd Derivative	59.1 (48.3)	67.1 (59.7)	73.2 (65.8)
		Original	61.1 (47.0)	71.1 (61.1)	75.8 (61.1)
2 (ROI)	No	2 nd Derivative	74.7 (62.1)	76.8 (61.1)	77.9 (66.3)
		Original	72.1 (60.6)	82.7 (66.3)	80.8 (67.3)
	Yes	2 nd Derivative	68.8 (47.7)	73.4 (53.2)	71.6 (58.7)
		Original	55.4 (41.5)	70.0 (56.2)	67.7 (57.7)
3 (VCA)	No	2 nd Derivative	44.4 (20.0)	43.3 (20.0)	48.9 (28.9)
		Original	44.0 (22.7)	41.3 (20.0)	40.0 (20.0)
	Yes	2 nd Derivative	46.2 (22.6)	54.8 (32.3)	48.4 (32.3)
		Original	53.9 (32.9)	56.6 (32.9)	59.2 (35.5)

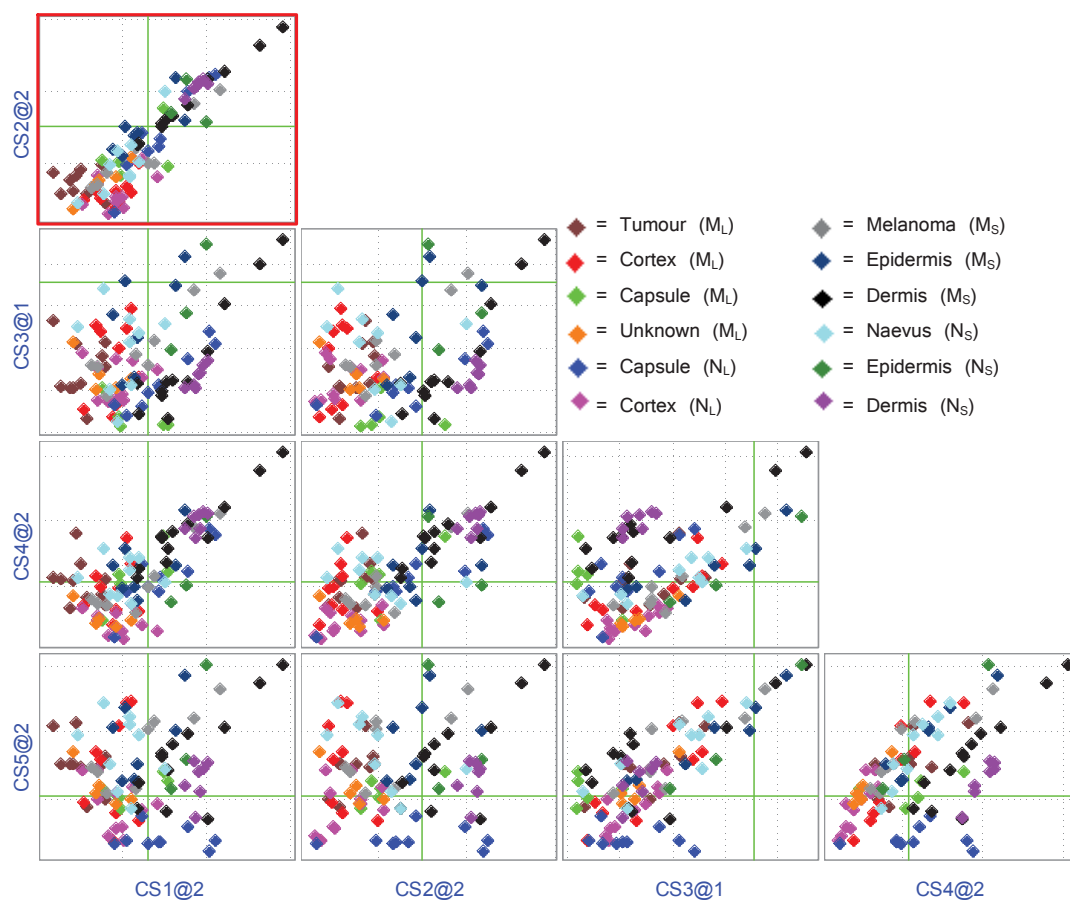


Figure 3-16: Multiplot of class distance for reference library Method 2 (ROI) utilising deparaffinised tissue and the second derivative (1012 - 3086 cm^{-1} spectral region). The tissue spectra have been sub-classified within the four main training tissue types of lymph nodes exhibiting metastatic melanoma (M_L), normal lymph nodes (N_L), skin tissue containing primary melanoma (M_S) and skin tissue with a naevus component (N_S).

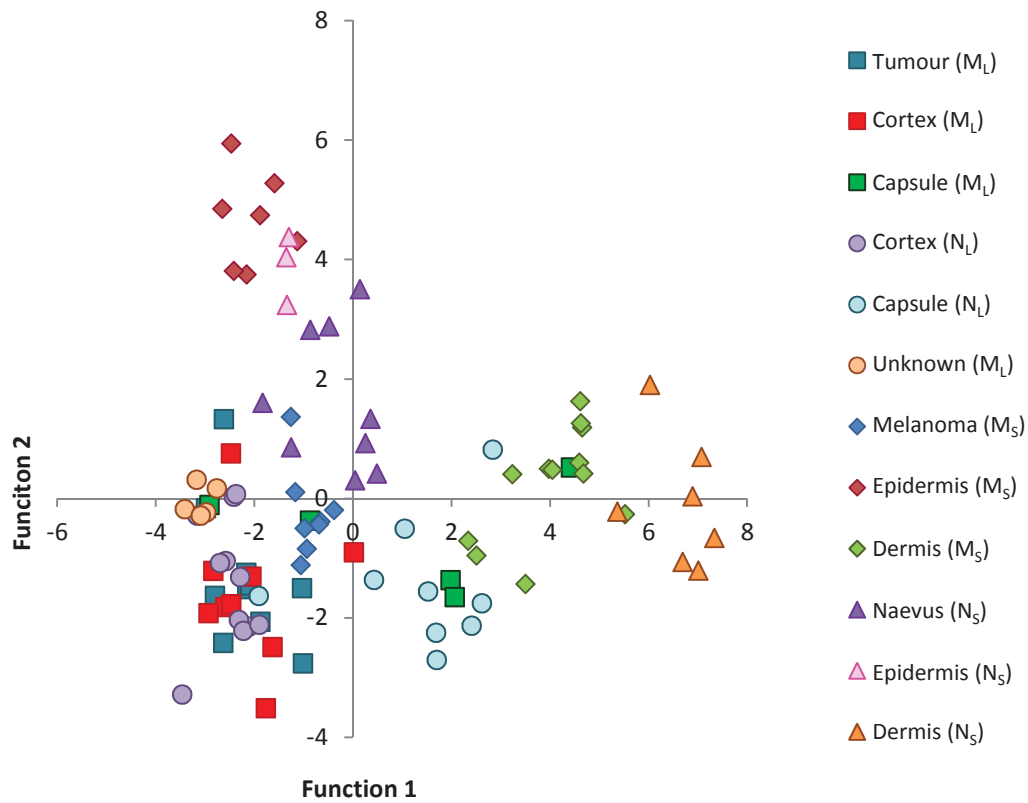


Figure 3-17: PCA/LDA discriminant score function plot for reference library Method 2 (ROI) utilising deparaffinised tissue and the second derivative spectra using the $1012 - 3086 \text{ cm}^{-1}$ spectral region. Although there is overlap between the different tissue types (i.e. skin and lymph node) there are distinct groupings of tissue from the same sub-classification type (e.g. epidermis spectra from both skin sample types).

3.1.6 Classification Results

Each collected image file to be tested using the spectral reference library was also subjected to data preprocessing (as outlined in Section 3.1.3) in CytoSpec for consistency and exported via MATLAB into an ENVI-ready image format in both an unprocessed and pre-processed form. These images were then available for subsequent SAM processing using the prepared spectral libraries.

While there are a number of methods that may be used to quantify the accuracy of pattern recognition, such as those explored by Bhargava, this study was simply concerned with assessing whether the SAM pattern-recognition approach was capable of detecting the subtle changes in tissue indicative of melanoma.^[13,15-20] Also, while methods have been explored in previous studies for the removal of the paraffin component in those samples imaged prior to deparaffinisation, in this study the impact of paraffin retention on classification was assessed.^[21] To do this, samples were imaged prior to and after removal of the paraffin embedding medium and the results of classification compared for each. Ideally a third sample group consisting of tissue images where the paraffin had been “removed” using spectral processing would also have been used in the comparison but there was unfortunately insufficient time to perform this additional step.

For each of the library methods examined, initial testing of the classification was performed on a small subset of the training tissue images. This approach was taken to investigate the robustness of the SAM classification algorithm on a relatively ‘simple’ image as the library contained spectra collected directly from that tissue sample. An example of the output results for a training lymph node image possessing tumorous melanoma growth has been given in Figure 3-18.

Following the imaging of a number of the training tissue images, test tissue images were then subjected to SAM classification (an example of this process has been given in Figure 3-19). For the ‘original’ spectral images, the data block which had undergone spectral quality assessment and initial spectral range truncation (1000 - 3100 cm^{-1}) was utilised. The results for each of the spectral reference library construction methods are presented in the following sections.

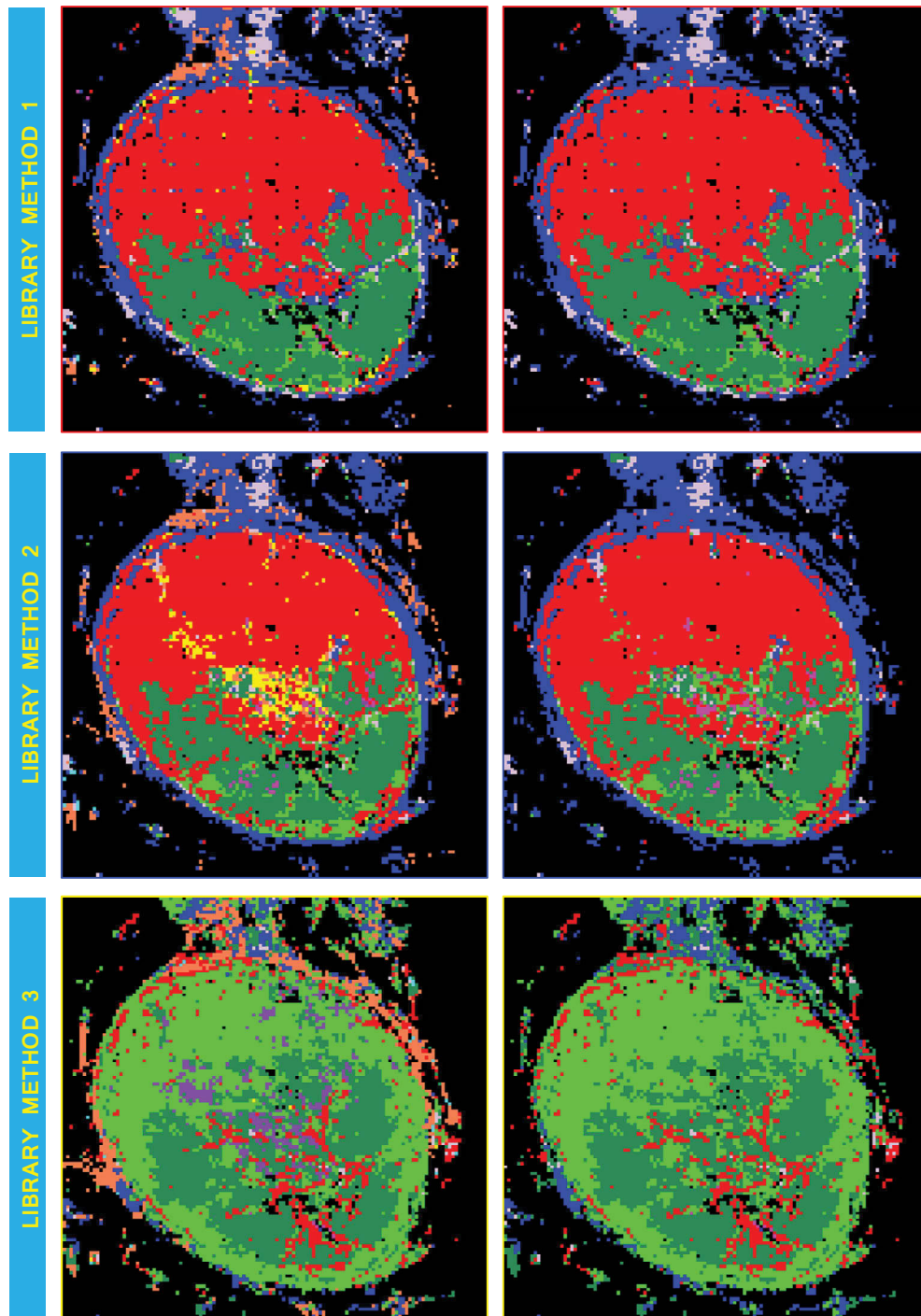


Figure 3-18: Demonstration of the SAM results using the various spectral reference libraries for a training image (lymph node tissue section exhibiting metastatic melanoma growth, deparaffinised tissue, second derivative data block). The left hand images use all library reference spectra, irrespective of tissue type source. The right hand images use spectra extracted only from lymph node training images.

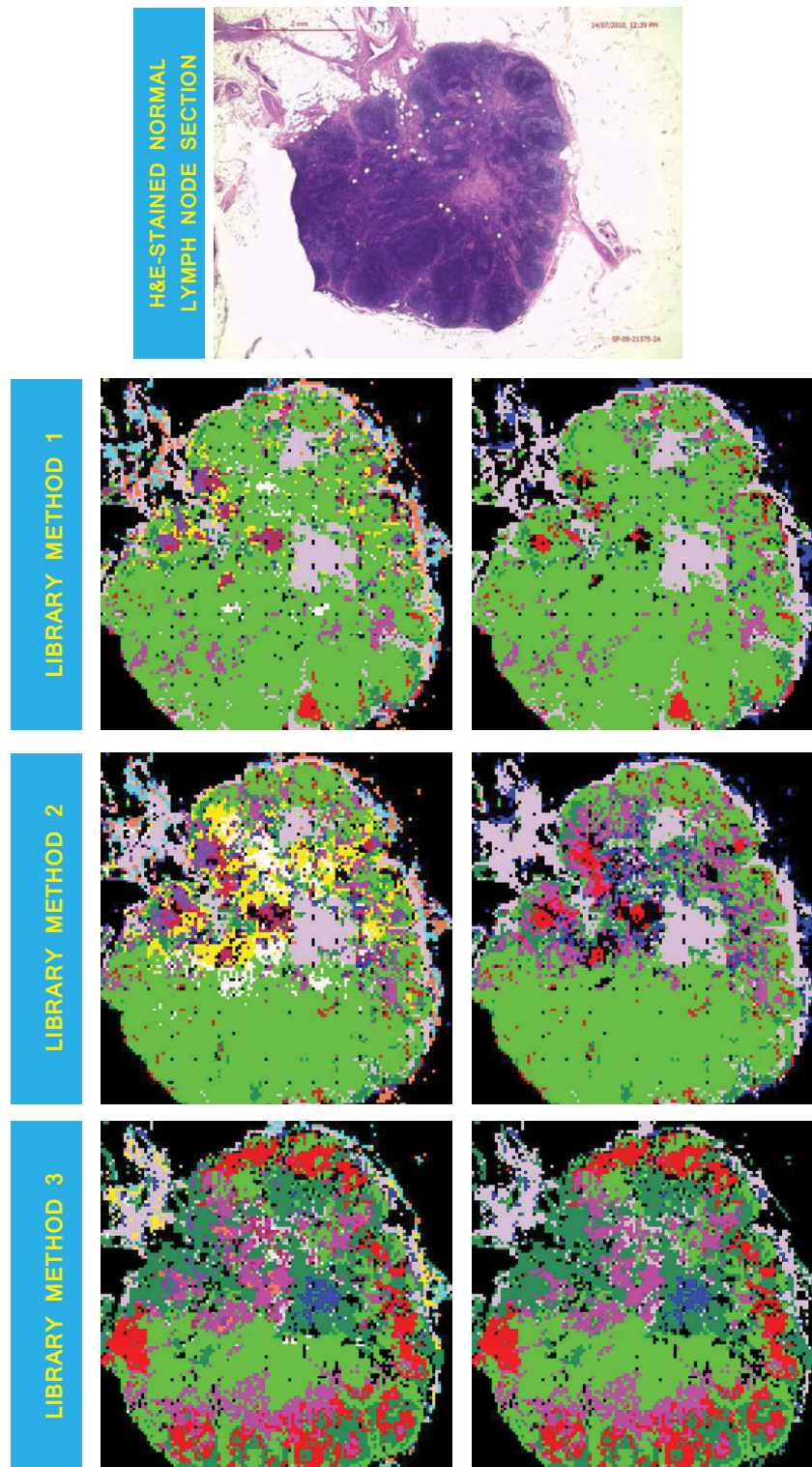


Figure 3-19: Demonstration of the SAM results using the various spectral reference libraries for a test image (normal lymph node, deparaffinised tissue, second derivative data block). Left images = all tissue library reference spectra; right images = lymph tissue library spectra only.

• Library Construction Method 1

The number of clusters required to obtain adequate tissue differentiation consistent with the regions observed in the H&E-stained images varied considerably (Table 3-7 provides a summary of the number of clusters employed in the training images), although in general between 8 and 13 clusters was sufficient for most training images. The retention of paraffin did not appear to affect the HCA classification as significantly as expected, and good tissue region discrimination could be achieved in many of the training sample images. Based on the results obtained, the greatest factor affecting tissue region discrimination was the spatial resolution employed, which was demonstrated in a small number of low spatial resolution images which were unable to be correctly differentiated using HCA.

Table 3-7: Range of cluster values employed in the training sample images for library construction Method 1.

Tissue	Paraffin Present?	Lowest Number of Clusters Used	Highest Number of Clusters Used	Average / Median
Lymph node displaying metastatic melanoma	No	8	15	11 / 9
	Yes	8	10	10 / 10
Normal lymph node	No	10	15	12 / 11
	Yes	10	15	13 / 14
Primary melanoma skin tissue	No	6	13	9 / 8
	Yes	6	17	8 / 8
Naevus skin tissue	No	4	14	8 / 10
	Yes	7	18	11 / 10

In relation to the SAM classification capability using this library method for the training sample images, this library approach worked well using both the entire library contents (i.e. all tissue spectra) but exhibited some improvement once the tissue-specific spectral library for the sample type was employed. The use of second derivative spectra produced a definite and clear improvement in classification. An example of a training sample classification using Library Construction Method 1 and SAM has been given in Figure 3-20.

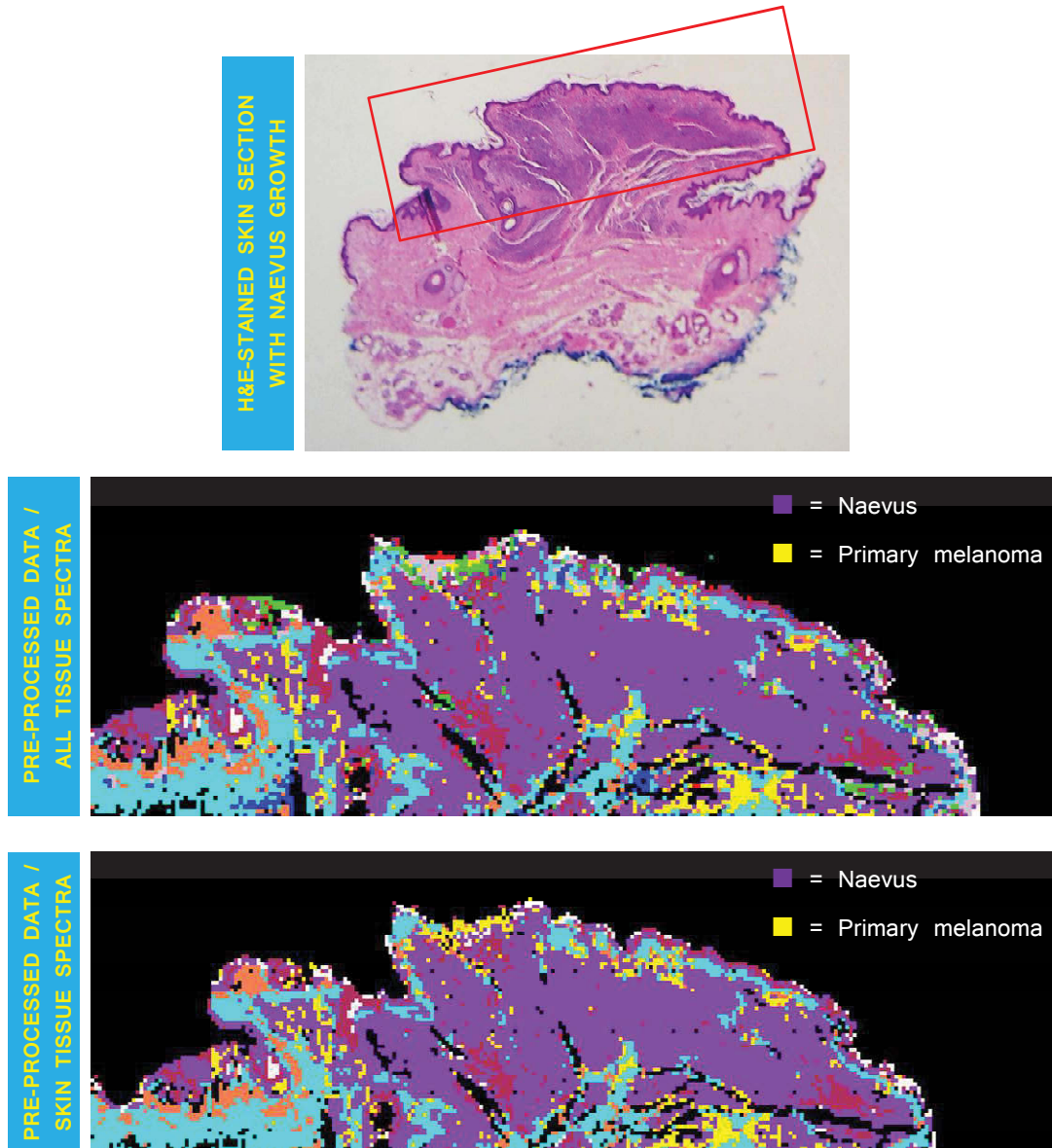


Figure 3-20: Classification of a naevus skin tissue section (deparaffinised, 2nd derivative data block) using reference library construction Method 1 and SAM. The presence of primary melanoma identified (and hence misclassified) pixels in a known naevus sample is a matter for concern and demonstrates clearly that the classification approach is imperfect but the majority of pixels have at least been correctly classified in this example.

Although the classification results for the training images using library construction Method 1 and SAM were generally promising, the variable classification quality obtained from the test sample images was disappointing. Classification quality ranged from excellent to extremely poor. The tiling effect mentioned earlier was noticeable in a number of samples, even where data preprocessing was employed. Truncation of the spectrum to the 1000 - 1585 cm^{-1} region appeared to reduce the tiling effect in the original spectra images in particular, which is to be expected, although in a small number of pre-processed data block images the tiling effect appeared only after spectral truncation. Again, as expected, the deparaffinised tissues classified better than their paraffin-embedded counterparts in general, as did the pre-processed images over the untreated.

Some examples of the classification results achieved for the test samples of different tissue types, showing the variety in classification quality achieved, have been given in Figure 3-20 to Figure 3-23.

- **Library Construction Method 2**

As with the first library construction method, this approach produced SAM-classified training images exhibiting tissue regions consistent with the corresponding H&E-stained sections in the majority of cases for the pre-processed data blocks (an example has been given in Figure 3-23). Nevertheless, as was observed in reference library construction Method 1, the use of the original spectral data block yielded very poor SAM classification results.

In contrast to the previous library construction method, the classification of the test sample images using library construction Method 2 greatly benefited from truncation of the spectra to the 1000 - 1585 cm^{-1} region in most cases (Figure 3-24 presents an example of this). As with the previous method, the pre-processed data block images produced consistently better classifications with SAM than the original spectra. The few exceptions to this occurred mainly with paraffin-embedded tissue samples (an example has been given in Figure 3-25).

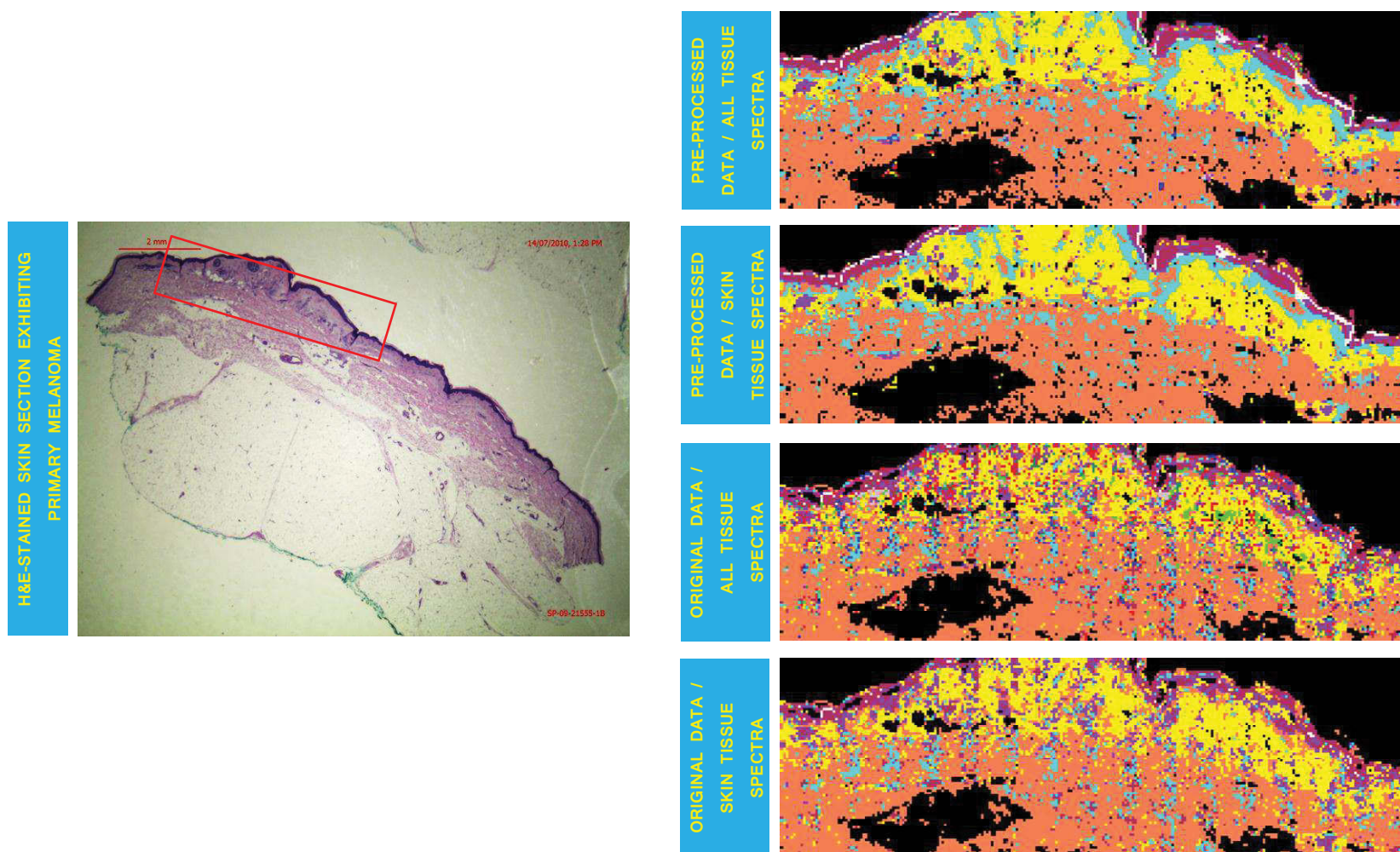


Figure 3-21: An example of a test skin tissue with primary melanoma present sample image exhibiting good quality classification using library construction Method 1 and SAM. Unfortunately the ‘tiling effect’ may also be observed to varying extents in the classified images.

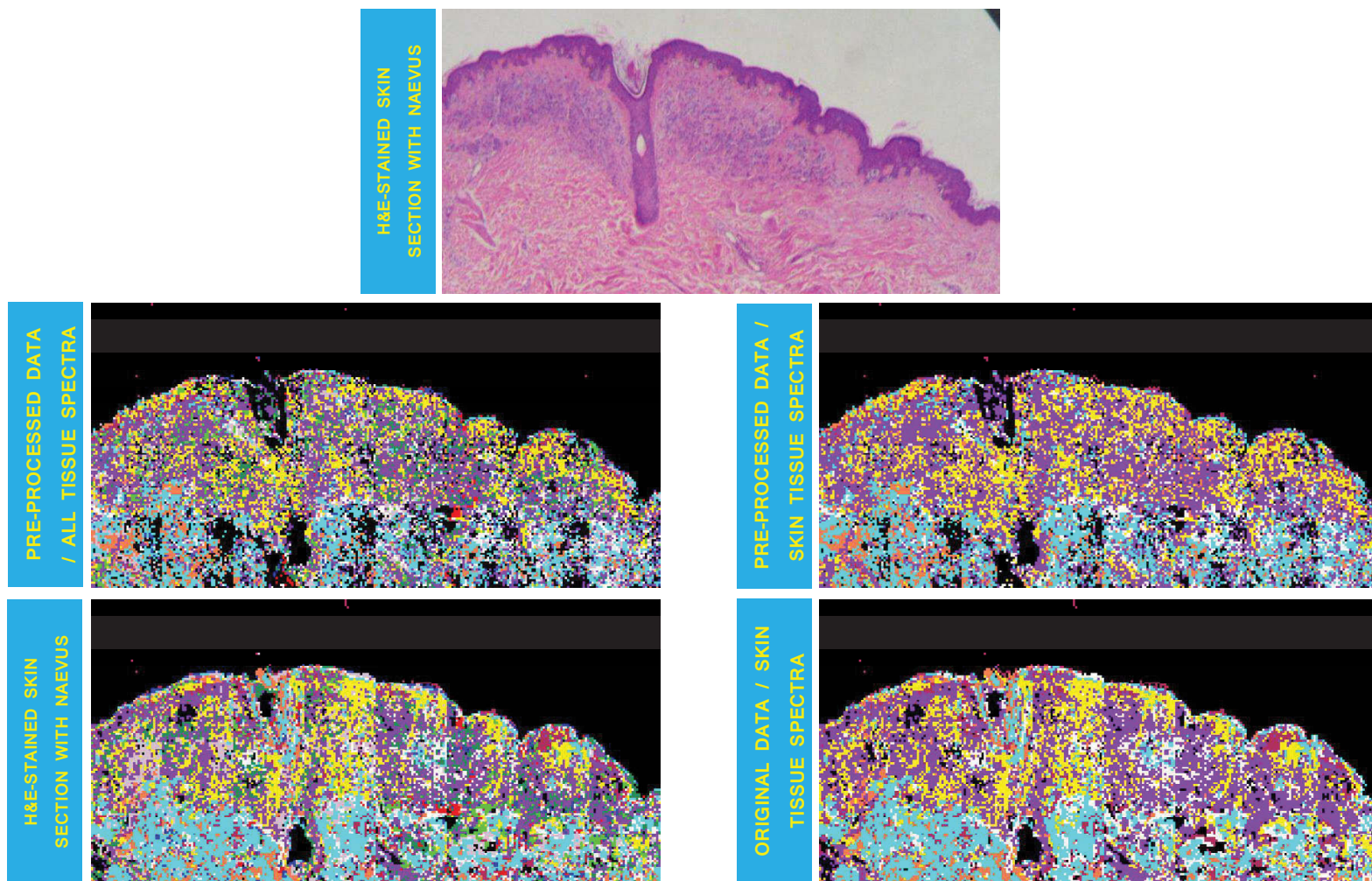


Figure 3-22: An example of a poorly classified test sample (skin tissue section exhibiting naevus growth) using the library construction Method 1 and SAM. A large number of pixels have been misclassified as primary melanoma (yellow).

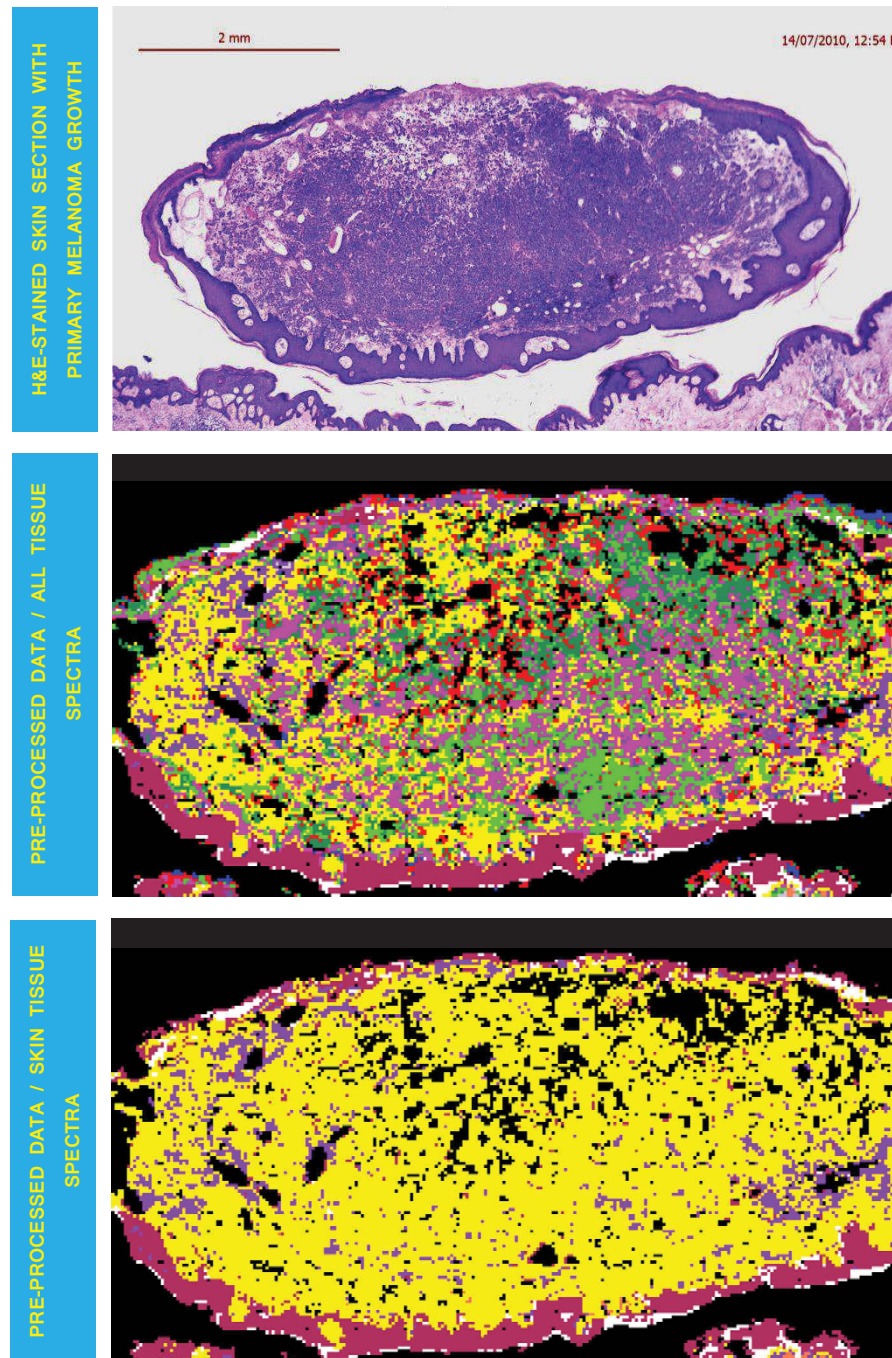


Figure 3-23: Classification of a primary melanoma skin tissue section (pre-processed data block) using library construction Method 2 and SAM. The use of the spectral library containing all tissue spectra clearly does not perform as well as the tissue-specific library, although the poor classification observed in the 'all' library for the training image classification was not so pronounced in the other samples examined.

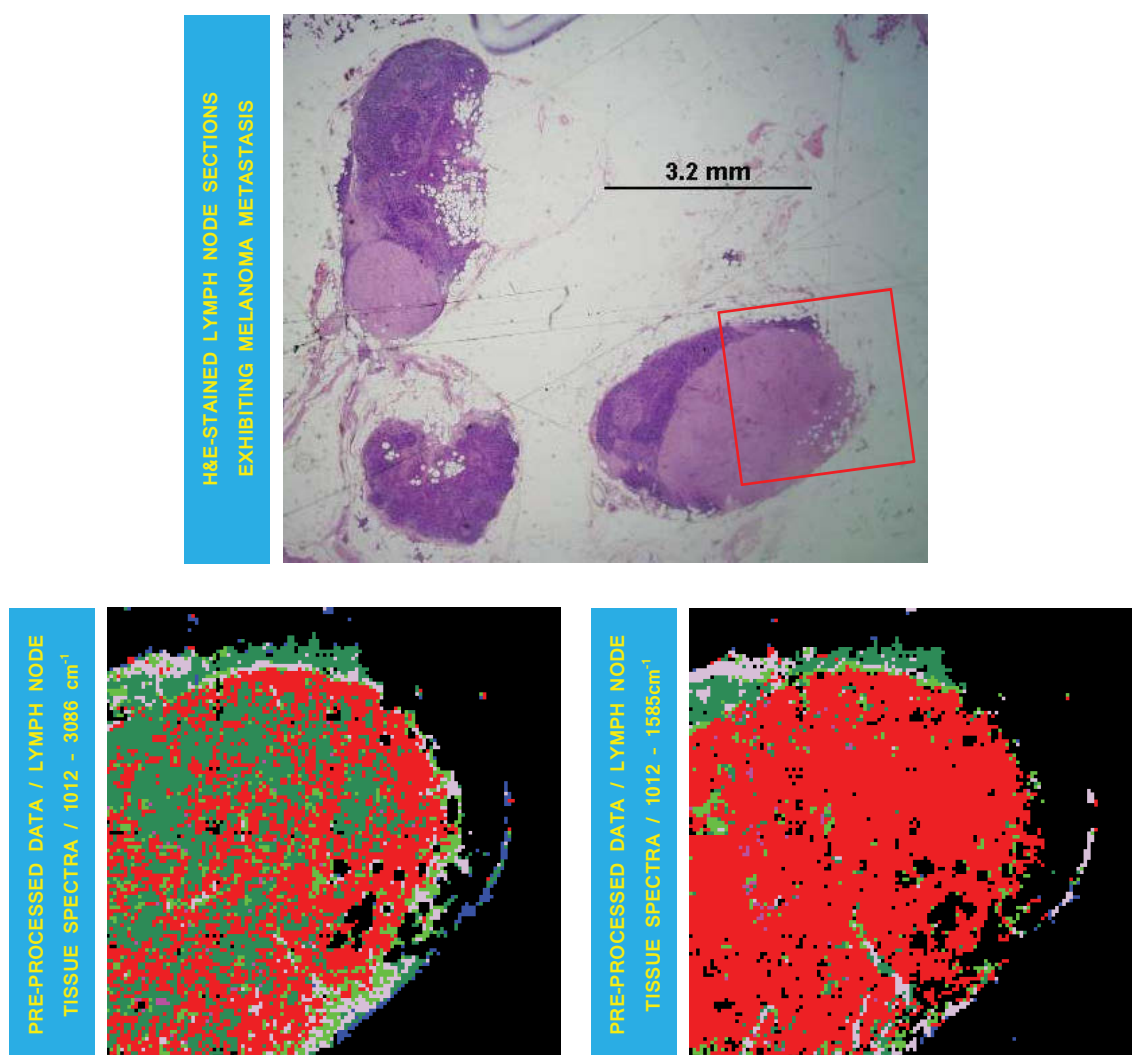


Figure 3-24: SAM classification using library construction Method 2 of a lymph node sample exhibiting melanoma metastasis (pre-processed data block). Red pixels indicate spectra classified as metastatic melanoma. Cortex spectra sampled from lymph nodes exhibiting metastatic melanoma involvement are indicated by pixels sea green in colour. Truncation of the image spectra to exclude the amide I peak resulted in overall classification improvement in a large number of the samples examined using this library method.

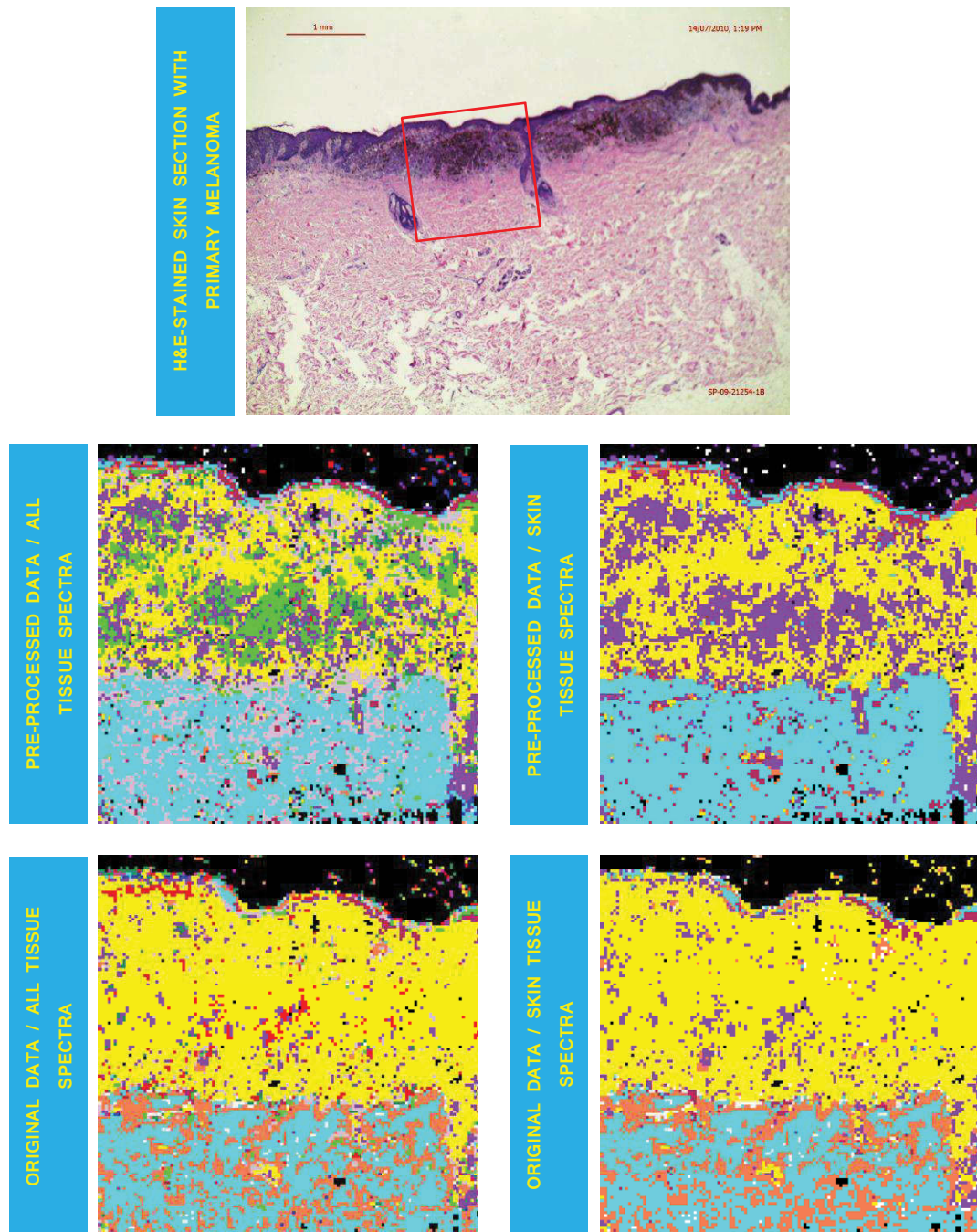


Figure 3-25: An example where the original data block produced a better image classification than that of the corresponding derivative data block. This result was generally uncommon and observed only in paraffin-embedded tissue images.

Compared to the results observed for library construction Method 1, the tiling effect was not observed as often using this library construction method and when it did occur it appeared to a lesser extent.

- **Library Construction Method 3**

Unlike the previous two library construction methods, the third one was only expected to have a limited success rate of SAM classification considering that the library spectra would be comprised of mathematically-derived component 'endmember' spectra extracted from pixels of mixed spectra. In addition to this, VCA assumes the presence of some spectrally 'pure' pixels in each image classified to assist in endmember determination, but in biological tissues these may be unattainable. Although selecting library spectra using VCA is sound in principle, assuming that there are pixels in the image of singular cell type (i.e. the pixel does not contain spectral contributions from different types of component cells or tissues), utilising SAM to then classify images may be of limited value unless the pixel spectra are comprised primarily of the endmember spectra (and are thus more spectrally similar).

Commencing with the reference library spectra collection, it was clear that there would be difficulties in using the VCA algorithm. For a large number of the training samples (approximately 13%) VCA image classification could not be successfully performed and no endmembers were extracted. For those remaining samples, where VCA did produce images consistent with the corresponding stained sections, representative endmembers could not be identified for all of the expected different tissue regions in all cases, resulting in the production of a library consisting of a smaller pool of spectra than the HCA and ROI-based collection methods. Both the original and pre-processed data blocks were used to obtain endmembers, with the number of endmembers required to visualise the expected tissue regions relatively consistent between both original and derivative data block images as well as paraffin-embedded and deparaffinised tissue samples. A summary of the endmember values required to classify the training images has been given in Table 3-8.

Table 3-8: Range of endmember values required for tissue region identification in the training sample images for reference spectra library construction Method 3.

Tissue	Paraffin Present?	Endmember values for Original data block		Endmember values for Pre-processed data block	
		Range	Average / Median	Range	Average / Median
Lymph node displaying metastatic melanoma	No	10 - 15	11 / 10	10 - 15	11 / 10
	Yes	10 - 15	12 / 10	10 - 20	13 / 12
Normal lymph node	No	12 - 20	16 / 15	10 - 15	12 / 11
	Yes	10 - 20	15 / 15	10 - 20	12 / 10
Primary melanoma skin tissue	No	8 - 20	14 / 15	8 - 20	12 / 10
	Yes	6 - 25	11 / 9	6 - 20	10 / 10
Naevus skin tissue	No	10 - 20	14 / 14	10 - 14	11 / 10
	Yes	10 - 20	12 / 10	8 - 23	12 / 10

The results obtained for the SAM classification of the training and test sample images were generally poor (an example is given in Figure 3-26). Often the tissue misclassification was extreme (poor tissue region definition as well as pixels incorrectly classified) and only a small number of images were produced that were considered consistent in appearance with their corresponding H&E-stained sections. Truncation of the spectral range to remove the amide I peak did produce some improvement in the image classifications, but the improvements were in general not sufficient to correct for the large amount of misclassification occurring.

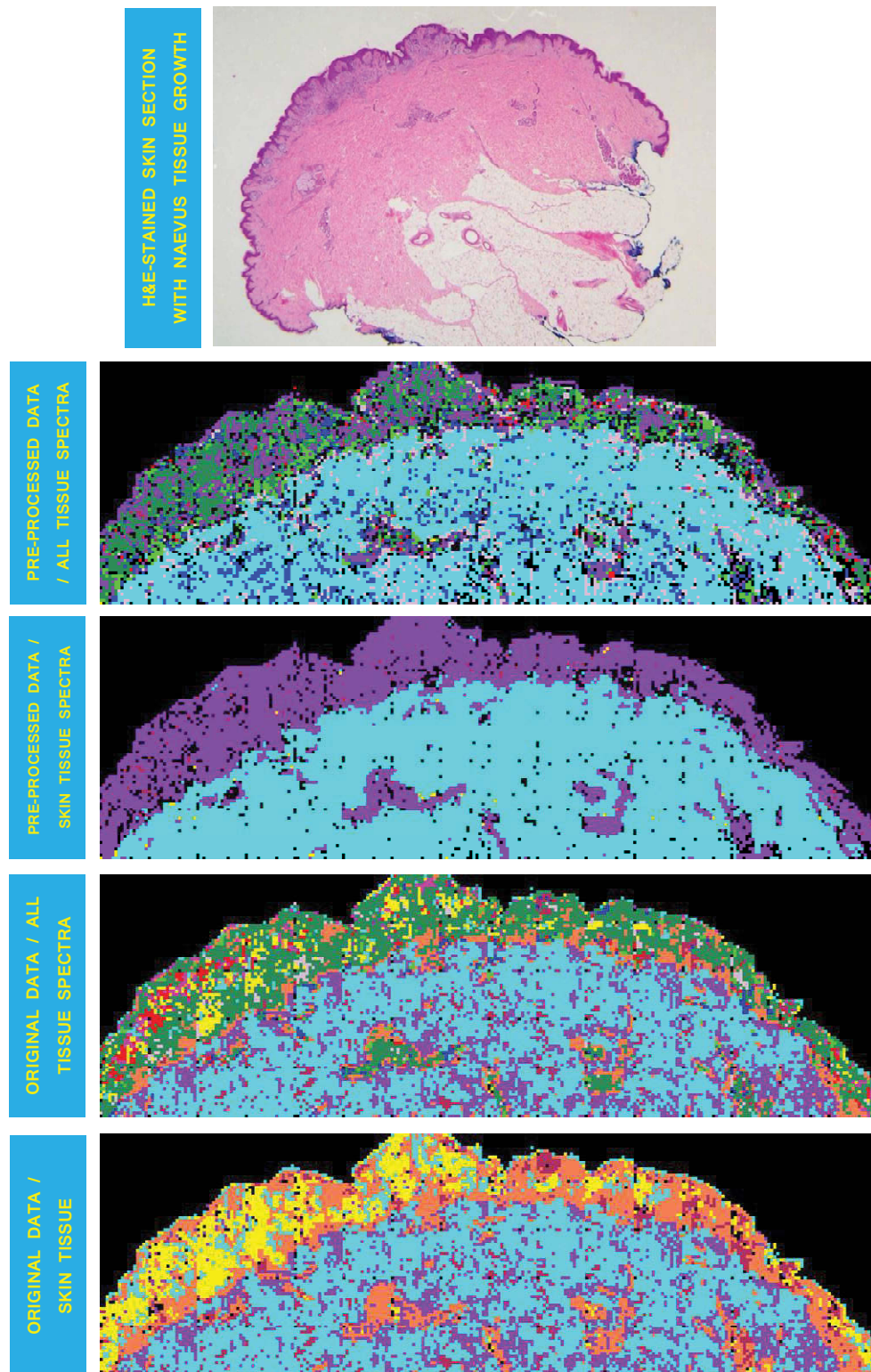


Figure 3-26: A naevus tissue sample processed using library construction Method 3 and the SAM classification algorithm. Purple pixels indicate classification as naevus tissue, yellow as primary melanoma. Note the generally poor overall tissue classification for both data blocks and in particular the original data.

3.2 DISCUSSION

3.2.1 Reference Spectra Analysis and Comparison

A number of infrared imaging studies of cancerous tissues have indicated that the spectral changes observed between the normal and diseased samples may in fact be related to a number of factors such as overall divisional activity of the cells, localised inflammation effects and the ratio of dividing to non-dividing cells.^[5] As mentioned earlier, a cursory inspection of the various average spectra for the various reference library tissue regions highlights how subtle the infrared variations are between the different tissues. According to Diem *et al* there is a general loss of structure in the 950 - 1100 cm^{-1} spectral region which may be associated with cancerous disease, an observation that was also noted in the spectra collected in this study.^[22] Weaker absorbance strength in the 1000 - 1500 cm^{-1} region (notably the phosphate peaks) has also been observed in tissues diagnosed with cancer and was also observed in this study, particularly in the case of the spectra obtained from the primary melanoma and lymph node tumours when compared to the 'normal' tissues.^[10,23] According to Sahu *et al*, the overall decrease in absorbance intensity in the 900 - 1800 cm^{-1} region is related to the smaller number of cytoplasmic components that occur in neoplastic tissues due to the increase in the ratio of nucleus to cytoplasm.^[24] Interestingly, the observed decrease in phosphate intensity is contrary to the observations recorded by Hammody *et al* who found that malignant samples have a higher absorbance in relation to the nucleic acid and DNA signals.^[25]

The most notable differences observed in the skin tissue spectra were between the dermis and the epidermal/naevus/primary melanoma tissues. The dermal spectra exhibited the greatest number of peaks with bands at 1200, 1270 and 1333 cm^{-1} that were not as clearly observed in the remaining skin tissue spectra, as well as a strong absorption at 1230 cm^{-1} which may be attributed to the PO_2^- anti-symmetric vibrations (see Figure 3-27).

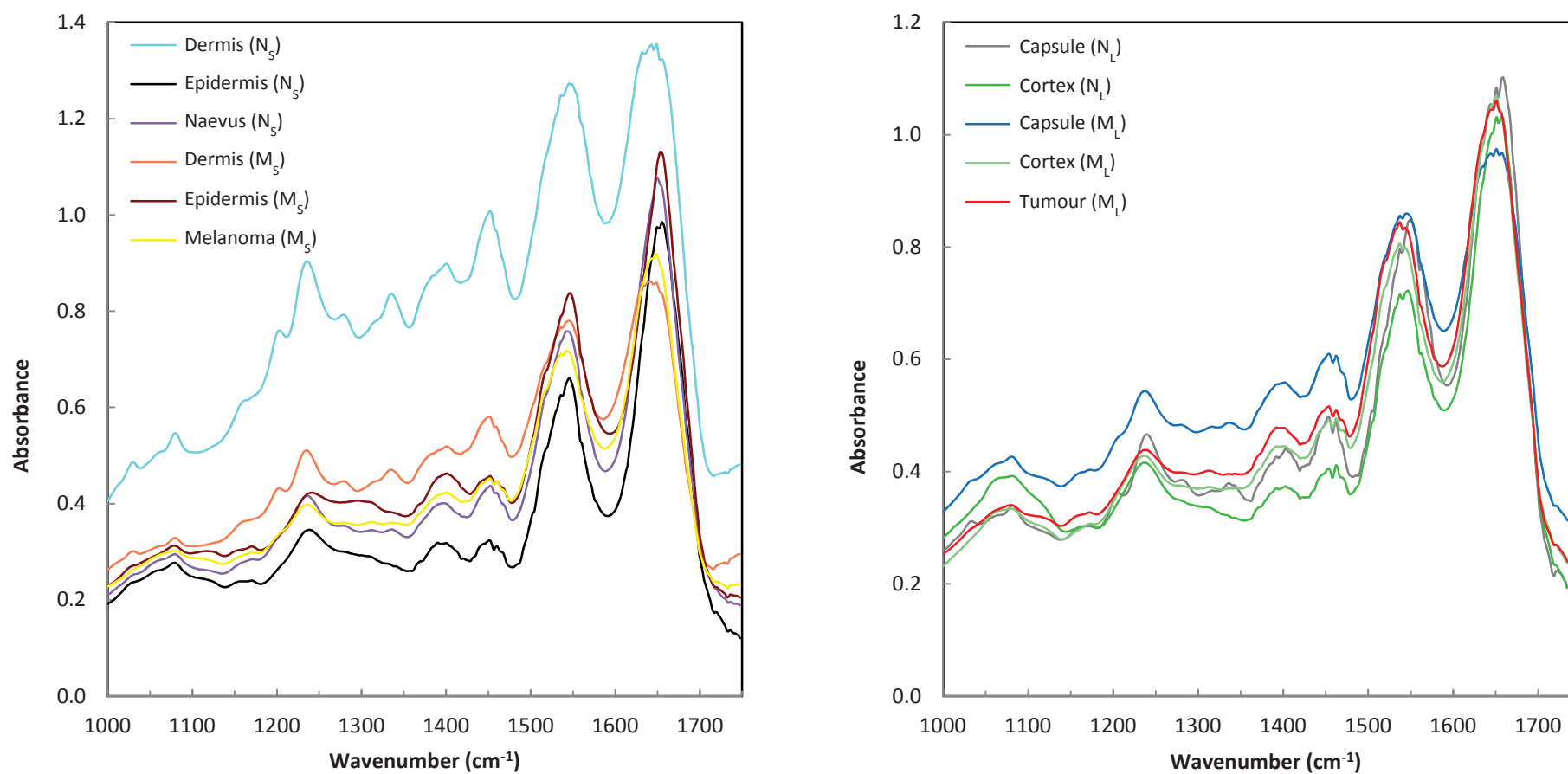


Figure 3-27: Original (i.e. unprocessed) average spectra of the skin (left plot) and lymph node (right plot) spectra obtained from the training tissue sample images. The spectra have not been subjected to any form of smoothing or normalisation at this stage and is in its most raw format.

According to McIntosh *et al*, collagen presence is generally associated with peaks at 1035, 1080, 1204, 1240, 1280 and 1330 cm^{-1} and may be observed in the dermis (which is naturally high in collagen content) but not in epidermal tissue or cultured melanoma cells.^[26] In addition to this, the ratio between the two peaks at 1390 and 1445 cm^{-1} (attributed to CH_3 symmetric and antisymmetric bending of the protein methyl groups respectively) was significantly different compared to that observed in the remaining skin tissues, with the former peak considerably weaker than the latter.^[15,23,25] Also, the peaks at 2916 and 2950 cm^{-1} were observed to be less distinct/prominent in the dermal tissue compared to the remaining skin tissue spectra.

For the epidermal tissue, the two CH_3 bending peaks were approximately the same height. In the naevus and primary melanoma tissue, the 1390 cm^{-1} peak was again smaller than the 1445 cm^{-1} peak, but the ratio was much smaller compared to that found in the dermal tissue. The amide I to amide II peak ratio was the smallest for the cancerous tissue. This drop in amide I band intensity and gross change in protein composition is consistent with the findings of other researchers regarding malignancy, which some infer is related to a decreased protein content in the melanoma which in turn is a result of the enlarged nuclei often observed in tumour cells (and therefore a decreased cytoplasm/nucleus ratio).^[11,27,28] According to the findings of McIntosh *et al* also, the intensity of the nucleic acid absorption is similar in epidermis and melanoma tumour spectra; this is consistent with the spectra obtained in this study.^[28]

According to Hammody *et al*, melanoma may be differentiated from epidermis based on nucleic acid, tyrosine, amide II and phosphate related parameters.^[25] Results consistent with this were observed in this study, with the greatest difference between epidermal and melanoma spectra occurring in the CH_3 symmetric and anti-symmetric bending regions as well as the amide I to amide II ratio. The large number of variations in spectral features between normal, malignant and dysplastic melanocytes obtained from skin samples has also

been reported in a paper by Tosi *et al.*^[10] Unfortunately, unlike the results achieved by Ttayli *et al* and Tosi *et al*, a clear distinction between melanoma and naevus tissue did not always occur during image classification.^[10,29] However, a much wider region of the spectrum was utilised in this study with no specific bands or narrow regions particularly targeted during spectral classification analysis.

Analysis of the lymph node specific spectra, and in particular the capsule spectra, revealed a number of peaks which were not as prominent, or not present at all, in the tumour and cortex spectra. These peaks occurred at approximately 1038, 1070, 1200 and 1327 cm^{-1} and may be attributable to the presence of collagen^[30,31] which is not surprising given that the capsule is composed of dense connective tissue (and therefore collagen), whereas the cortex mainly contains aggregates of lymphocyte cells.^[32,33] Inspection of the cortex spectra in comparison to the tumour spectra revealed very minimal difference, with the only clear observation being in relation to the amide I to amide II peak ratio, which was higher in the cortex tissue than that found in the tumour tissue. Interestingly the cortex sample obtained from the tumour nodes also exhibited a lower ratio than the normal cortex value, perhaps indicating that this region had overlap with the tumour spectra (this result was consistent for both the HCA and ROI extracted library spectra).

It was also observed that for the lymph node tumour spectra, the peak at 1230 cm^{-1} was less prominent. The peak at 1070 cm^{-1} was also broadened and flattened in appearance, a feature observed in cancerous tissue by Bird *et al*, although a similar feature was also recorded in the cortex spectra.^[11] As expected spectra obtained from the lymph node capsule spectra exhibited prominent peaks in the protein region of 1200 - 1300 cm^{-1} consistent with increased collagen content in relation to the other lymph node tissues sampled.

3.2.2 Spectral Similarity Investigation

The purpose of using SIMCA and PCA/LDA analysis to inspect the reference library spectra was two-fold. Firstly, purely as a quick quality control check, performing the chemometric analysis on the reference spectra ensured that all spectra were imported into the correct reference library (i.e. only pre-processed data were present in a pre-processed library, etc.). This step minimised another potential source of human error which might be difficult to detect at a later stage during image classification.

The second reason for performing a chemometric analysis of the library spectra was to produce additional data, such as modelling and spectral comparison information, which may be then used to assist in assessing the variation between spectra from different tissues as well as the limitations of the spectra compiled for each library. This approach was also performed by Tosi *et al* to examine the classification quality of different tissue spectra as well as the spectral similarity of the various tissues sampled.^[10]

Analysis of the SIMCA data obtained from the various libraries, as well as that of the PCA/LDA, show the strong spectral similarity between the various cell types sampled. As may be observed from the data in Table 3-2, SIMCA classification of the reference library spectra for library construction Methods 1 and 2, based on the four tissue types (lymph node with melanoma metastasis, normal lymph node, skin tissue with primary melanoma, and skin tissue with naevus growth), indicated that good discrimination could be achieved between the four tissue sources (85 to 99% correct classification accuracy). These values dropped for the VCA-derived library spectra, which only produced correct classification accuracy values of between 58 to 80%. Inspection of the values for correct classification of library spectra using PCA/LDA given in Table 3-3 revealed a similar trend, with the third reference library spectra collection method performing poorly in comparison to the other two.

An examination of the canonical distribution function output graphs obtained for library construction Methods 1 and 2 displayed a loose clustering of samples around their respective group centroids and a considerable amount of overlap in the cloud of samples for each group (an observation also visible in the PCA/LDA discriminant score function plot provided earlier in Figure 3-15). Interestingly the same sample groups were not always the closest positioned centroids, nor were the centroids of spectra from the same tissue type (i.e. metastatic and normal lymph nodes) necessarily found closest together. The reason for this is most likely due to the fact that the spectra for each of the four groups were from a combination of different cellular types, and hence overlap would be expected. What these results did indicate was that there is not a clear and easy distinction in the spectra obtained from the skin as opposed to lymph node tissue images. This observation was supported by inspection of the modelling and discrimination power graphs (such as those given in Figure 3-13 and Figure 3-14) from the same data's SIMCA analysis results which did not indicate any particular variables which may be helpful for spectral discrimination.

The subsequent analysis of the twelve nominated tissue groups sampled from the training images using the various library construction methods provided more of an insight into the discrimination of the infrared tissue spectra collected. Once again, the SIMCA and PCA/LDA analysis revealed that the VCA-gathered reference spectra possessed limited discrimination capability, which is interesting considering the fact that they are supposed to represent 'pure' and representative spectral components. Based on the SIMCA analysis results (provided in Table 3-5), the reference spectra library collection method which produced the best classification (93% correct) of tissue spectra was that of library Method 2, utilising the "full" spectral region of $1012 - 3086 \text{ cm}^{-1}$ in the pre-processed spectra of deparaffinised tissue. The PCA/LDA results given in Table 3-6 also support the quality of the library Method 2 collection method, although the original spectra following spectral truncation collected from paraffinised tissue would appear to produce the best spectral discrimination. For this study, original spectra were not exposed to any form of smoothing

or normalisation prior to library construction, although all spectra were normalised prior to SIMCA and PCA/LDA analysis in Pirouette, and it would be interesting to analyse the impact of preprocessing (aside from derivatisation) on the classification results that may be obtained.

Interestingly the PCA/LDA data analysis indicated that for the HCA-derived reference spectra the use of pre-processed or paraffin-removed spectra did not produce any clear advantage, but truncation of the spectral range did, a result mirrored in the SIMCA analysis for the derivative spectra only. However, for the ROI-selected spectra, LDA and SIMCA classification was improved when using deparaffinised tissue, but not with spectral truncation. Graphical examples of the group separation achieved using PCA/LDA on the ROI-selected spectra (reference library Method 2) have been given in Figure 3-16 and Figure 3-17 and show that while significant overlap is observed between the twelve tissue groups, spectra from tissue of the same sub-classification type (e.g. epidermis from both skin tissue containing primary melanoma and that only with a naevus component) are grouping together and in some cases are distinctly separated from the other tissue sub-classes.

3.2.3 Image Classification

3.2.3.1 *Library Construction and Image Classification Observations*

Based on the results obtained using a combined library approach (i.e. all tissue types) compared to a library containing only spectra from that of a similar tissue type (i.e. lymph nodes or skin tissue), it is clear that the combined library containing spectra from all tissue types does not perform as well as would be hoped with the SAM algorithm for image classification. Examination of the results obtained for both the training and test sample images using the combined library unfortunately show a reduced performance by SAM to consistently distinguish highly spectrally similar tissues.

The limited ability of the SAM algorithm to discriminate using all tissue spectra is an interesting problem when one considers that the two tissue types (skin and lymph node) are so physiologically different in their biological roles and architecture. One might reasonably expect a high collagen content in the skin tissue spectra as opposed to that obtained from the lymph nodes which are comprised primarily of lymphocytes. Also, in a study conducted by Kong and Park on mouse skin tumours using fluorescence, it was observed that in relation to the spectral signatures of normal and malignant tissues, there is a decrease in collagen and elastin as the tissues move from normal to malignant, as well as a decrease in nicotinamide adenine dinucleotide (NADH) levels.^[34]

It is clear from the image classifications obtained using SAM, as well as the inspection of the library spectra conducted in Section 3.1.4, that some tissue spectra are more similar than others (primary melanoma-coloured pixels appearing in lymph nodes exhibiting melanoma tumour growth). In general most images demonstrated correct tissue spatial regions (clear architectural differentiation between dermal and naevus regions for instance) although within-region classifications were often a mixture of correctly classified pixels and their commonly observed misclassification pixel colours. Misclassifications were observed to be more extensive using the original data as opposed the pre-processed image spectral data blocks using the one library approach, a result consistent across the library construction methods. Spectral truncation to remove the amide I peak and potentially minimise the impact of the dispersion artefact on classification was shown to be of assistance in some cases, improving tissue classification. Nevertheless, the results did not indicate a consistent positive effect across all test samples for any of the reference library construction methods employed.

For the extraction of the reference spectra from the training images, HCA performed well for the majority of the original and pre-processed images. In general, the test images subjected to pre-processing (second derivative, vector normalised) classified the best using

library construction Methods 1 and 2. The difference in quality between the two library methods regarding tissue classification was relatively small (when compared to the VCA results), with Method 2 performing slightly better overall. This is most likely due to the exclusion of boundary pixels which would be comprised of cells from the neighbouring regions as well as perhaps cells exhibiting a transitional phase (this latter possibility applies to primary tumours only).

Based on the results obtained in this study, the VCA approach taken underperformed compared to the alternative methods explored. As mentioned earlier, VCA experienced limitations in obtaining endmembers for each of the expected tissue types present in the training samples, particularly when the original data block was used. However, unlike the HCA method, no preliminary study was performed using VCA to determine the optimal preprocessing parameters as well as to investigate the most effective spectral range for training image classification. The results obtained here indicate that further studies need to be conducted to understand why some tissues did not classify well in comparison to others and which, if any, data preprocessing methods affect the classification capability of VCA.

The quality of a number of the training image classifications using VCA obtained indicate that this high-speed algorithm is capable of some tissue discrimination, a result supported in the literature.^[35-37] However, each of these studies examined only performed within-image classifications, and did not attempt to use VCA-derived endmember spectra to then classify a separate test image. For this study, a spectral unmixing approach was not used on the test samples. This means that the reference library spectra are in a sense component spectra which the SAM is then trying to compare with mixed spectra. Ideally one needs to perform spectral unmixing on the test images and then compare the endmembers obtained to those gathered from the training samples and look at similarity measures between endmembers. This was not attempted due to a lack of time, but given the speed of VCA it is not an unreasonable approach to take in future studies.

3.2.3.2 Impact of Paraffin on Image Classification

As may be observed in Figure 3-28, the paraffin peaks are inconveniently located in the typical regions of interest (the C-H stretching region of $2800 - 3000\text{ cm}^{-1}$ and the C-H deformation region of $1350 - 1490\text{ cm}^{-1}$) for biological tissues and as Ly et al point out, may in fact mask the molecular vibrations of importance in the tissue being analysed.^[21]

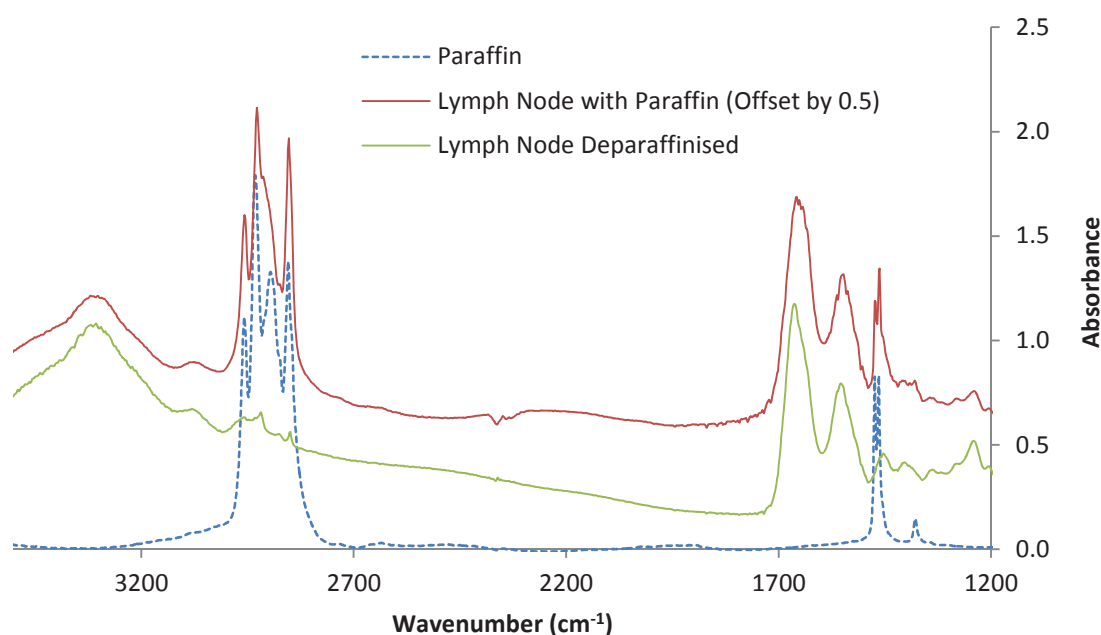


Figure 3-28: A comparison of spectra taken from the approximately the same region of a lymph node prior to and following deparaffinisation. The paraffin spectrum was obtained from the tissue block residue present on a tissue section slide prior to deparaffinisation. The large bands attributable to paraffin are an obvious problem considering the frequencies of interest in biological tissues.

Based on the results of this study, as well as observations by Ly *et al*, it is clear that the sample variability is less than that of the paraffin component and that the paraffin contribution needs to be removed by some means.^[21] As mentioned earlier, one has the choice of physically removing the paraffin from the tissues, and consequently risking the

removal of biochemically important components from the sample during the washing process, or somehow subtracting paraffin's spectral contribution. One form of paraffin subtraction was suggested in a paper by Ly *et al*, in which the 'electronic' removal of paraffin in the tissue using a process, known as extended multiplicative signal correction (EMSC), was performed.^[21]

It is clear from the HCA analysis of the training images that differentiation between different tissues within a sample is possible, but the results obtained in this study clearly show that constructing a spectral reference library using unprocessed paraffin-containing spectra is not recommended. The reason for this latter observation may be due to a number of factors such as the varying concentration of the paraffin within each tissue sample (which is difficult to normalise between spectra from different tissue samples), as well as the variation in the possible contaminants present in the paraffin between samples (in some institutions paraffin is re-used when large volumes of tissues need to be embedded). Ideally the images obtained in this study from the paraffin-embedded samples should be subjected to a more specific form of data preprocessing which addresses the regions of the spectrum affected by paraffin (such as the approach taken by Bird *et al* and Tfayli *et al*), or using some form of chemometric paraffin 'removal' tool such as EMSC, and the resulting classification results compared to those obtained from the corresponding deparaffinised tissue images.^[11,21,29]

3.2.3.3 Method Challenges and Future Directions

A number of challenges were encountered in the development of the FTIR CI methodology employed in this study. Areas requiring essential improvement in regards to sample analysis efficiency and quality were easily identifiable: sample spatial resolution limitations, sample image collection times, initial training sample image classification times and importantly correct reference spectra selection. Aside from conducting studies to examine optimisation of the image collection specifications (e.g. minimising the number of scans,

spectral resolution determination, etc.), alternative instrumentation and operating software calculations may also be explored.

One approach to improving spatial resolution has been the development of a 'super-resolution' concept by Offory *et al.*^[38] This technique involves fusing several low-resolution images of the same sample obtained from an FPA detector to form one higher resolution image. The authors claim to have observed a 30% improvement in spatial resolution, although further research remains to be conducted to explore the impact such a method has on images obtained from using a spectrometer with a higher magnification objective and an FPA with a higher density pixel value.

Another approach to improving image collection may involve changing to a different instrumentation setup. For example, Nasse *et al* developed an infrared imaging approach which was claimed to have acquired diffraction-limited high spatial resolution infrared images with very fast collection times by combining twelve low emittance synchrotron beams into one 3 x 4 matrix beam.^[39] Such instrumentation, when commercially available, may make such infrared imaging approaches such as those explored in this study more appealing to large throughput laboratories such as those commonly encountered in public health systems.

While the SAM classification may be performed on large sample images very rapidly, it was the collection of the reference library spectra that was a yet another time-consuming and as labour-intensive step in the methodology used in this study. The HCA step utilised in library construction Methods 1 and 2 meant that further restrictions in file sizes for images were encountered (even when employing a 64-bit version of CytoSpec) aside from those imposed by the instrumentation operating system. Large file sizes in turn meant longer HCA calculation times (in the region of hours, and depending on the computer used, sometimes days). In addition to this, HCA is costly in regards to the demands placed on computer processing resources. Despite technological advances in computing, the reference

library gathering techniques employing HCA remain a limiting factor in performing quick and extensive optimisation studies at this point in time.

Another obvious and major limitation of the method developed in this study was the author's lack of pathology knowledge. This problem was identified at the outset but could not be addressed during the course of this research as pathology is a specialised field requiring years of specific training and practice. Although assistance in the examination of the samples provided was obtained, this could by no means replace the quality of tissue region identification that a trained pathologist with years of experience could perform. Considering that the reference library spectra were collected by an individual with extremely limited pathology knowledge, the results obtained in this study were very encouraging.

The concept of using the "gold standard" of pathology (i.e. the findings derived by a certified pathologist) to identify targeting regions of tissue samples is itself problematic. For example, is it possible that a tissue exhibiting no physical changes observable using conventional histopathology techniques may in fact be in the early stages of disease at a biochemical level? If one uses such tissues as exemplar 'normal' then the risk of skewing any reference library is high. Also, diagnosing diseases such as cancer can be even more difficult if there are only isolated cells exhibiting irregular growth interspersed amongst a mass of 'normal' cells. Such cancerous cells may be overlooked by the pathologist, or if found, will be difficult to resolve in an image if the spatial resolution is incapable of resolving to the single cell level. Hence, any imaging method requires most importantly the construction of a reference spectral library of a considerably large sample number and extensive breadth, which in turn means obtaining access to the many tissue states that inform the various diagnoses of the cancer. Ideally the diagnosis for each exemplar should be independently verified by a second pathologist to ensure that, within the limits of human perception and knowledge, the sample is a truly suitable reference standard.

Another problem observed from the study of the classified images was that of the tiling effect. As mentioned earlier in Section 3.1.3, the causes of this problem are fundamentally related to instrument performance and the manner in which background images were size-restricted. While stage and infrared spectrometer calibrations were performed prior to every image being collected, the extent of the tiling effect was observed to vary between samples across time. Preprocessing the data did, to some extent, remove some of the tiling effect, but in a number of images the problem remained. This would suggest that the instrument operating variation was considerably greater than the within-sample variation, and that preprocessing of the images could only remove or minimise the tiling effect within limits.

According to Kong and Park, as the spectral angle mapper is measuring the size of the angle between pixel and target spectra to assess similarity (utilising vector direction), it should be relatively insensitive to variations in illumination which only change vector length.^[34] Gaining access to an infrared source which completely and evenly illuminates the sample area, as well as ensuring that the stage employed is as near to perfectly level as possible and collecting a background image of equal size to the sample image, would hopefully assist in addressing the tiling problem in any future studies performed.

Although the tissue sample thickness of 6 μ m was selected based on observations regarding spectral quality in the preliminary component of this research, it was clear from the spectra obtained that the dispersion artefact (the derivative-like line shape observed on the high wavenumber side of the Amide I band) was present in a large number of the spectra collected. The artefact is mainly observed in mid-infrared spectra collected in transflection mode, particularly in spectra collected from the edges of tissues and cells, and has been attributed to a combination of physical phenomena such as resonant Mie scattering, electric field standing wave (EFSW) artefacts and ultimately sample thickness.^[40-44]

Besides causing the observed sharp, sloping baseline, the dispersion artefact also affects the amide I to amide II band ratio, and shifts the amide I band. Algorithms have been

developed to reduce the impact of, or in some cases, remove the effect with some success and have been even built into software such as CytoSpec.^[41,45] The effectiveness of these algorithms is still being assessed and for the purposes of this feasibility study it was decided that such additional data preprocessing would be omitted to reduce the number of data pretreatment steps at this stage. A future assessment into the dispersion artefact correction algorithms available in the literature and their effectiveness in potentially improving image classification using SAM is highly recommended.

During the HCA and SAM classification of tissue images it was often observed that 'poor' classifications occurred in images possessing noisy spectra. The primary source of the noise was difficult to conclusively identify in each image, and it is believed that it was often a due to a combination of factors such as instrumental noise (variations in the global source, etc.) and water vapour. This latter factor was difficult to control, particularly in the Sydney region even with the assistance of constant air conditioning. As Wood and McNaughton point out, although the infrared spectrometer is purged with dry air constantly, the area above the sample beneath the microscope objective is not.^[5] Variations in day to day humidity levels in turn mean that the water vapour contribution could in turn change during the course of a day, and in the case of long imaging times, across the image itself. This problem would be further compounded by the fact that the background image was often collected hours before the completion of the sample image collection. In a presentation by Lasch in 2007, the construction of a chamber around the microscope sampling area is shown to reduce the water vapour contribution significantly.^[46] Unfortunately such a device was not available to the author during this study.

In relation to the classification algorithm aspect of the FTIR CI approach, future work could focus on the testing of recently published alternative chemometric approaches. In an infrared imaging study conducted by Tan *et al* on exfoliated oral mucosa cells, the authors analysed their image data using a combination of multivariate chemometric tools, such as

constrained hierarchical clustering analysis and band-target entropy minimisation (BTEM) curve resolution algorithm.^[47] Such an approach exploited the spectral vector properties (SVP) of the infrared spectra and during this study two novel unsupervised clustering algorithms were developed and tested. Tan *et al* also propose a method for the removal of outlier spectra (such as the random noise spikes with an absorbance value of '8' mentioned earlier in Section 2.2.4). Another recent development is the application of frequent pattern mining, a method in which data is first discretised, which then permits a form of feature selection that identifies the sub-groups of data most useful for classification.^[19] This approach was effectively applied to segment histologic classes in prostate cancer specimens and showed considerable promise.

3.3 CONCLUSION

According to Binder and Menzies, who explored the concept of computerised cancer recognition, "for an automated instrument to be useful in an expert setting it should achieve a high sensitivity (exceeding 90%) and have a benign:melanoma ratio lower than 12."^[48] Although the results obtained during this examination of human tissue sections for melanoma diagnosis using FTIR CI in combination with the SAM algorithm did not consistently achieve such high automation standards, they have shown that this imaging analysis approach certainly warrants further investigation. Like many chemical imaging methods explored as pathology-assisting tools, there are a number of limiting factors in effectively applying such chemometric techniques, the greatest of which were identified as spectral reference library collection and spectral noise recorded during image collection. Given that this author was in no way skilled in pathological diagnosis of melanoma or human tissues, the fact that some images were produced reflecting the known tissue diagnosis was highly encouraging.

Future work should focus on examining if classification accuracy can be improved when a trained pathologist is enlisted for reference library spectra collection. Exploring faster

imaging techniques, as well as working towards higher image quality (e.g. the use of a synchrotron) and reducing data noise (e.g. exploring apparatus to reduce water vapour present in the spectra, employing the minimum noise fraction (MNF) transform, etc.) should also be investigated. Use of the VCA algorithm for endmember spectra collection, while only examined to a limited extent in this study, produced some excellent tissue classification images in some instances and its use for reference spectral library construction should be further explored. However, the use of endmember spectra obtained from the VCA in a subsequent classification routine may require a slightly different data analysis approach to be developed than the one used in this study.

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CHAPTER 4:

WOUND STUDY PART 1

(BACKGROUND AND METHOD)

4.1 WOUND THEORY

4.1.1 Definition of a Wound

An injury, or wound, is defined as a disruption in the normal continuity (anatomic structure and function) of a body tissue, usually produced by physical means.^[1] For example, a wound may be a bruise, cut, laceration or abrasion. The severity of a wound can range from a minor scratch which the bearer may not even sense the existence of, to those of a fatal nature, such as the severing of a major artery or a haematoma in the brain. Wounds are generally classified as either acute or chronic.^[2] Acute wounds in general occur due to a sudden insult to the body, such as that incurred by surgery or trauma inflicted from a gunshot, and generally proceed through the wound healing process in a predictable manner. In contrast, chronic or 'nonhealing' wounds are open wounds that do not heal in a normal or timely fashion and are considered to be clinically stagnant.^[3] Examples of chronic wounds include diabetic ulcers, radiation injuries, infected burn wounds and pressure sores.

The drive to improve on our knowledge of wounds is motivated by the painful truth of mankind's own fragility and the ultimate goal of prolonging life. It is the ease with which humans may exploit this vulnerability in one another that unfortunately necessitates the involvement of medical professionals to not only understand the healing process of wounds in the living, but also in the dead.

Wound recognition, treatment, and in the cases of death, interpretation, has existed, even if in a most rudimentary form, since mankind first became aware of physical harm. Wounds are often classified according to the mechanism involved in infliction, and may be considered as belonging to one of the following types:

- **Incised:** these wounds are the result of sharp force injury, such as that from a blade of a knife or scalpel, which has been moved tangentially across the skin.^[4] Often described as cuts, slashes or slices, the resulting wound generally exhibits regular, clean edges, minimal damage to the surrounding tissues and is longer than it is deep.^[5]
- **Penetrating:** inflicted by a thrust of a pointed object, or by the falling or pressing of a body against a rigid object, and possessing a physical description as being deeper than they are wide, penetrating wounds may be further divided into three categories:^[6]
 - 1. *Puncture:* caused by a sharp, thin object (e.g. syringe needle or snake bite)
 - 2. *Perforation:* caused by a blunt-ended, elongated object (e.g. sharpened post)
 - 3. *Stab:* caused by a flat, sharp object (e.g. knife).

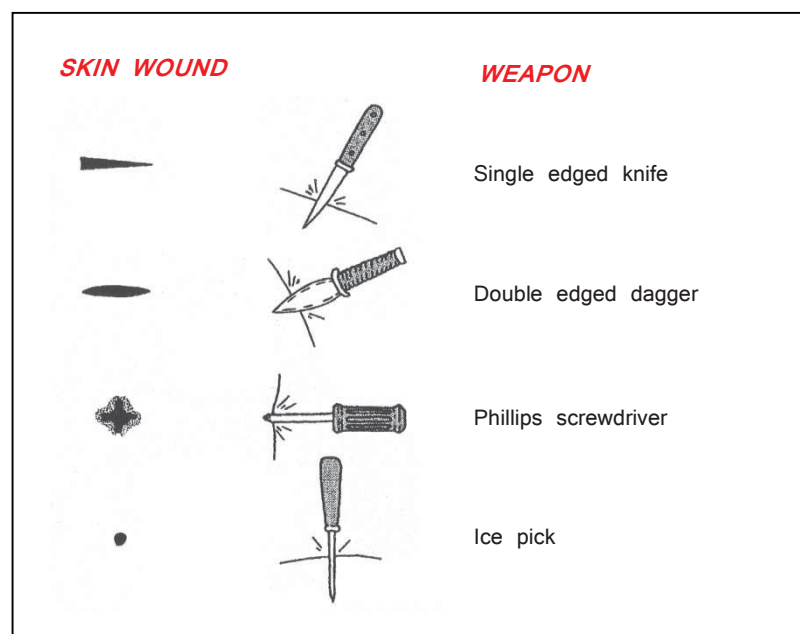


Figure 4-1: Some examples of pointed and sharp edged weapons and the appearance of the skin wounds they produce (adapted from Di Maio).^[7]

Penetrating injuries result in damage to the underlying tissues below the skin and generally produce minimal external haemorrhage. However, in those penetrating wounds which do prove fatal, severe internal haemorrhage almost always is observed.^[8,9]

- **Abrasions:** an injury to the surface of the skin, abrasions are caused by friction which results in the superficial layers being removed and exposing nerve endings.^[10] Depending on the force experienced, and the coarseness of the surface encountered, the wound may be considered deep or superficial.^[11] Abrasions include injuries such as grazes, imprints and scratches, and in some cases may prove to be the only external indication of a severe internal injury having occurred.^[12]
- **Lacerations:** Caused by pressure on the skin surface which inflicts a tearing, shearing or crushing action on the tissue, lacerations are most easily thought of as a split or tear in the tissue. Lacerations are often found in victims of violence such as those suffering injuries caused by blunt force trauma (e.g. the tearing of the scalp tissue which results from a blow to the head from a hammer), as well as individuals subjected to falls or pedestrian/vehicle collisions.^[5,12] Unlike the clean edges of an incision wound, there are often bridges of tissue connecting the wound sides,^[11] and there may be significant tissue loss and bruising.
- **Burns:** extremes of heat can cause cellular injury if a tissue or body is exposed to temperatures above 42.5°C for a sustained amount of time. If a tissue or body is exposed to a degree of heat greater than that compatible with normal metabolism cellular injury will result.^[9] The greater the temperature of exposure, the less time the temperature can be tolerated without damage by the tissue. Aside from extremes of heat, burns can be caused by friction, exposure to chemicals (e.g. acids and alkalis), radiation and electricity.

- **Bruises and contusions:** Unlike the previously mentioned injuries, bruises and contusions involve no skin breakage, and may be a result of blunt physical force on the skin, or the product of some diseases.^[5] Force brought to bear on the skin's surface ruptures the vascular system (usually capillaries) beneath, causing bleeding into the surrounding tissues. Initially a dark red to purplish colour, the bruise noticeably changes colour in lighter-skinned people due to the gradual break down of the haemoglobin of the escaped blood. Whilst often considered the same thing, the term contusion may be used to represent a more severe form of bruise in which greater energy has been inflicted causing the wound to exhibit separation of the tissue layers.^[10]
- **Ulcers:** described as a depressed lesion in the skin's surface, ulcers exhibit a loss of the epidermal surface along with a portion of the connective tissue in the upper dermal region.^[10] Cutaneous ulcers do not simply appear in normal skin but are preceded by another pathologic lesion, such as an infected wound.^[13]
- **High velocity projectile:** this wound type may be inflicted by any missile travelling at high velocity and includes gunshot wounds as well as the shrapnel expelled from an explosive device. The extent of damage to tissue and organs is dependent on a minimum of five variables: projectile mass, projectile shape, projectile construction, projectile striking velocity and the target tissue type.^[14]

Based on the descriptions given above, it is clear that each of the above wound types disrupts the tissue structure in varying manners and to differing extents. In turn, the amount of tissue damage experienced and the impact of this damage on an individual's overall wellbeing will heavily depend on a number of factors, such as the severity of the wound (e.g. one would expect an individual hit by a rapidly moving vehicle to experience more tissue damage than if they were hit with a human fist), the area of the body wounded (organ or superficial tissue damage), the structural integrity of the tissue prior to damage, and the overall health of the individual at the time of wound infliction.

4.1.2 The Importance of Wound Examination and Age Determination

Forensic science may be broadly defined as the application of science to the law.^[15] Although most associate the process of autopsy with the medical pathologist attempting to ascertain the *cause* of death (the injury or disease which initiates the sequence of biological events leading up to death),^[11] the determination of all other factors which may or may not have been involved is also required to assist the investigator. Examining wounds to determine if they are consistent with the investigative evidence, identifying the fatal wound in cases of multiple wound sites, as well as determining how long the deceased could have lived (survival time) after the assault, are all important tasks which may assist in the reconstruction of the circumstances under which wounds were inflicted.^[16] Hence, wound examination can play a critical role in forensic investigations, providing information on not only the cause, but also the surrounding circumstances, or *manner*, of the individual's death and accordingly assist the court in reaching its conclusions.^[9]

According to the text of Gradwohl's Legal Medicine,^[6] the facts one is attempting to ascertain when examining a wound are:

- *whether it was caused before, at the time of, or after death*
- *how it was caused*
- *what caused it*
- *what amount of force was required to produce it*
- *what degree of injury has resulted from it and whether it has influenced death or caused disability.*

An example of the value of detailed wound analysis may be found in a case reported on by Gill *et al.*^[17] In this example, the body of a deceased 53-year-old man was found in a

locked apartment and was exhibiting advanced putrefactive change (e.g. skin slippage, bloating, purged blood, fly/maggot infestation, etc.). An autopsy revealed the presence of three skin defects with similar morphology to stab wounds on the front of the abdomen suggestive of foul play. Further investigations revealed that the skin defects were actually caused by dehiscence of an old surgical wound brought about by the putrefactive gases produced during decomposition and were not antemortem injuries at all. The cause of death was ruled to be an accidental overdose of opiates and demonstrated the need to collate as much antemortem and postmortem information as possible when determining wound etiology.

Forensic pathology is deeply concerned with the time dimension of events, particularly the sequencing of the various activities conducted by the victim in their final moments of life. The time of an individual's death can be critical in assisting the investigator to identify or discount potential suspects, and ultimately may assist the court in reaching a conclusion as to an accused individual's guilt or innocence. Sometimes of greater importance to the investigator is the timing of the injuries incurred, even those of little or no medical significance, particularly in the reconstruction of events where perhaps numerous offenders are involved or when the chronology of events supplied by a suspect is questionable. Shkrum and Ramsay cite a study in which it was found that suicide victims averaged 14.4 sharp force injuries (stabbing and/or cutting injuries), whereas homicide victims possessed a mean of only 8.8.^[18] Therefore, identifying the injury responsible for the death of an individual may involve the examination of numerous wound sites inflicted over an unknown time interval. In cases where an individual may have suffered a number of potentially lethal injuries, obtaining an estimate of the survival time for each may assist in ascertaining the sequence in which they were sustained and assist the jury in apportioning blame to the correct defendant.

Raekallio cites an example where the timetable of wounding was crucial in a criminal investigation.^[19] A man in a jealous rage hit his fiancée on the head with a stone and mistakenly believed he had killed her. In an effort to cover up his crime, the man placed his fiancée's body on a railway track in an effort to simulate a suicide or accidental death. A train inflicted fatal injuries two hours later. In this example, the time between the man's actions and that of the train would be difficult to elucidate without application of specialised wound ageing techniques, and hence potentially the nature of the man's crime may have gone undiscovered.

Polson *et al*/ describe a case in which the ability to conclusively determine if a wound occurred ante or postmortem led to the judge dismissing an injury from a criminal hearing.^[12] In this case, the body of a two-year-old child was observed to have sustained numerous bruises, thought to be caused by a studded leather belt, with the cause of death being given as due to a subdural haemorrhage. Also observed during the autopsy were lacerations on the gums and the absence of two teeth. Close visual examination of the gum in the region of the missing teeth did not reveal any bleeding, but based on this observation it was impossible for the pathologist to determine if the child had been alive or dead at the point in time when these injuries had occurred.

Wound timing information may prove invaluable in court situations, assisting in the answering of such questions as whether a graze was inflicted on an individual in a struggle prior to death, or if such wounds were a result of movement of the body after death. Therefore, the timing of wounds is critical in establishing the sequence of events surrounding such criminal activities as murder and assault, and the development of examination protocols and techniques sensitive enough to detect the ageing of the wound inflicted will assist the pathologist in reaching their conclusions.

4.1.3 The Wound Ageing Process

Time of death studies may involve the application of numerous scientific fields, such as pathology, physical anthropology, entomology and botany. Although various biochemical and physical indicators have been documented in the literature, the conviction with which a forensic pathologist may assert the order of events becomes tenuous when the wounding occurs within a narrow time frame around the point at which life is defined as extinct. It is this survival time period, in the antemortem stage of pathological examination, and the supravital period beyond, that is of considerable interest to forensic researchers.

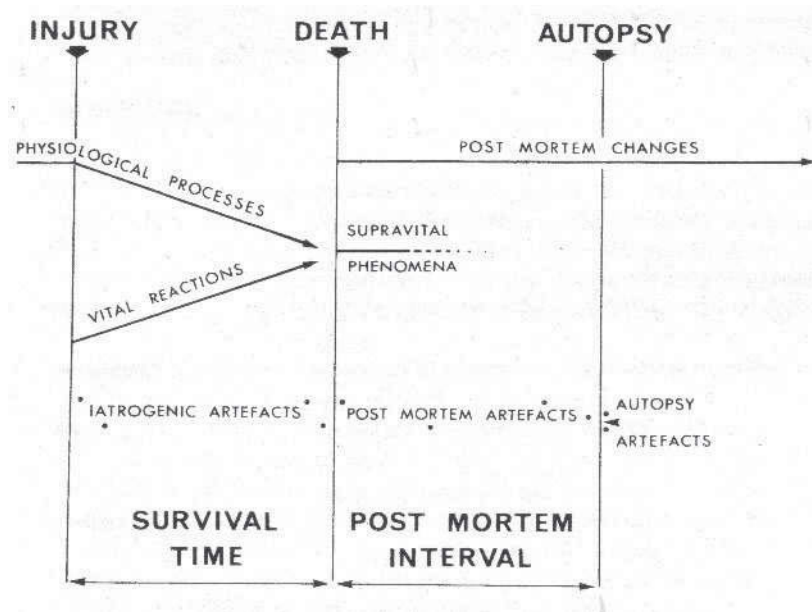


Figure 4-2: A diagram showing the time course from injury to autopsy.^[5]

To accurately determine the age of a wound, one must assess the vitality of the wound. This becomes difficult due to the fact that clinical death does not result in the cessation of all cellular activities within every tissue of the body at the same time. Some tissues, particularly those that do not require a constant blood supply, may cease to function some

period of time after death, a situation referred to as molecular death.^[6] In order to establish wound vitality, an understanding of how the arrest of respiration and circulation will affect tissue function, as well as a thorough knowledge of the pathophysiology of skin wound healing, is required.

Each type of wound exhibits a slightly different healing process due to the varying types of tissues affected. For example, a surgical incision will disrupt the continuity of the tissue layers through which the blade passes by virtue of the cutting process, but produces no loss of tissue and minimal structural damage away from the immediate wound site. However, a full-thickness burn destroys a large amount of the epidermis and dermis (including the skin appendages) and subcutaneous tissue, making it more difficult for the body to enable the various chemicals and biological entities to access the wound site, as well as requiring a significant amount of tissue regeneration.

4.1.4 Skin - Function and Structure

Whilst wounds may involve damage to multiple tissue structures such as bone and organs, for the purposes of this introductory section, the discussion of the wound healing process focuses mainly on injuries to the various layers of the skin. Prior to detailing the various cellular and biochemical events which take place during the biologically complex wound repair process, it is prudent to provide an initial brief description of the function and structure of skin.

4.1.4.1 Function

The skin is a large, highly evolved and complex organ system possessing a wide range of biological, chemical and physical functions. The average adult human skin covers a surface area of 2 square metres and weighs approximately 4.5 - 5 kilograms.^[20] This external covering performs a multitude of vital homeostatic and protective functions:

- **Protection:** the tough outer dead layer protects the delicate inner living tissues by providing a barrier against physical trauma, infection from pathogens, chemical exposure, and thermal and electromagnetic radiation damage.
- **Excretion:** the skin secretes small amounts of water, salts and wastes via sweat to prevent toxin build-up.
- **Sensation:** sensory receptors are located throughout the skin to enable the detection of pressure, pain and temperature changes.
- **Regulation:** by changing the blood flow in the capillaries and altering the amount of sweat secreted, the skin can regulate water and heat loss from the body.
- **Storage:** The subcutaneous fat layer stores nutrients.
- **Synthesis:** the skin is responsible for synthesising a number of biochemicals (e.g. Vitamin D₃ production).

4.1.4.2 Structure

Skin is comprised of two major layers: a thin, outer **epidermis** and a thicker, inner **dermis** (refer to Figure 4-3 and Figure 4-4). Embedded in the dense connective tissue of the dermis are lymph vessels, nerves, blood vessels, specialised exocrine glands (eccrine, apocrine and sebaceous - depending on skin region) and hair follicles.

The **dermis** gives skin its strength and elasticity, and possesses two distinct sub-layers:

- **Reticular layer (bottom layer):** this layer is composed of dense connective tissue with interlacing elastic and collagen fibres, and contains blood vessels, exocrine glands, phagocytic cells and deep pressure receptors.^[21] This layer rests upon what is sometimes referred to as the third, innermost layer of the skin, known as the subcutaneous layer.

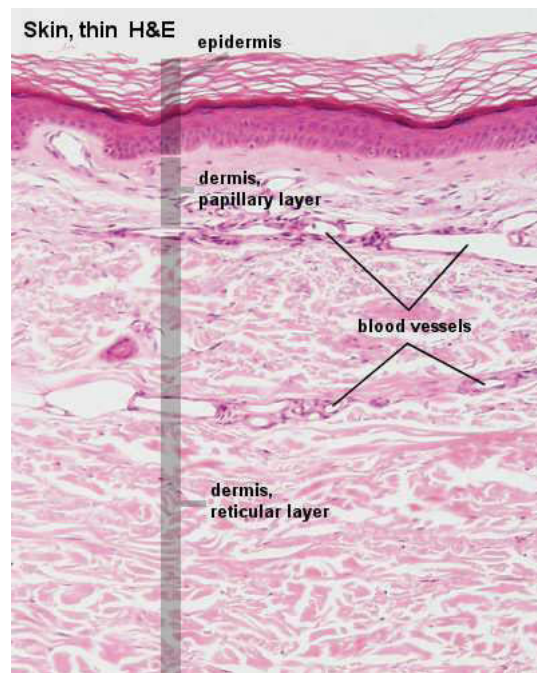


Figure 4-3: An example of an H&E-stained section of skin showing the epidermis and dermis.^[22]

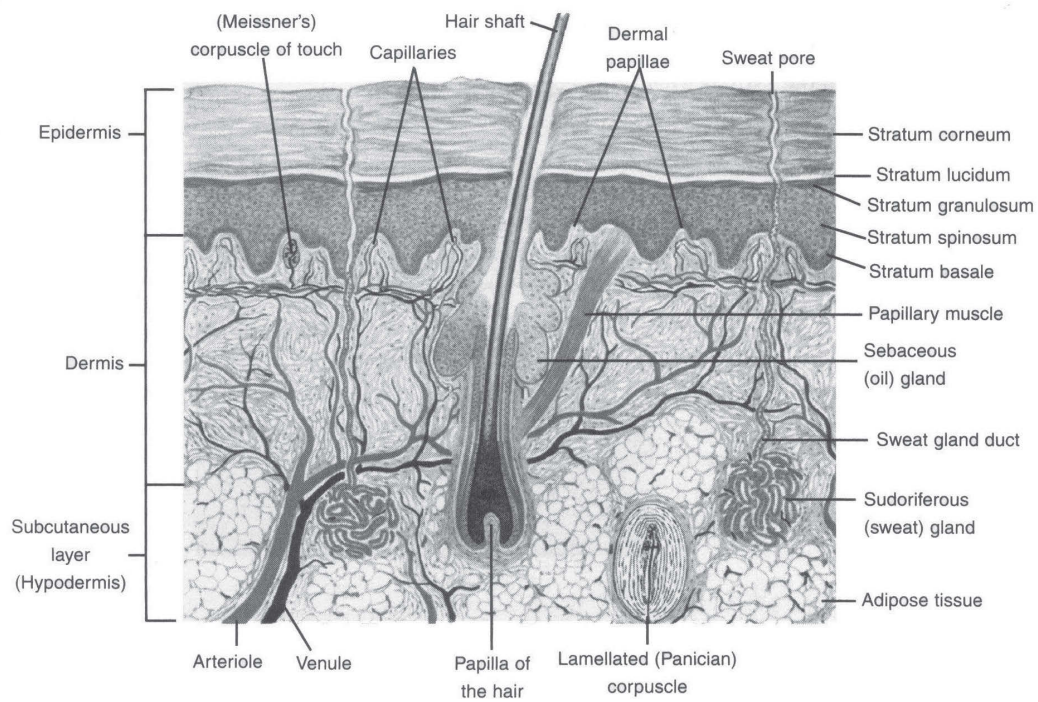


Figure 4-4: The multiple layers of the skin.^[20]

- **Papillary layer (top layer):** this layer is composed of loose connective tissue. The top-most component of this layer is shaped in a series of peak and valley configurations, forming finger-like projections (known as dermal papillae), which interlock with the epidermal tissue layer above, strengthening the dermal structure (Figure 4-5).

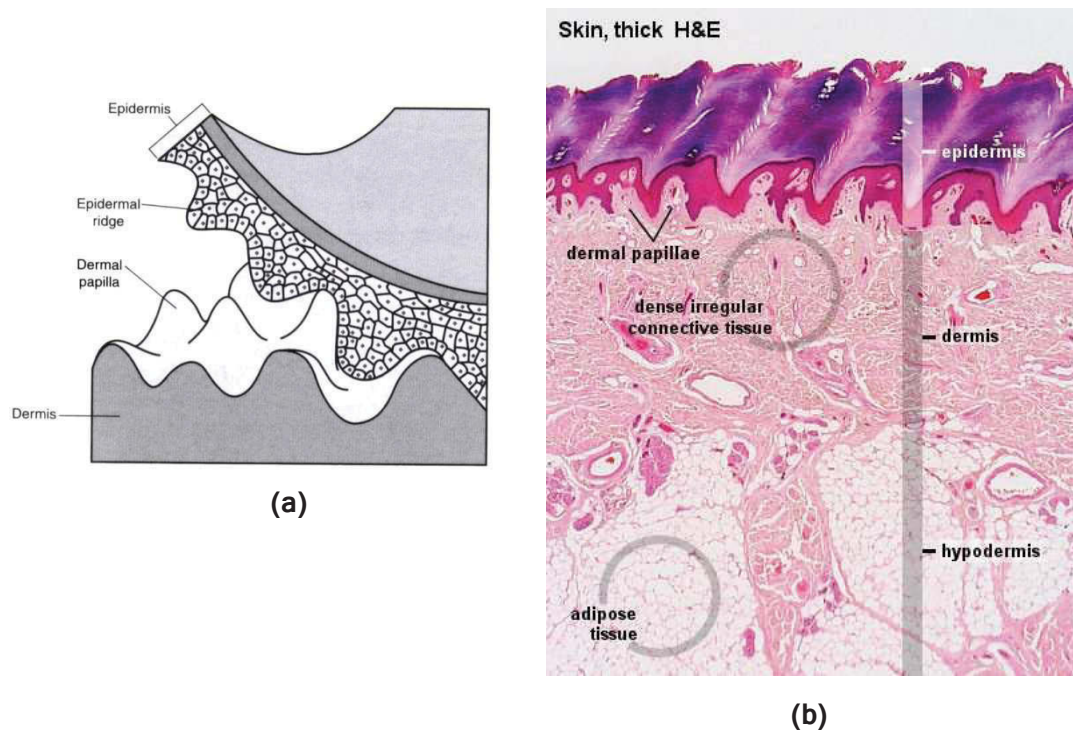


Figure 4-5: (a) Diagrammatic representation of the arrangement of the dermal papillae,^[20] and (b) an H&E-stained section of skin showing the papillary and reticular layers.^[22]

This arrangement maximises the surface area and blood supply to the epidermal layer directly above, as well as providing a suitable location for nerve endings.^[20] It is the dermal papillae which give rise to the corrugated appearance, known as *friction ridge skin*, of the epidermal layer on the hands (palmar) and feet (plantar) contact surfaces.

The **epidermis** is described as being constructed from five layers (strata) of epithelial cells -

the stratum basale (generating layer; rests directly on the dermal layer), stratum spinosum (spinous layer), stratum granulosum (granular layer), stratum lucidum (hyalin/transparent layer) and the uppermost stratum corneum (horny layer). The epidermis is avascular and relies on its close proximity to blood vessels in the dermal layer to provide essential nutrients for sustenance and continual growth. Because of constant wear and tear on the exterior surface of the skin, the cells beneath are constantly growing to replace the sloughed outer cells, and as these daughter cells travel towards the outer layers and away from their nutrient supply they fill with keratin protein (keratinisation), gradually hardening, changing shape and eventually dying before being sloughed from the skin's surface. This constant renewing cycle takes on average 28 days to complete.

Regions such as the hands and feet, because of their constant physical use and pressure-bearing employment, possess a thicker layer of skin, to assist in cushioning and protecting the delicate tissue underneath. Therefore, it is not surprising to discover that the epidermis will be thicker on the palms of the hands (1.5 mm) in comparison to the eyelids (0.05 mm) for instance. This also is reflected in the observed variation of dermis thickness across the body.^[23]

4.1.5 Wound Healing

Homeostasis is the process employed by the human body to maintain the environmental constancy required for essential cellular processes despite external changes in a person's immediate environment. If the surroundings of cells are subjected to change, then various biological mechanisms are activated to return the area to a state of balance and enable resumption of normal cellular processes. As described earlier, the integumentary system has numerous vital functions, and a loss of tissue integrity (from wounds such as incisions) activates a number of homeostatic mechanisms along various intracellular and intercellular pathways to restore order.^[24] A closely orchestrated triggering of numerous complex

biochemical events must be performed to restore tissue integrity and prevent further blood and fluid losses, including disposal of any necrotic tissue to make way for the formation of new tissue, and the activation of inflammatory pathways and the immune system against any potential pathogenic substances introduced by the wounding process.

When the body is wounded, an inflammatory response is initiated, which is a vascular and cellular response designed to protect the body against foreign substances. The inflammatory response also assists in the disposal of necrotic tissue, creating an environment conducive to new tissue formation. The acute wound healing process and its component cascade of cellular and extracellular events is a dynamic, interactive process which is normally restricted in both time (i.e. proceeds over a number of days to weeks) and space (usually takes place within a few millimeters of the wound site in the case of incisions).^[25] The various biochemical events which take place during the wound healing process tend to proceed in a known sequence and overlap in time (refer to Figure 4-6), and are classically divided into three groups, or phases:

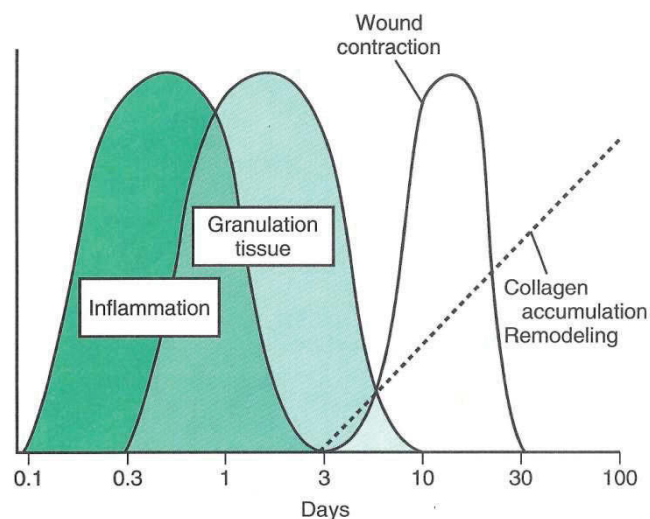


Figure 4-6: The three phases of wound healing and the order in which they occur.^[26]

Note the overlap, particularly between that of the first two phases.

- **Phase 1:** Inflammatory stage
- **Phase 2:** Proliferative stage (granulation tissue formation)
- **Phase 3:** Remodelling stage (scar formation)

Each of these phases and their component biological processes involves a wide variety of biochemical and cellular participants, such as soluble mediators, blood cells, parenchymal cells and extracellular matrix.^[27] A brief list of the major biochemicals involved in the wound healing process have been given in Table 4-1. A description of the various biochemical processes, as well as the cellular mediators of each wound phase, has been given below.

Table 4-1: The principal growth factors, chemoattractants, and vasoactive substances involved in the wound healing process (other than those involved in clotting and complement cascades) (taken from Iocono *et al*).^[28]

Wound Healing Biochemicals	Examples	Abbreviation
Growth factors	Platelet-derived growth factor Fibroblast growth factor Insulin-like growth factor Epidermal growth factor Transforming growth factor	PDGF FGF IGF-1, JGF-2 EGF TGF α , β
Arachidonic acid metabolites	12-hydroxyeicosatetranoic acid Thromboxanes Leukotrienes Prostacyclin Prostaglandin	12-HETE TxA ₂ PGI ₂ PGE ₂ , F _{2α} , 6-keto F ₁₂
Cytokines	Tumour necrosis factor Interleukins Interferon gamma	TNF α IL-1, IL-6, etc. IFN γ

Phase 1 - Inflammation

The inflammation phase represents the initial phase in wound repair, and in healthy humans generally lasts about 4 - 6 days,^[13] although some consider it to last only 2 days ^[24]. Due to the large number of processes occurring during this phase, only the main ones have been listed below.

1. *Haemostasis*

An incisional wound will generally involve damage to the epidermis and dermis, which includes the underlying blood vessels, resulting in blood constituents leaking into the surrounding extravascular tissues. The exposure of the blood to the collagen of extravascular tissues, along with the rupturing of the damaged blood vessel cell membranes, triggers haemostasis. This process commences immediately with the injured lymphatic and blood vessels rapidly constricting (vasoconstriction) for a period of a few minutes to decrease bleeding.^[13] During this time platelets in the blood begin to secrete biologically active substances into the wound environment, such as inflammatory factors and cytokines (including tissue growth factors), and also aggregate along the endothelium of the damaged vessels, initially forming a platelet plug.

Meanwhile, the blood coagulation (clotting) cascade process has also been activated, resulting in the formation of strands of polymerised fibrin and fibronectin. A clot, known as a haemostatic plug, comprised of platelets and various blood cells embedded in a matrix of fibrin,^[13] is then formed at the wound site to control active bleeding of the blood vessels and provides a matrix, or scaffold, through which a number of cells necessary for wound healing, such as inflammatory cells, may then move.^[25]

2. *Vasodilation and Increased Permeability*

Besides secreting chemicals such as thromboxanes and prostaglandins, platelets also release other chemical mediators which directly influence the permeability of the blood vessels (e.g. anaphylatoxins produced as a result of the initiated complement cascade - a biochemical process which employs a variety of proteins to assist antibodies and phagocytic cells to clear away pathogens) which can then cause vasodilation and an increase in permeability of blood vessels near the injury site. The generation of C3a and C5a anaphylatoxins also stimulate the release of histamine and leukotrienes (C4 and D4) from mast cells.^[3]

3. *Chemotactic Growth Factors and Phagocytosis*

Damage to blood vessels and/or parenchymal cells (the main functional cells of a tissue), along with the degranulation of platelets and the products of bacterial degradation, causes the release of cell-attraction factors (e.g. thrombocytes secrete growth factors) and the activation of complement, which in turn attracts blood-borne inflammatory cells to the injury site (chemotaxis). Within minutes of the wounding, white blood cells infiltrate the wound site via the blood capillary pores, followed a few hours later by neutrophils, which become the most abundant cells present at this early stage of wound healing, peaking in concentration 1 to 2 days following wounding.^[13] Assuming there is an absence of infection, their number diminishes in the following days. The role of the neutrophils is to cleanse the wound site of bacteria and any foreign particles. As the neutrophils degranulate and die, monocytes are recruited from the circulation and the surrounding area, appear in the wound and then differentiate into macrophages, with their population reaching a maximum 4 - 5 days following wounding.^[13] Along with the neutrophils, the macrophages phagocytose any invading pathogenic microorganisms and debris, and debride the area of any necrotic tissue.

T-lymphocytes are recruited to the wound site, although usually later than neutrophils and macrophages, reaching a maximum number approximately six days post-wounding.^[13] Macrophages also bind to specific proteins in the extracellular matrix, stimulating monocytes to metamorphose into either inflammatory or reparative macrophages. Macrophages release a variety of chemoattractants and growth factors (including platelet-derived growth factor [PDGF], tumour necrosis factor alpha [TNF- α] and fibroblast growth factor [FGF]).^[25,27] Macrophages synthesise and secrete proteinases which are required to remove the material comprising the initial fibrin clot. In addition to this, macrophages secrete instructional factors that direct the activity of fibroblasts, endothelial cells and keratinocytes, which are responsible for the formation of the new tissue matrix. Hence, macrophages are responsible for the movement of the wound healing process into the next phase - tissue proliferation.

An example of a skin wound experiencing the inflammation phase of wound healing has been given in Figure 4-7.

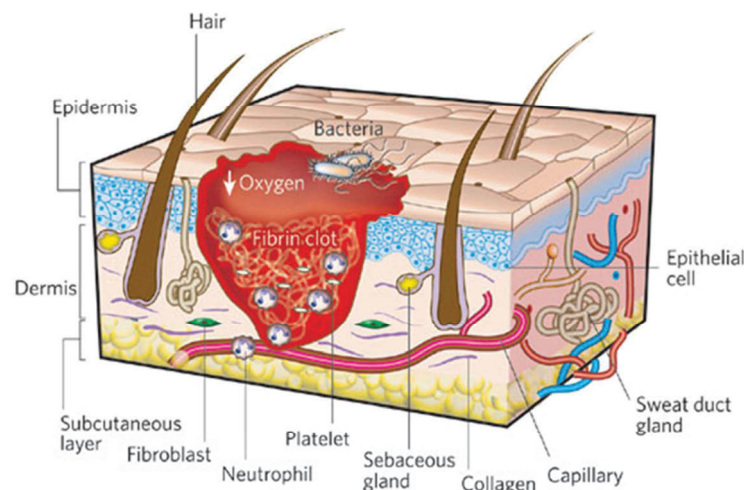


Figure 4-7: A skin wound 24 - 48 hours after injury demonstrating the cell groups typically present during the inflammation stage of healing.^[24] A fibrin clot has filled the wound area and the wound environment is ischaemic.

Phase 2 - Proliferation

Characterised by the formation of granulation tissue and later re-epithelialisation and wound contraction, the proliferative phase can be considered to start anywhere from within hours^[27] to several days^[13] of injury. Some authors consider the commencement of the proliferative phase to be characterised by cellular proliferation and migration of different cell types, such as fibroblasts and keratinocytes.^[24] Regardless, the proliferative phase is best considered as a combination of overlapping events: angiogenesis and granulation tissue formation, followed by fibroplasia and re-epithelialisation.

1. *Angiogenesis and granulation tissue formation*

Particularly occurring during the later days of the inflammatory phase, the growth factors released by macrophages encourage endothelial cells to commence cell division and form new, branching capillaries. These capillaries then grow to surround the wound and push through the extracellular fibrin matrix into the wound centre, thus resulting in the formation of new blood vessels (angiogenesis) at the wound site to form a branched and complex vascular network.^[13]

A tissue matrix consisting of the new blood vessels, macrophages, and granulation tissue (a tissue comprised of collagen and 'ground substance', in which is embedded white blood cells, young endothelial cells and fibroblasts),^[13] forms approximately four days after injury to fill any void left by the wound.^[27,29] The presence of the macrophages ensures a continuing supply of growth factors, and the extensive blood vessel network provides the oxygen and nutrients required for the extensive cell activity occurring in the wound area.

2. *Fibroplasia*

Whilst angiogenesis is occurring, and usually within two to three days of wounding,^[13] fibroblasts also migrate into the wound site. Within a few days, the fibroblasts start to produce matrix proteins, predominantly collagen type III, which are deposited into the wound area to create some structural alignment to assist in skin integrity development and to increase its tensile strength.^[30] In addition to collagen, fibroblasts also secrete compounds such as proteoglycans which are essential for the formation of the extracellular matrix necessary to support cell growth and movement within the wound site.

3. *Re-epithelialisation*

Keratinocytes from the epidermis, attracted by the secretion of various growth factors such as TNF- α and epidermal growth factor (EGF), first by platelets, and later by macrophages, migrate between the fibrin clot and the collagenous dermis.^[29] One of the main roles of the keratinocytes in wounds is to activate plasmin and produce collagenase-1, two factors vital for the dissolution of fibrin and collagen (and therefore the extracellular matrix), so that epidermal cells may migrate between the fibrin eschar and collagenous dermis.^[27] Additionally, after proliferating and maturing (usually beginning one to two days after injury), the keratinocytes at the wound margins then begin to restore the barrier function of the epithelium,^[24] with epidermal cells being observed to detach themselves from the basal lamina and migrate from the margins of the wound towards the wound matrix.^[13] Within a few hours of their initial migration, growth factors stimulate the epidermal cells to proliferate, enabling re-epithelialisation to be completed and the firm attachment of the newly established basement membrane and the underlying dermis.^[27] In the case of simple incisional wounds, this re-epithelialisation process can be completed within 24 hours.^[13]

4. Wound Contraction

Later in the proliferative phase of healing (during the second week of healing generally), fibroblasts are stimulated by the growth factors secreted by macrophages (particularly $\text{TGF-}\beta_1$), with some differentiating into contractile myofibroblasts.^[13] With features similar to those observed in smooth muscle cells, these contractile cells help to bring the edges of the wound slowly together, gradually reducing the size of the wound, a process which can take days to weeks. Fibroblasts and myofibroblasts also interact and produce extracellular matrix which assists in further contraction of the wound and collagen remodelling, resulting in a wound gaining approximately 20 percent of its final strength during these first few weeks of the healing process.^[27] This extracellular matrix, which is primarily comprised of collagen, is what will form the initial material of the mature scar tissue.^[24] As the wound contraction ends, the myofibroblasts commit apoptosis (programmed cell death), and the provisional matrix within the wound breaks down, signaling to the fibroblasts to stop migrating to the wound site.

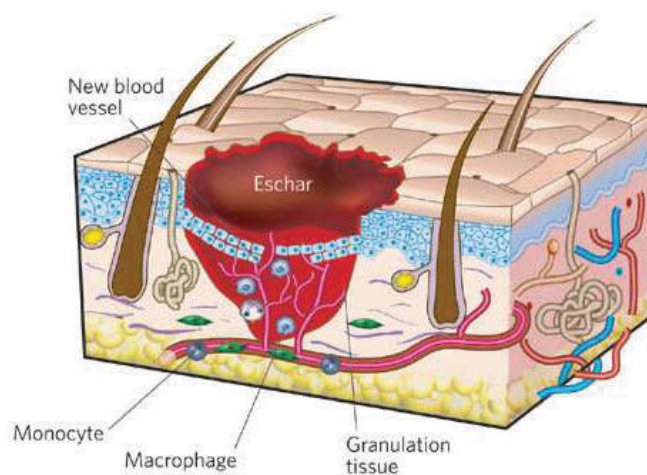


Figure 4-8: A skin wound undergoing the proliferation phase of wound healing. In this diagram the skin is shown 5 - 10 days after injury showing the eschar (scab) formed over the wound surface.^[24] New blood vessels have formed in the wound region and the basal cells of the epidermis are migrating across the wound gap.

Phase 3 - Remodelling

Remodelling of the wound tends to begin 2 - 3 weeks after injury, with this maturation phase lasting up to a year or longer.^[24] The remodelling phase is generally considered to have commenced when the injury-activated biological processes begin to wind down and cease, signaled by the gradual disappearance of the myofibroblasts, macrophages, endothelial cells, excess newly formed vessels and the extracellular matrix,^[24,29] leaving an almost acellular mass comprised of collagen and other extracellular matrix proteins.^[24] During the many months comprising this phase, a continuous process occurs consisting of the synthesis of a new, more stable and organised arrangement (with improved tensile strength) of type I collagen, and the gradual breaking down by tissue collagenases (matrix metalloproteinases, MMPs) of the original type III collagen backbone of the original wound matrix. This transition of granulation tissue into scar is also assisted by other enzymes such as hyaluronidase, bacterial collagenase and lysosomal proteases.^[29]

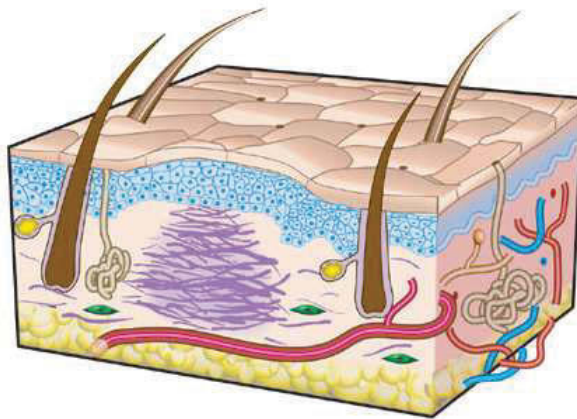


Figure 4-9: A skin wound after 1 - 12 months of healing (undergoing the remodelling phase).^[24] Note that the wound has contracted near its surface and the new epidermal layer appears slightly higher than that of the surrounding surface. Within the dermal region of the wound collagen (still disorganised in configuration) has been deposited by fibroblasts, but there are no normal skin appendages present.

Whilst the gradual replacement of the less stably arranged type III collagen with the stronger, cross-linked type I collagen fibres slowly improves wound tissue durability, it will never regain the properties of the original uninjured skin, attaining, at most, only 70-80% of its original tensile strength.^[13,24,27]

To put all of the wound phases into perspective, a timeline showing how each of the cellular events overlap, and a simplified overview of the wound healing process, has been given in Figure 4-10 and Figure 4-11 respectively.

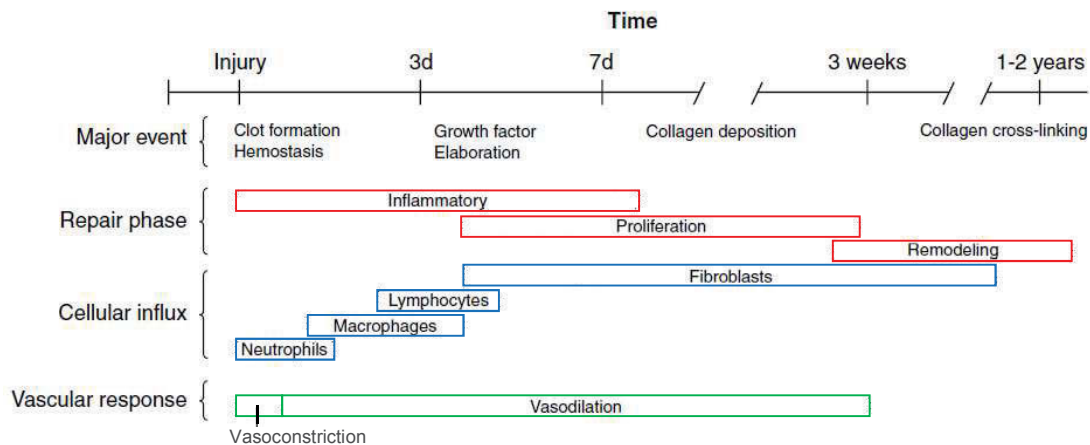


Figure 4-10: Timeline showing the various wound healing phases and some of the major cellular groups involved, taken from Lorenz and Longaker.^[3]

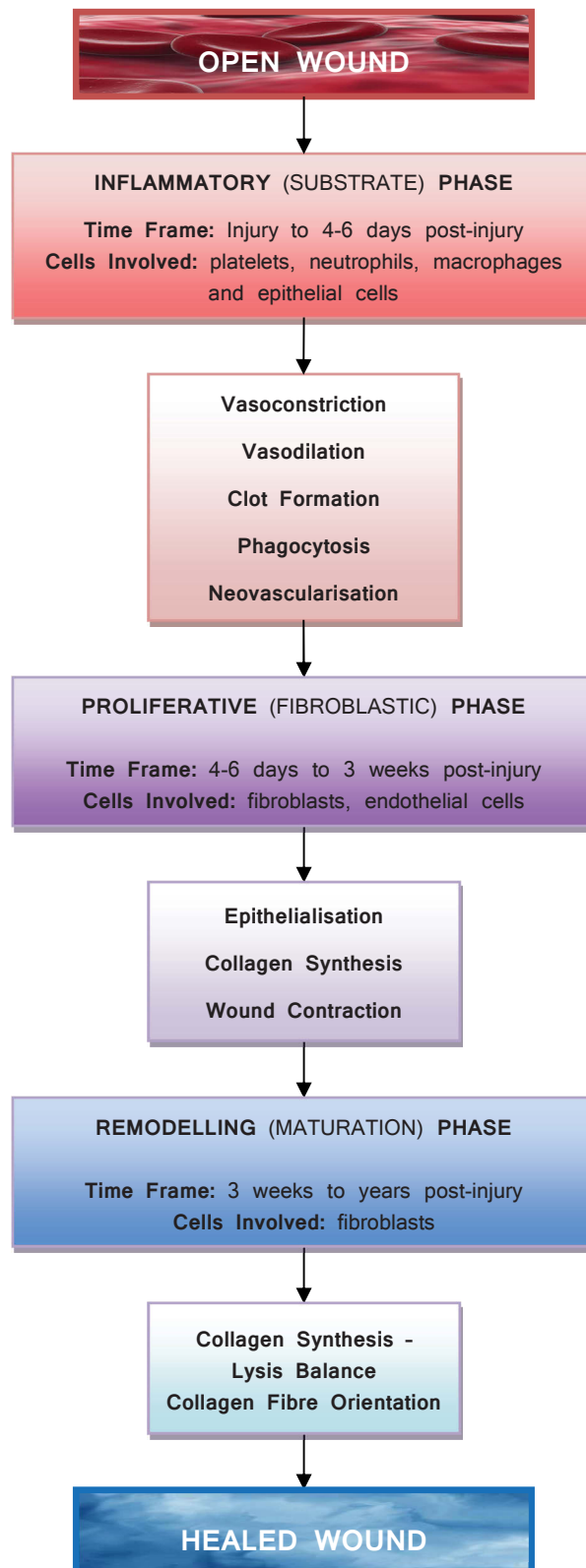


Figure 4-11: A flow chart showing normal wound healing, adapted from Mandracchia *et al.*^[31]

4.1.6 Types of Wound Repair

In surgical terms, there are three modes of wound management:

1. Healing by Primary Intention

Also known as healing by primary union, wounds which may undergo healing by this method tend to have experienced only localised damage to the epithelial basement membrane and little tissue loss (such as that of a clean surgical incision). The wound edges are directly approximated to each other with the assistance of sutures, staples or some form of adhesive material. Because of the minor disruption to the basement membrane continuity, and small epithelial and connective tissue loss, the scarring produced by this healing mode tends to be minimal and the wound healing process quite rapid.

2. Healing by Secondary Intention

In chronic wounds, such as ulcers, or generally in wounds where the tissue loss has been extensive (and therefore at higher risk of developing infection), the repair process is much more difficult as it is no longer a simple matter of the parenchymal epithelial cells regenerating and restoring the skin's original architecture.^[32] Larger or chronic wounds tend to incur a more intense inflammatory reaction in order to remove the large amount of fibrin, exudates and necrotic debris in the wound space. To fill this larger void, more granulation tissue is formed, and a larger scar (compared with those formed in first intention wounds) results.

In wounds such as these, the wound edges are not approximated, with the treating physician relying on the natural contraction process which occurs during the later phases of wound healing. When treating such wounds, a medical professional will need to perform wound care on a daily basis (e.g. ensure adequate drainage of

infection by-products, re-dressing, etc.).

Figure 4-12 shows a comparison of the wound healing process between primary and secondary intention treatment methods.

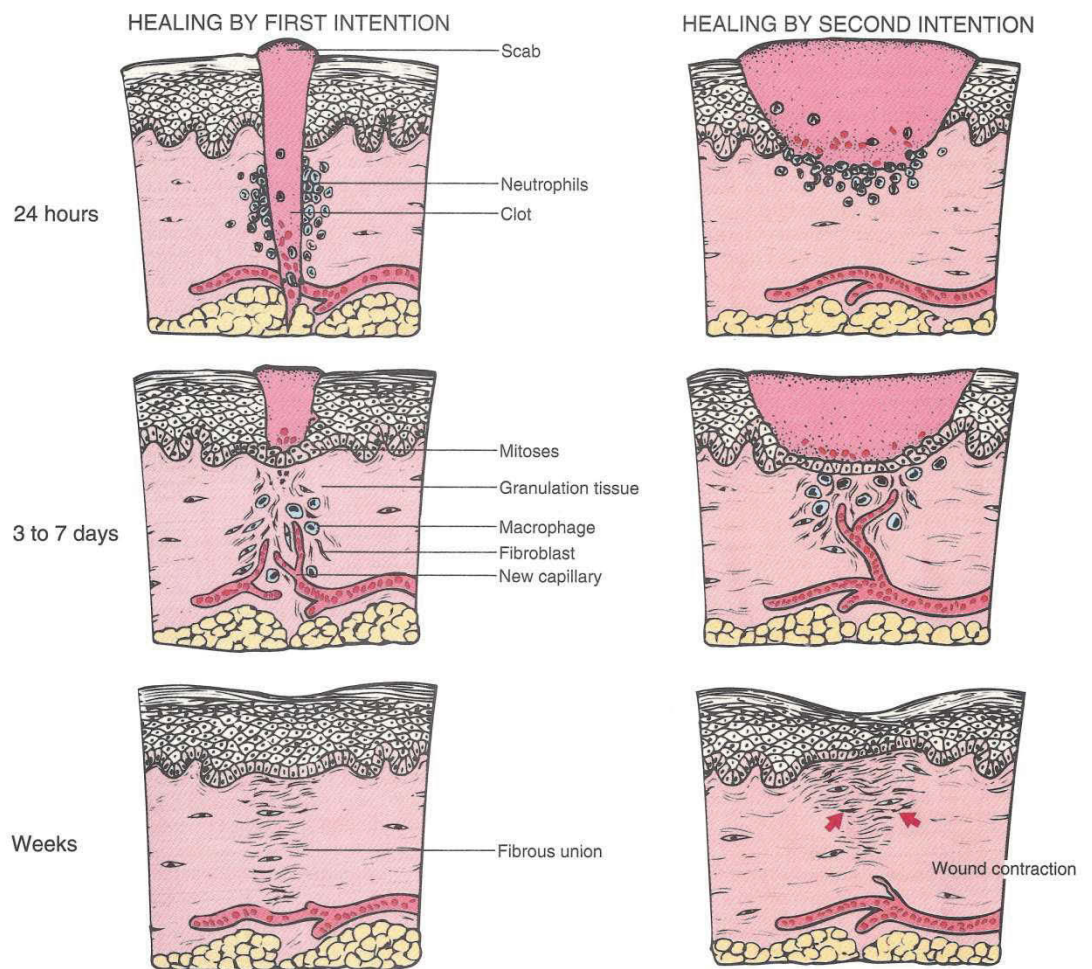


Figure 4-12: Healing by Primary (First) and Secondary (Second) Intention.^[26]

3. Healing by Tertiary Intention

Also referred to as delayed primary closure, wounds in this situation are managed in a two-step process. After initially cleaning and removing any dead tissue, the

wound is then left open by the treating doctor for a few days to permit various physiological processes to take their course, such as exudate drainage or swelling reduction.^[13] Following this, the wound is then closed by the surgeon.

4.1.7 Previous Wound Ageing Studies

Wound healing is a complex process involving numerous stages, cellular activities and physical changes; however, it generally proceeds along a rough timeline of biochemical events. It is then reasonable to conclude that if one can observe or record variations in the biochemical and cellular entities present in a wound over time, the ageing of a wound is theoretically possible. Separate from the issue of wound age, it is sometimes more important to determine which wounds were inflicted prior to, or after, an individual's death, and therefore the question of wound vitality becomes critical. Hence, it is necessary to develop robust techniques for establishing wound vitality and ageing in forensic pathology which can withstand scrutiny not only by other scientists but also meet the legal standards required in a court of law.

The easiest and most immediate examination performed on a wound by the forensic pathologist employs the use of the naked-eye and the wound *in-situ*. Based on average conditions, red and swollen wound edges are often evident after a lapse of twelve hours following wounding.^[19] Scab formation may occur in a small wound after approximately 24 hours, and is a simple way of identifying survival following injury. After 36 to about 72 hours epithelium growth around the wound edges is clearly visible (may occur as early as 30 hours in superficial scrape-like abrasions),^[33,34] and complete re-epithelialisation of a small, clean wound can occur within 4 - 5 days. Another naked-eye observation may be the presence of bleeding into the tissue, which is often taken as indicative of the deceased having been alive at the time of the injury being incurred. However, trauma inflicted on a body of a recently deceased may also cause bleeding into the soft tissue.^[33]

Although gross examination of the wound may reveal considerable amounts of information as to the wound's age, examination at the microscopic level is highly recommended, to assist in the confirmation of naked-eye observations and in narrowing the timeline in cases where no clear visible changes to the wound edge are evident. Excision of the wound and analysis of sections under the light microscope may be used to further age the sample. Leukocytic infiltration of a wound site may be evident between 4 to 24 hours after wounding.^[19] Raekallio also describes the formation of wound zones in the vicinity of the wound edge after 8 - 12 hours (refer to Figure 4-13), with the peripheral zone consisting of polymorphonuclear leukocytes, macrophages and activated fibroblasts, and the central zone exhibiting necrosis in the connective tissue after approximately 32 hours.

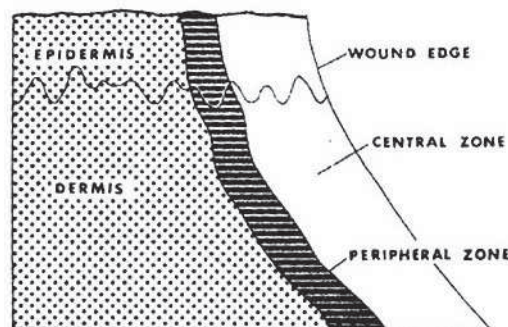


Figure 4-13: Schematic diagram showing the zones of an antemortem injury.^[19]

Many of these physiological changes can be more easily visualised by employing standard histopathology stains. It is here that a vital point in wound examination must be stressed: the absence of reaction is not conclusive in determining that a wound has been inflicted post-mortem. Some tissues do not exhibit an inflammatory reaction that can be detected using light microscopy and staining unless the victim has survived for several hours after injury.

Two common stains employed by forensic pathologists to assist in visualizing tissue morphology are H&E and Perl's Prussian blue stain. H&E, as discussed earlier in Chapter 1, stains cell nuclear material blue and cytoplasm, collagen, red blood cells and keratin a pink/red in colour. Cellular structures containing iron are stained blue by Prussian Blue, and it has been employed in wounding studies to detect tissue components such as deposits of hemosiderin 3 or 4 days after wounding.^[35,36] Tarran, as part of her PhD work, examined the effectiveness of these two stains in assisting a forensic pathologist in their wound age determinations.^[37] Blinded to wound age and the number of wound age groups, an experienced pathologist was presented with 130 wound tissue samples ranging in age from 5 minutes to 12 days. The pathologist correctly identified all of the control (unwounded) tissue sections, and obtained good accuracy with the wound sections aged 5 days or greater. However, for the earlier wound ages, it was noted that the large variability in inflammatory infiltrate between samples of the same age in this group made wound age estimation very difficult. In the absence of additional visible markers in the tissue (e.g. degree of epidermal regeneration), standard stains were proven to be inadequate in assisting the pathologist to estimate wound age.

Techniques commonly addressed in the literature to assist in the identification of antemortem injuries include specific biochemical and enzyme histochemistry methods, as well as immunohistochemistry and more sophisticated and instrument-based chemical analyses, some of which are described below.

Further Wound Examination Methods

To narrow the time of injury further, particularly in cases where the wound is inflicted only a few hours prior to death, techniques such as enzyme histochemistry and immunology-based techniques may be employed. Enzyme histochemistry was employed by Raekallio to demonstrate changes in enzyme activity as early as 1 hour after injury.^[38] Biochemical changes in serotonin and histamine levels were also demonstrated by Raekallio as evidence

of antemortem injury origin.^[19,39] A summary timeline of Raekallio's findings is given in Figure 4-14.

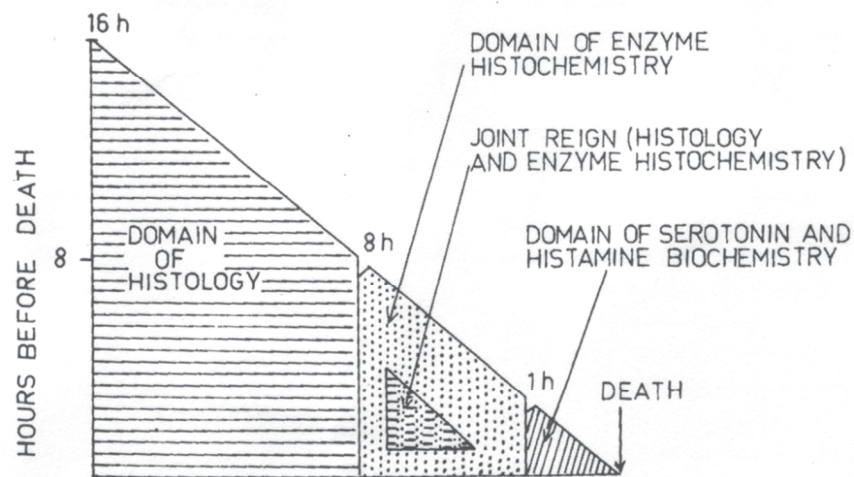


Figure 4-14: Timing of injuries.^[19,40]

Additional enzyme histochemical methods such as those employed by Betz^[41] offer additional means by which wound age differentiation may be achieved. Betz claimed that an increase in non-specific esterases could be observed as early as 1 hour after wounding, and was followed by the expression of such enzymes as acid phosphatases (~ 2 hours), ATPase (~ 4 hours), aminopeptidase or alkaline phosphatase (both ~ 4 hours). However, positive reactions were not consistently obtained, and Betz warned of the need to exercise caution in using enzyme histochemical results when applied to human tissue, indicating at best a minimum post infliction interval of a few hours. Again, the absence of a result was highlighted as not necessarily being clearly indicative of post mortem infliction. Research conducted by Fieguth *et al*^[42] also warns of the need for immunohistochemical researchers to be aware of the possibility of false negatives introduced by damage to the sample due to postmortem changes such as those brought on by autolysis and mechanical forces.

Immunohistochemical studies have also included the analysis for the expression of fibronectin and tenascin in rat skin wounds by Ortiz-Rey *et al.*^[43] expanding on previous research performed by Betz^[44] and Grellner *et al.*^[45] In this study, wounds were found to stain positive for the presence of fibronectin and tenascin in wounds inflicted as early as 5 minutes prior to death, and showed very little reactivity in wounds inflicted postmortem. In addition to this, the results obtained from this research highlighted the differences in vital reactions exhibited in rats as opposed to humans, with the conclusion being reached that animal studies can be used to help to understand the problem but are not directly applicable to other mammals.

As may be seen from the small selection of examples given in the previous paragraphs, the analysis of wound tissue to determine vitality and age has been a popular pursuit in the forensic pathology field for many decades. Additional studies on wound vitality using various histological analysis techniques have included (and are not limited to) the examination for the time-dependent appearance of myofibroblasts in granulation tissue,^[35] chemokines (e.g. IL-8, monocyte chemoattractant protein and macrophage inflammatory protein) expression,^[46] localisation of nonspecific esterase,^[47] detection of intercellular and vascular cell adhesion molecules,^[48-50] proliferation of epidermal cells,^[51] proinflammatory cytokines,^[52] expression of p53 protein (tumor suppressor gene),^[53] fibrocyte detection,^[54] ubiquitin (protein present in eukaryotes),^[55] mast cell enzyme activity,^[56,57] expression of oxygen-regulated protein 150 (ORP150)^[58] and detection of peroxidase activity.^[59] However, all of these techniques require specialised treatment of the tissue sections and are generally limited to individual biological markers at specific times.

ELISA (enzyme-linked immunosorbent assay) has been explored by a variety of researchers as a wound-ageing tool. Kondo and Ohshima in 1996 examined the applicability of ELISA and immunostaining for the determination of wound age in mouse skin.^[60] Kondo and Ohshima's study demonstrated the important role inflammatory cytokines have as chemical

mediators in a healing wound and their potential as wound age markers. ELISA has also been successfully applied in a recent study by Sheta *et al* to examine the levels of interleukin-6 (IL-6), fibronectin and lipoxin A₄ (LXA₄) in rat wound samples aged between 30 minutes and 24 hours.^[61] Consistent with previous studies, Sheta found that the levels of fibronectin increased within 30 minutes of wound infliction, and both fibronectin and IL-6 were elevated in the 3 and 6 hour old wounds. LXA₄ levels were also noticed to have increased in the 24 hour old wounds and the fibronectin and IL-6 levels remained high. None of the three mediators displayed any significant increase in levels in the 24 hour postmortem-wounded tissue, indicating that a vital reaction is necessary for their release into wound tissue. ELISA has also been employed to detect the time-related expression of IL-2 and TNF- α in rat wound tissue^[62] as well as prostaglandin E₂ (PGE₂) levels in mouse skin,^[63] producing yet further potential biomarkers for wound vitality and age determination.

The more recent focus in wound ageing studies has included assessing the application of methods which may analyse for more than one biological marker using the same tissue sample. Takamiya *et al*^[64] in their 2008 publication proposed utilizing a method known as a bead-based immunoassay, an analysis technique that permits the simultaneous detection and quantitation of up to 100 different proteins and peptides molecules in a single sample.^[65] Unlike ELISA assays which may only analyse for one molecule at a time, bead-based immunoassay is likened to conducting multiple ELISA assays simultaneously. By employing the bead-based immunoassay technique, Takamiya's team were able to quantify IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN γ), and TNF α in a variety of human skin wounds ranging in age from 6 minutes to 31 days. Cytokine expression per total protein (as opposed to per tissue weight) analysis results gave data showing the most significant changes in the cytokine levels. The researchers were able to observe peaks in levels of each of the cytokines analysed, how long they were expressed for, and during which wounding phase they were present. Further investigations are planned, as the authors acknowledge the fact that variables such

as differences in their donor subject's ages, gender and wound type could not be taken into account because of the limited number of samples analysed to date.

Other multiple-assay methods examined have included atomic absorption spectroscopy (AAS) and ultraviolet-visible (UV-Vis) spectroscopy, which were both employed by Girela *et al* in the analysis of marker stability of intra-vital wounds.^[66] In this study, the diagnostic value of several wound vitality markers known to be able to diagnose wounds inflicted in pigs up to five minutes prior to death - cathepsin D and several metal ions (Ca, Mg, Cu, Zn and Fe) - was examined in samples exposed to putrefaction. It was found that the ions analyzed retained their diagnostic ability after autolysis and even putrefaction had set in; however, the same could not be said for the cathepsin D marker. Both of the methods employed were also destructive and did not permit further examination of the sample using alternative methods.

High performance liquid chromatography (HPLC) was used by He and Zhu to detect the presence of leukotriene B₄ (LTB₄)^[67] in human wound tissue, expanding on an earlier rat wounding study conducted by Zhu and Sun,^[68,69] in which a linear relationship was found to occur between wound age and LTB₄ content at the wound edge within one hour of wounding. In He and Zhu's study, skin wound tissue was obtained from the corpses of a small number of individuals who had died from a variety of known injuries such as traffic accidents, and both ante-mortem and post-mortem wounds were analysed. The tissue was homogenised with ethanol and prostaglandin B₂ (internal standard), centrifuged and the resulting supernatant extracted. Using this method, LTB₄ was detected only in antemortem wounds and was found to be detectable in tissues which had been fixed in formalin for less than 10 days.

Ohshima and Sato have examined the use of reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ hybridisation techniques for the analysis of the temporal expression of messenger RNA (mRNA) for a number of inflammatory and inhibitory

cytokines. In their 1998 paper, Ohshima and Sato found that interleukin-10 messenger RNA (IL-10 mRNA) demonstrated an increased expression in vital skin wound reactions of mice; this could even be detected in putrefied tissue.^[70] In 2000 Sato and Ohshima, following on from an earlier study by Kondo and Ohshima,^[60] conducted an analysis of the temporal expression of inflammatory cytokines such as IL-1 α , IL-1 β , IL-6 and TNF α using RT-PCR and *in situ* hybridisation (ISH) techniques.^[71] The findings of this latter study clearly demonstrated the importance of increasing levels of IL-1, IL-6 and TNF α in early wound healing. IL-6 mRNA levels peaked at 6 hours post-wounding, followed by those of IL-1 α , IL-1 β and TNF α between 48 and 72 hours. All of the cytokine mRNA levels observed appeared to have almost normalised by the ten day mark.

In 2009, Kagawa and co-workers also conducted an investigation into the use of RNA analysis for wound age determination.^[72] mRNA expression was analysed in ten genes in mouse skin wounds aged from 15 minutes to 21 days. Three of the genes examined in the tissue samples showed no significant changes in levels, but the remaining seven exhibited differential expressional patterns with time course, but across differing wound age spans. For example, for the 12 to 24 hour age bracket, increases in the CD14 and CCL9 transcripts suggested their usefulness as an early wound age indicator, whereas the MCP-5 and PIGF transcripts could be employed in wounds aged 2 to 5 days. Further work using this method on human skin wounds is planned by the authors.

In situ end labelling (ISEL) of DNA fragments was employed by Betz *et al* to detect the presence of nuclear DNA which had been degraded during apoptosis of fibroblastic cells in human skin wound tissue.^[73,74] This study found that values obtained from fibroblastic cells located adjacent to necrotic areas, as well as those in the granulation/scar tissue could provide reliable information in regards to how many cells were exhibiting DNA degradation as a result of the initial wounding. Using this method, Betz and coworkers could clearly see the increase in the number of cells possessing degraded DNA with increasing wound

age. However, a negative or low value could not be used to draw any conclusions as such results were found in wound tissue of advanced age.

In a paper produced by Tarran *et al* in 2007, proteomic analysis was also examined for its suitability in wound age determination.^[75] Proteomics is the study of complete complements of proteins and includes the identification and quantification of proteins as well as the determination of their localisation, interactions, modifications, activities and function.^[76] As wound healing is heavily dependent on the complex interplay of proteins, proteomics offers the ability to perform mass screening of the protein types present within a wound tissue sample.^[77] Of particular interest are the proteins appearing in a wound before the inflammatory infiltrate. The protein which displayed the most prominent change in levels was that of haemoglobin, which was detectable at an elevated level in wounds aged 5 minutes to 3 hours. Levels of haemoglobin were observed to start decreasing in wounds aged 12 hours and had returned to values similar to those observed in the control tissue samples by 2 days. Combined with other testing schemes (for example immunohistochemistry), the detection of haemoglobin using proteomics could hold some merit, although used alone as a wound age marker it was considered unreliable as the protein may be overabundant in some samples. Nonetheless, the results obtained demonstrated the potential of proteomics, particularly if the method may be improved to detect additional proteins, especially those in low abundance.

In addition to the proteomic research, Tarran has also examined the application of flow cytometry to wound tissue samples for ageing determination.^[37] Flow cytometry detects the light-absorbing or fluorescing properties of cells or subcellular fractions which have been passed in a narrow stream through a laser beam. A multi-parameter data profile of the sample composition is produced based on the absorbance or fluorescence properties of the sample, and can be used to analyze particles and cells in the size range of 0.5 μm to 40 μm in diameter.^[78] The sample may then be fractionated, or 'sorted', based on these

properties. Tarran examined incisional wound tissue from rats with wound ages ranging from 5 minutes to 12 days and was able to identify trends in the percentages of macrophages, CD4+ and neutrophils present with increasing wound age. Whilst trends were observed using these target cells, it was determined that flow cytometry did not offer any clear advantage over immunohistochemistry as it was a subjective analysis method (one needs to determine which cells are to be analysed) that relied on the presence of inflammatory cells (inadequate for early wound age detection) and was destructive to the sample.

The review given above is by no means an exhaustive list of the wound-ageing research conducted to date and simply provides some examples of the various biomarkers assessed for wound age detection suitability. Summaries of research into vitality and wound timing have been given over the years by Raekallio,^[34] Oehmichen,^[79] Hernandez-Cueto *et al*^[80] and more recently by Kondo.^[81,82] Table 4-2 provides a compilation of information obtained from the summaries listed above as well as some additional example studies on the chemicals and cellular components examined, primarily using histopathology methods, by various researchers around the world.

4.1.8 Practical Considerations in Wound Ageing Studies

For the most accurate wound timing estimation to be obtained, a combination of approaches is recommended. In addition to this, biochemical events which may overlap in the wound healing time-line may be used to narrow the wound age further. The time lapse permissible between time of death and postmortem may also be of concern, although some studies have attempted to address this in cases of particular wound vitality markers, with some claiming the stability of their respective tests to be sufficient for several days.^[52] In addition to this, the pathologist is very aware of the fact that biological death does not occur at a precise time, and is a continuous process (molecular death).^[6] Factors which

Table 4-2: Examples of potential wound age and vitality markers examined in the literature.

Post-traumatic interval (approximate)	Earliest appearance	Regular appearance	Latest appearance	Marker	Species used in studies	Related References
< 30 min	10-20 min	4 hours	Months	Fibronectin	Human, pig	[44,45,61,81,83,84]
	-	-	-	Cathepsin D	Human, Pig	[66,85,86]
	-	-	-	D-Dimer	Human	[68]
	15 min	-	-	TNF- α	Human, mice	[52,87,88]
	-	-	-	IL-1 β	Human, mice	[87,88]
	-	-	-	Leukotriene B4	Human	[67]
	Few minutes	-	7 hr	P-selectin	Human	[89,90]
> 1 hr	1 hr	-	17 days	E-selectin	Human	[90]
> 1.5 hr	1.5 hr	-	3.5 days	ICAM-1 (CD54) (intercellular adhesion molecule)	Human	[48]
> 3hr	3 hr	-	3.5 days	VCAM-1 (DD106) (vascular cell adhesion molecules)	Human	[50,81]
> 4 hr	-	-	-	IL-1 α	Human, mice	[49,87]
3 - 6 hr	3 hr	-	-	IL-6	Rats, mice, human	[61,87,88]

Table 4-2 (continued): Examples of potential wound age and vitality markers examined in the literature.

Post-traumatic interval (approximate)	Earliest appearance	Regular appearance	Latest appearance	Marker	Species used in studies	Related References
> 1 day	2-3 days	> 6 days	Months	Collagen III	Human	[44]
	3- 4 days	> 6-8 days	Months	Collagen IV	Human	[44,91]
	3 days	6 days	Months	Collagen V	Human	[81]
	3 days	> 6-7 days	Months	Collagen VI	Human	[44]
	4-6 days	> 7 days	Months	Collagen I	Human	[44]
	1.5 days	6 days	Months	Myofibroblasts (mf) positive for laminin	Human	[44]
	1.5 days	-	Months	mf positive for Heparin sulfate proteoglycan (HSPG)	Human	[44]
	4 days	-	Months	mf positive for collagen IV	Human	[44]
	2 - 3 days	> 5 days	Months	Tenascin	Human	[44,92]
	5 days	5 - 31 days	< 2 Months	mf positive for α -actin	Human	[35,44,81]
> 72 hrs	4 days	> 13 days	< 22 days	Epithelial basement membrane fragments	Human	[44]
	3 days	1 week	-	p53 protein	Human	[53]
> 200 hrs	> 5 - 13 days	> 19 - 23 days	-	Cytokeratin 5	Human	[44,93]

also may affect wound healing include a victim's age and general state of health (e.g. diabetic, malnourished), infection at the wound site, and even the presence of various drugs in the body.^[94,95] According to Betz *et al.*,^[93] there appears to be no significant difference in the time taken for a wound to heal in regards to where it is localised on the body, although it could not be ruled out that a faster re-epithelialisation phase may occur in skin appendage-rich regions (e.g. head).

Ultimately it is the pathologist who must decide how many methods he or she may wish to employ before satisfactory wound age estimation may be obtained. Although the use of multiple ageing methods is encouraged, one must also factor in workloads, labour time involved, financial constraints and access to materials and instrumentation.

Routine analysis often involves only gross and microscopical examination,^[33] as many additional wound vitality examination methods are technically demanding and labour intensive. In addition to this, many of the techniques which claim to reduce the wound ageing uncertainty period to zero have not been applied comprehensively to human tissue samples, mainly due to the ethical restrictions and practicalities of obtaining human tissue wound samples of an exact known age. Hence, a method which can analyse for multiple tissue components is preferred over that which is limited to only one or a few chemical species or cell types.

4.1.9 Infrared Spectroscopy and Wound Ageing

Hyperspectral imaging, with its ability to provide both spatial and spectral information, possesses great potential for wound ageing studies. Depending on the spectroscopic technique employed, the researcher may obtain data on the quantity and/or spatial distribution of one or more particular molecular entities in a sample, a capability of considerable relevance to wound analysis when one considers the large number of tissue components that may be present in any one sample.

Hyperspectral imaging has been employed in a considerable variety of medical and pathology studies into skin and wounds over the last decade. For example, an area of difficulty commonly encountered by medical professionals is the ability to effectively and objectively assess the condition or 'health' of a subject's skin. In 2001, Attas *et al* examined the epidermal and dermal layers of human and rat skin using near-infrared reflectance spectroscopic imaging in an effort to develop an objective method for assessing skin health.^[96] Attas' method was capable of measuring hydration and oxygenation in tissue without the need for skin contact.

The employment of visible reflectance hyperspectral imaging, (ie. the spectroscopic source and detector measure light in the visible range around 500 - 725 nm) has been explored by researchers such as Zuzak *et al* in an effort to develop non-invasive dermal tissue oxygenation measurement methods.^[97,98] By utilising light in the visible range rather than that of the near infrared, one is aiming to overcome the problems of overlapping water bands and vibrational overtones common to NIR. The ability of visible hyperspectral imaging to non-invasively monitor changes in physiological states (which are often quickly manifested in the large and very accessible organ of the human skin), such as that of shock, has been successfully demonstrated in studies such as those by Gillies *et al*^[99] and Cancio *et al*,^[100] and found to hold considerable promise.

Another area of particular interest to medical professionals has been the examination of the wound healing process of ulcers in diabetic patients. Diabetes is believed to cause changes in the large vessels and microcirculation of the foot, resulting in impairment of hyperaemic response.^[101] This reduction in blood flow, and therefore alteration in oxygen delivery and tissue metabolism, is thought to be a major factor in ulcer development of the lower extremities and a possible reason for slowed wound healing. Diabetic foot ulcers can be particularly difficult to treat and, if inadequately assessed in the early stages, may result

in the need for prolonged hospitalisation and even the amputation of limbs.^[102] One technique which has been explored to assist in diagnosis is that of hyperspectral imaging.

In their 2005 paper, Greenman *et al* employed an imaging system consisting of a liquid crystal tuneable filter combined with a CCD camera which could collect *in vivo* images of foot ulcers of diabetic patients in the 500 - 600 nm region.^[101] Using the spectra collected, it was possible to measure the differential absorption of oxy- and deoxyhaemoglobin in the wounds, and in turn determine the total tissue haemoglobin across the ulcer area sampled. Greenman found that skin haemoglobin saturation is reduced in diabetes sufferers, particularly in their feet. Further studies by Khaodhiar *et al*^[103] and Nouvong *et al*^[104] have shown that hyperspectral imaging in the visible light range, again measuring for oxy- and deoxyhaemoglobin absorption, may even be used to predict ulcer healing based on its ability to identify ischemic complications before they may be seen by the naked eye.

In general, the examination of cutaneous tissue for the signs of injury requires considerable time and a well-trained pathologist. In 2003, Shah *et al* successfully employed the combination of a hyperspectral imaging method (wavelength region between ~ 430 and 920 nm) with that of correlative bright-field analysis to examine uniform, full-thickness porcine skin wounds, in an effort to develop a means to automate the process of wound region identification and delineation.^[105] Shah's goal was to find a method which could be used to assist in the rapid evaluation of a wound (i.e. region of tissue affected - including wound area and perimeter) and provide quantitative data for clinical or commercial research projects. Whilst the study was of limited size, Shah's results clearly demonstrated yet again another useful aspect of hyperspectral imaging for wound analysis. The results obtained indicated that hyperspectral imaging possessed significant promise, particularly if magnification and resolution could be improved and chemometric data analysis techniques employed.

The ability to monitor wound healing progression, as discussed earlier, is in practicality a difficult task for medical professionals, particularly when one considers that a patient may be seen by a number of different health service practitioners over a substantial length of time. In an effort to obtain an objective wound examination and progression diagnostic method, Chen *et al* developed a combined thermal and hyperspectral imaging system (referred to as THSIS) field-portable prototype instrument.^[106] THSIS was comprised of an IR camera (for thermal imaging), two monochrome cameras with seven narrow bandpass filters (for the 'hyperspectral' data) and a standard colour camera to document the wound for the physician's reference. In this study, wound appearance and volume were simultaneously recorded with thermal and hyperspectral images, in an attempt to correlate the local wound metabolic activity and total patient metabolic activity. The results obtained were considered promising and yet again demonstrated the valuable information on wounds that may be obtained using hyperspectral methods.

A suggestion for the application of hyperspectral imaging for forensic investigations into wound ageing/repair analysis can be found as early as 2000 in an article by Malkoff and Oliver.^[107] An area of interest for hyperspectral imaging has been the examination of wounds such as bruises, as well as their age determination, for medicolegal investigations. The extent to which a bruise appears (i.e. time-frame of first appearance, colours observed in the affected tissue, and the rate of the biochemical changes occurring within the wound) is difficult to objectively describe and quantify. The ability to distinguish the bruised from unwounded skin using spectral methods is dependent on the presence and amount of various skin and blood chromophores, with the relative chromophore concentration being an underlying factor in bruise colour and age determination.^[108,109] Hyperspectral analysis of wounds using a variety of wavelengths ranging from the visible, near infrared and short-wavelength infrared has been attempted by a number of research groups^[108,110-112] and met with moderate success, although the high variability that may be exhibited across a single bruise surface must be taken into consideration when examining particular areas of the

bruise. In addition to this, bruise healing is a form of immune response and the rate at which the healing process proceeds is dependent on a large number of factors, and hence the precise ageing of a bruise may prove extremely difficult, particularly in groups such as the elderly, where bruises can take exceedingly long times to resolve.^[111]

In regards to infrared spectroscopic wound examination applications, the analysis of skin tissue by FTIR has been performed by a number of research groups, but primarily with the goal of differentiating diseased from healthy tissue^[113-118] or to develop models to assess and predict the rate of absorption of various chemicals by the skin.^[63,119-121] A study conducted by Gentner *et al* examined the use of FTIR microspectroscopy for the analysis of human arterial tissue.^[122] However, no spectroscopic distinction could be made between samples of fresh and simulated post-mortem arterial tissue.

The ability of spectroscopic imaging to track changes in the skin during the wound healing process was assessed in a study conducted by Chan *et al*.^[123] Using a combination of infrared and confocal Raman spectroscopic imaging, Chan *et al* developed a molecular structure-based characterisation of several factors (mainly the major skin proteins). This in turn enabled the researchers to study the temporal sequence of events occurring during the wound healing process by tracking the changes in these major skin proteins and their spatial distribution. With a similar goal in mind to that of Chan *et al*, a more recent study conducted by Crane and Elster has directly investigated the application of vibrational spectroscopy imaging (both Raman and FTIR) to monitor changes in wound tissues with a goal of identifying characteristic markers.^[2] It is hoped that by identifying these markers medical practitioners will achieve a more thorough understanding of the mechanisms involved in the wound healing process that will then enable a more objective assessment of wounds and improve clinical outcomes.

To date, the specific application of FTIR CI to skin wound age analysis in a forensic context does not appear to have been explored, and as explored above offers exciting

potential in the examination and ageing of wounds, as it may be used to provide both information on the chemical species present in wound tissue as well as their relative spatial distribution around the wound edge. The potential advantages and disadvantages of using FTIR imaging to examine wound healing have been listed below:

Advantages of using FTIR CI for wound ageing:

- Non-destructive
- Offers the potential for wound age classification to be automated and hence not require any human interpretation of results (removing subjectivity which is a common problem in pathology)
- One preparation method and minimal chemicals required
- Samples are stable and easily archived
- Method could potentially be validated and optimised
- The large data sets made available by this analytical technique may permit the inherent error of the method to be mathematically estimated. This will enable the forensic expert to state the accuracy of the method when providing evidence to the court.

Disadvantages of using FTIR CI for wound ageing:

- The wound age classification capability of the method could highly depend on the tissue preparation method (e.g. deparaffinisation of formalin-fixed, paraffin-embedded tissue may result in a loss of key chemical markers)
- Requires access to expensive instrumentation
- Requires database establishment (i.e. large sample numbers for training and testing of the classification method to ensure the method is statistically robust)
- Skin tissue, particularly that in the dermal region, produces generally poor spectra in the mid-IR range in transfectance mode due to the loose cellular structure of the tissue.

There are two approaches which may be taken for the analysis of the FTIR images obtained from the wound samples. Whilst intensity maps may be used to highlight the presence of particular chemical entities across the sample image, this is impractical when one considers the number of different cellular and biochemical entities that may be present in any one wound at any particular healing age. In addition to this, the chemical variation between the same region of skin tissue (e.g. dermis) of wounds of varying ages may be quite subtle. Therefore, wound age classification of samples will need to be performed using one or more multivariate statistical techniques.

To determine the most suitable classification method, a library of infrared spectral data obtained from the samples may be processed using a variety of statistical data treatment techniques performed by specialised chemometric software, and the most suitable data treatment procedure determined. As described earlier, a large body of work has been conducted on wound examination using hyperspectral imaging, with many involving the analysis of univariate data (e.g. monitoring changes in one factor, such as changes in tissue oxygenation levels).^[110] Examples of multivariate data analysis methods employed for wound age classification have included independent component analysis (a method which estimates the source signals that combine to form the observed signals) to classify bruises of varying age,^[108] linear discriminant analysis (LDA) to establish a threshold for the differentiation of ulcers based on their healing capability,^[103,104] and a combination of LDA and *K*-means clustering to discriminate between healthy and diseased aortic tissue.^[124]

Methods worth considering for the classification of wound ages from infrared hyperspectral data could include Principal Component Analysis (PCA) combined with Linear Discriminant Analysis (LDA), Soft Independent Modelling of Class Analogies (SIMCA), and Artificial Neural Networks (ANN), just to name a few. The primary goal is to be able to develop a data processing technique capable of objectively distinguishing between features within a sample so that differences between samples of different wound ages may be consistently detected.

Once developed, the method may then be used to obtain a time of infliction estimate from a wound tissue sample of unknown age.

4.1.10 Wound Type Selection

When undertaking a wound timing study one cannot realistically control all of the possible variables that affect the wound healing process (e.g. wound type, participant age and health, extent of tissue putrefaction, etc.) but one must aim to, at the very least, identify and minimise them somewhat. Due to the differences in wound healing processes between wound types, this study has focused on the incised wound type only. This wounding method was selected as it is one of the easiest wound types to 'control' in the sense that, if one is inflicting the wounds, then the manner in which the incision is made (e.g. cutting tool, time, date, location, depth, length) can be carefully reproduced. Alternatively, if archival / case work samples are available, incised wounds in the form of surgical incisions are well-documented through medical records, providing samples of known age and patient health prior to their death.

4.1.11 Animal Subject Selection

To accurately study the wound healing process over a specified period of time, it is necessary to ensure that the method of wounding is standardised and that the exact timing of the wounds is known. Whilst *in vitro* assays, such as cell monolayer cultures and three-dimensional cell cultures (e.g. skin explants) may be used to approximate normal wound physiology *in vivo*, these methods require considerable care to maintain the cell/tissue viability and assay conditions between donors.^[125] *In vivo* studies, on the other hand, offer the investigator the ability to examine wound healing in a complete system, where the wounding site is exposed to the large number of biochemical and physiological processes present in the mammalian organism and their respective chemical and cellular participants.

For the purposes of a criminal trial, the method used by an expert witness (to produce evidence) must be proven to be scientifically sound. It should have been subjected to peer review, and any results obtained using the method should ideally be able to be verified by a second party. Any wound ageing determination should be based on a thorough knowledge of wound healing in the human body. The pathologist providing evidence in court will often have spent years looking at tissue samples from large numbers of decedents and will have accumulated a vast knowledge base. For the purposes of simply establishing the feasibility of a new wound-ageing method, one requires tissue samples of known wound age of a statistically sound sample size. According to Perez, a sound histopathological wound examination method (so that one can observe the entire healing process) should involve obtaining biopsies from a number of humans at multiple time points with similar wound morphologies.^[125] Unfortunately, obtaining wound tissue samples of known age from deceased subjects is fraught with ethical and social issues.

Whilst some studies have obtained human wound tissue from deceased individuals who were known to have undergone documented surgical procedures in the past (hence providing a known date and time of wound infliction),^[42,49,73,83,126] in Australia the consent for the tissue must be provided by the decedent's next of kin.^[127] In the study undertaken by Tarran in her 2007 PhD thesis research, whilst ethical approval was granted and six suitable candidates identified, permission to collect and use the samples for research was not granted in all cases.^[37] Tissue and organ donation remains a sensitive topic for many Australians, and the emotionally charged environment in which permission must be sought to obtain tissue for research purposes makes the *in vivo* study of human wound tissue a difficult and complicated process.

An alternative to using human wound tissue is to conduct an *in vivo* animal model. Besides enabling the scientist to conduct a study on the multi-factorial nature of the wound healing process, animal models can also have the added advantage of requiring shorter

study times due to the accelerated nature of their wound healing processes compared to humans.^[128] Employing an animal model enables standardisation of experiments (i.e. animal age, living conditions, post-operative treatment methods, controlled euthanasia methods and timing) and, in the case of small animals, may enable access to a large number of subjects in one location, reduced material costs, direct and rapid sample collection, as well as less emotionally and ethically complex dilemmas.

The choice of animal used will depend on numerous factors, particularly those of research budget and study duration. Large mammals, such as pigs, are advantageous in that their skin is very similar to that of a human's in respect to epidermal thickness and dermal to epidermal thickness ratios, as well as possessing similar hair follicle and blood vessel structure and dermal collagen and dermal elastic content.^[125] Unfortunately, pigs can be costly (particularly in respect to their ongoing maintenance), and their speed of growth and final adult size can make their housing and regular handling quite difficult. Wound location on the animal may also be an issue, as pigs exhibit differing wound contraction attributes across their body surface and studies utilizing them will therefore require more rigorous wound infliction protocols.^[125]

Alternatively, small animals such as rats are relatively inexpensive, more tractable in nature, and less demanding in their requirements for food, water and space. However, their small size also translates into a reduced surface area available for wound sites, and therefore the cost advantages gained by using the smaller animal may be eclipsed by the need to employ a larger number of animal subjects. Whilst possessing fast healing modes (requiring only days for wound healing processes which take weeks in humans), the researcher must also be aware that rat skin morphology differs considerably from that of a human. Rats have what is described as a "loose" skin type (highly elastic and without strong adherence to the underlying tissue structures) and a subcutaneous muscle known as the panniculus carnosus, the combined effect of which permits rapid wound contraction and

collagen formation (i.e. wound closure and healing), contrasting significantly with the tighter skin structure of a human.^[128] A comparison of the physiological attributes of rats and humans has been summarised in Table 4-3.

Table 4-3: Physiological comparison of rats to humans from Dorsett-Martin.^[128]

Characteristic	Rat	Human
Epidermis	Yes	Yes
Basement membrane	Yes	Yes
Dermis	Yes	Yes
Panniculus carnosus	Yes	No
Hair growth	Patches	Mosaic
Apocrine glands	No	Yes
Eccrine glands	No	Yes
Vitamin C source	Endogenous	Exogenous
Thermoregulation	Peripheral vascular circulation especially in the tail, respiratory system, physical movement	Peripheral vascular circulation, respiratory system, physical movement

It must be stressed that the results from any animal model cannot be directly applied to the equivalent human situation unless the model can be proven to possess all of the complexity of a human wound, a seemingly impossible task. Nevertheless, animal wounding models may be effectively employed to formulate concepts and assess the feasibility of a wound ageing technique. It must also be noted that differences in wound healing processes have been observed between neonatal and adult rats, with adult rats demonstrating slower wound healing rates and scar tissue formation.^[129] Hence, the choice of animal age can also become a factor in the wound ageing study.

Based on a number of the factors listed above, skin wound tissue from a rat animal model was determined to be suitable for assessing the possibility of employing infrared imaging for wound ageing.

4.1.12 Sample Size

A fundamental requirement of any statistical study is the sound selection of a suitable minimum sample size. Whilst collecting data from a large number of samples minimises the prediction error of a method, the researcher is constrained by many factors, not the least of which is time and cost. It is often prudent to perform a small scale or pilot study initially to assess if the range of variables being tested, as well as the sampling technique and analysis methods, will be suitable.

The samples employed in this study were originally obtained for the purposes of an earlier PhD project conducted by University of Sydney researcher Sarah Tarran (the outcomes of which were discussed earlier in section 4.1.7). In the case of Tarran's research, a pilot study using 13 male specific pathogen free (SPF) Wistar rats was performed to determine if the wound age time-points selected were acceptable and whether the surgery techniques proposed would be tolerable to the rats. To assist in determining the number of replicates to perform for each of the 13 wound age groups chosen (control; 5, 15, 30 and 60 minutes; 3, 6, 12 and 24 hours; 2, 5, 7 and 12 days), Tarran performed an A-priori power analysis, a statistical test that is capable of measuring the sensitivity of a testing method to detect an actual difference in parameters if one in fact exists,^[130] utilising a significance level of 0.05, power value of 0.8, effect size of 0.4. Using the results obtained from this pilot study, combined with the power analysis, Tarran determined that 130 samples (13 wound ages with 10 replicates of each) would be required.^[37]

4.2 EXPERIMENTAL METHOD

4.2.1 Ethics Approval

All tissue sections were acquired from the rat wound samples generated by Sarah Tarran as part of her PhD project conducted at the University of Sydney. The collection of rat wound tissue by Tarran was performed in accordance with the National Health & Medical Research Council's guidelines for the care and use of animals. Ethical clearance for the pilot and subsequent larger full animal study was obtained from the University of Sydney Animal Ethics Committee, with the study approval reference numbers being K16/10-2003/1/3823 and K16/4-2005/2/4112 respectively. The use of these samples, along with information regarding the original University of Sydney's Animal Ethics Committee ethical clearance, was also communicated to the Royal North Shore Hospital / UTS Animal Research and Ethics Committee in late 2004.

4.2.2 Animal Model

Prior to launching into the main large-scale, full animal study (henceforth referred to as the **"Main Study"**), an initial smaller study (referred to in the following sections as a **"Pilot Study"**) was conducted using a small number of samples to assess the feasibility of using FTIR imaging to age incisional wounds in rat skin tissue.

PILOT STUDY

As outlined in Tarran's thesis,^[37] full-thickness incised skin wounds were obtained from a number of male specific pathogen free (SPF) Wistar rats. Although Tarran examined twelve different post-wounding ages during her pilot study, only four were selected for this initial component of the FTIR CI study:

- **60 minutes** - representing a 'short' post-wounding interval where no or minimal cellular infiltration would be expected;
- **3 and 12 hours** - representative of the 'mid' post-wounding interval where cellular inflammation results in the production of increasing numbers of neutrophils, and possibly the earliest appearance of macrophages, at the wound site;
- **5 days** - to represent the proliferative phase during which wound closure and re-epithelialisation is observed.

Six control samples (unwounded) were also obtained in addition to the wound tissue. A summary of the wound ages examined has been given in Table 4-4.

Table 4-4: Wound ages used in the Pilot Study.

Wound Timing Group	Wound ages	Number of Replicates
Short	60 minutes	6
Mid	3 and 12 hours	6
Long	5 days	6
Control	Unwounded	6
Total Number of Samples		30

MAIN (FULL ANIMAL) STUDY

As mentioned earlier, it was determined that 130 wound samples were required for the study conducted by Tarran at the University of Sydney (twelve time points and a control with ten replicates each). It was determined that each rat could be wounded twice quite safely, reducing the number of animals required for the study. In total, 65 SPF Wistar rats were used, aged between 7 to 12 weeks and with a weight range of between 380 to 550 g. The wound ages examined have been given in Table 4-5.

Table 4-5: Wound ages used in the Main Study.

Wound Timing Group	Wound ages	Number of Replicates
Short	5, 15, 30 and 60 minutes	10
Mid	3, 6, 12 and 24 hours	10
Long	2, 5, 7 and 12 days	10
Control	Unwounded	10
Total Number of Samples		130

Wounding and Tissue Collection Process (as performed by Tarran)^[37]

Two incisional wounds were inflicted on either side of the dorsal region of skin of the rats using the method listed below:

- Each rat was first administered the anaesthetics ketamine (60 mg/kg) and medetomidine (0.5 mg/kg) via intraperitoneal (IP) injection.
- Following anaethetisation, the dorsal hair was then removed and the underlying skin disinfected with iodine.
- A 3 cm long, full thickness incised wound (penetrating down to the fascia and including the panniculus carnosus) was then inflicted on the back of the rat (with two being created for each rat).
- The wounds were then immediately closed with three interrupted sutures made with 4-0 polypropylene thread, with the sutures remaining in place until the animal was sacrificed.

- Atipamezole (0.5 mg/kg) was employed to bring the rats out of anaesthesia immediately after surgery, and buprenorphine (0.01 mg/kg) was then administered subcutaneously for pain relief.
- Euthanasia of the rats was performed using carbon dioxide. Each wound was left uncovered and no local treatment was used on the site at any stage prior to rat sacrifice.
- The wound tissue was excised from the deceased rat and placed into phosphate-buffered saline (PBS) and then stored in formalin. A tissue processor, using a number of graded alcohol and xylene treatments, was then employed to embed the tissue into a paraffin block.

Figure 4-15 provides a diagrammatic explanation of the wounding technique. The animal handling and tissue collection process has been outlined in Figure 4-16.

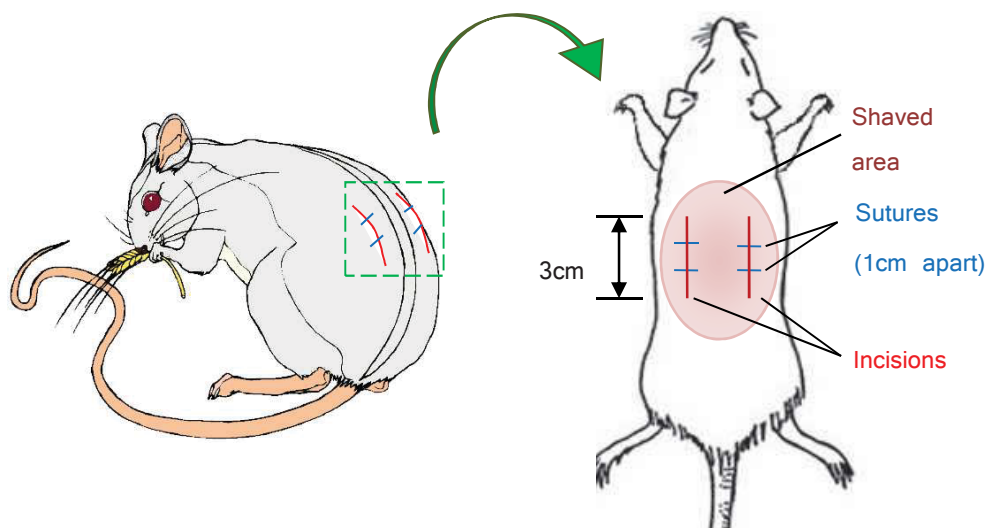


Figure 4-15: Diagrammatic representation of the rat wounding sites (adapted from Tarran).^[37]

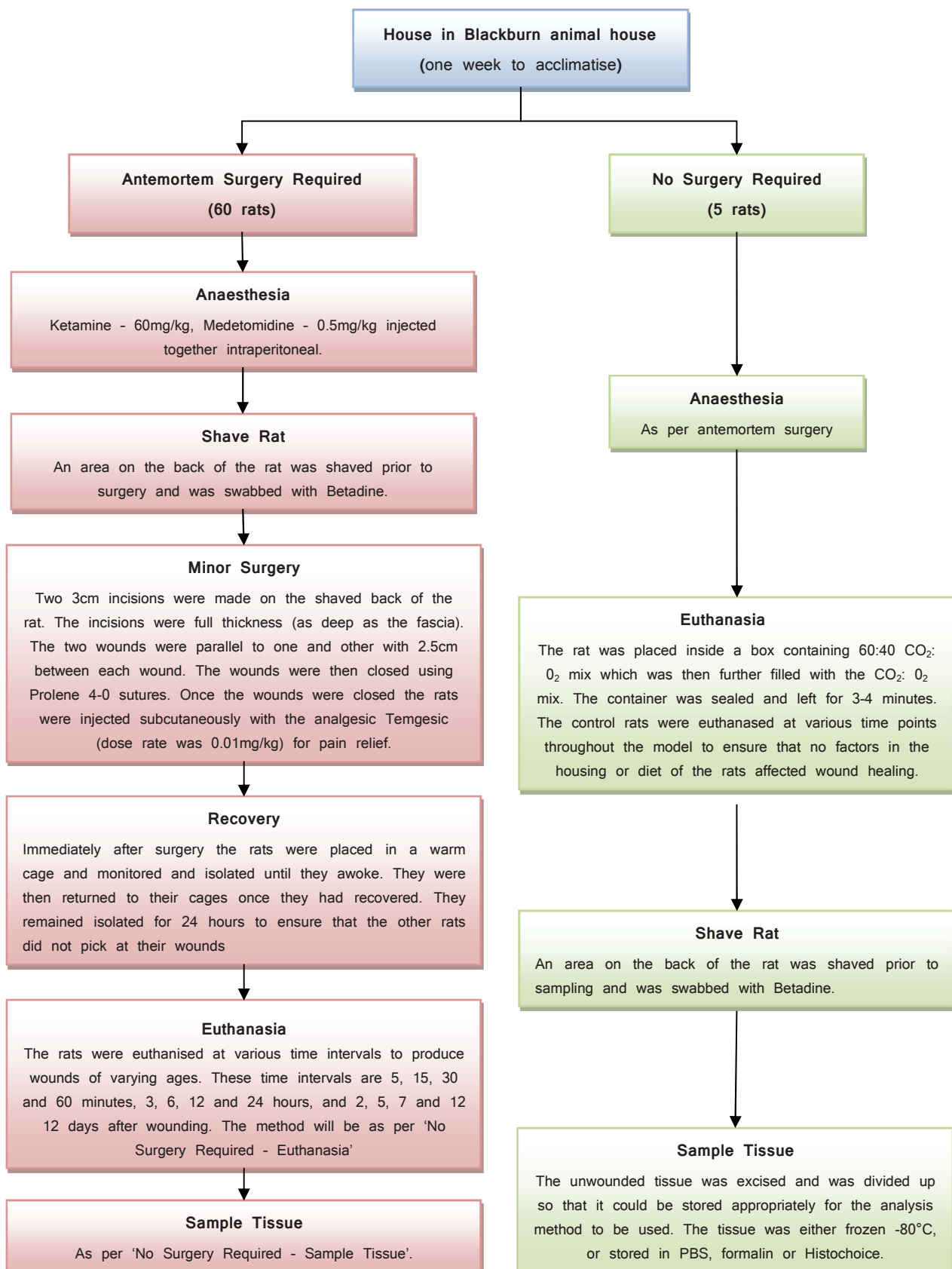


Figure 4-16: Flow diagram of Animal Model procedures (obtained from Tarran).^[37]

4.2.3 FTIR Study Sample Preparation

PILOT STUDY

For each of the tissue blocks, 4 μm thick microtomed sections of each wound were cut and mounted on infrared reflective glass microscope slides (Low-e Microscope Slides, Kevley Technologies). The tissue was then deparaffinised using xylol and a series of alcohol solutions using the method outlined earlier in section 2.2.2. Corresponding H&E slides were also obtained for each of the samples using a serial section to enable the comparison of the morphological structures elucidated using the FTIR imaging with those of the actual tissue sections.

MAIN STUDY

Due to observed problems with tissue fragility and a high number of poor spectra obtained, it was determined that a thicker tissue section could improve the quality and number of usable spectra. Hence, for each of the 130 tissue blocks, an 8 μm section was cut and mounted on the Low-e slides. The tissue was then deparaffinised using the same method as that used in the Pilot Study.

The dried tissue slides were then stored in a dessicator in a temperature-controlled laboratory prior to and following their use.

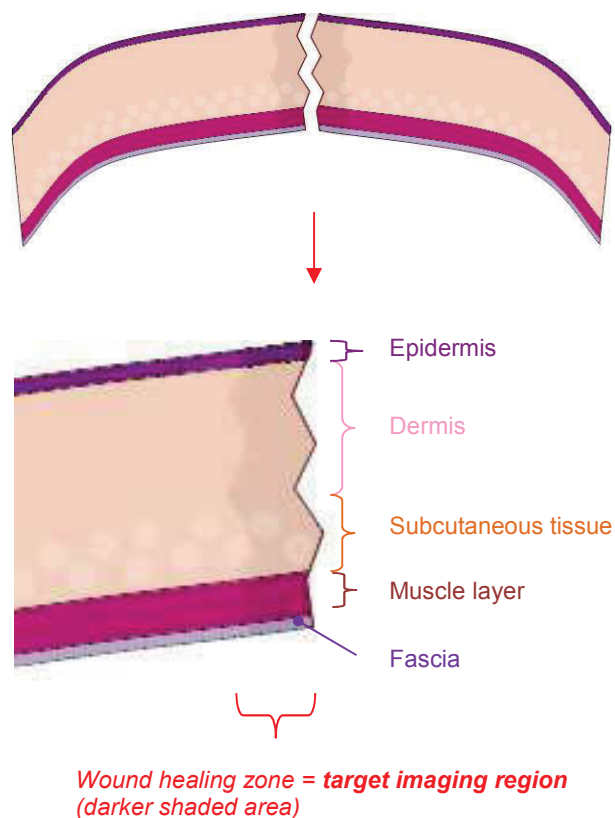
4.2.4 Instrument Parameters and Image Collection

To conduct the FTIR imaging, a Digilab Stingray IR Imaging System with 64 x 64 Focal Plane Array MCT detector (S/N 02-2134) and UMA600 Microscope (S/N 010-0232-0092) was employed (software operating system: Digilab Win-IR Pro v 3.4.0). For the IR operating parameters used during the Pilot and Main studies, please refer to Table 4-6.

Table 4-6: Instrument operating parameters.

Study	Spectral Resolution (cm ⁻¹)	Scans
Pilot	4	64
Main	4, 8 & 16	64

The region of the wound edge in the dermal layer of each skin section was imaged in reflectance mode in six regions selected per wound (see Figure 4-17 for an example of the tissue region targeted for each sample).

**Figure 4-17:** Region of wound tissue targeted for imaging.

4.2.5 Data Pre-processing and Analysis

PILOT STUDY

Each FTIR image was imported into ENVI (ITT Visual Information Solutions, version 4.7), a hyperspectral imaging software program, where 120 spectra were sampled from the image (excluding spectra from void areas and visible tissue structures such as blood vessels where possible). These spectra were then exported as a text file and imported into Excel (Microsoft, 2010) where they were then subjected to a variety of data pre-processing steps such as normalisation (minimum/maximum or vector), derivatisation (first or second order), median-interquartile range (MIQR) and averaging (Avg) to produce a single, representative “feature vector” spectrum^[131] or group of spectra.

PCA and SIMCA analyses were performed on the resulting spectra using Pirouette (Infometrix Inc, version 4.0). The PCA scores obtained were then used as the basis of an LDA classification model (with cross-validation) in SPSS (SPSS, release 14.0.0). Five of the six wounds at each time point were used for the model data, and the remaining wound from each time point was used as a test set sample.

MAIN STUDY

To minimise the number of individual files being handled at each stage of the data pre-processing, the multiple images from each tissue section were combined, or ‘tiled’, to provide a composite image file in ENVI. The tiled image was then exported in an ASCII format to Microsoft Excel, where noise could be removed from the data (using an averaging method) along with extraneous file information. Wavenumber labelling was then performed to obtain a file which conformed with the importing requirements of the hyperspectral imaging program CytoSpec (version 1.4.02, build 00327b), the software which would be employed for the majority of the data pre-processing methods being assessed (e.g. removal

of 'void' spectra using a tissue thickness testing algorithm, normalisation, spectral sub-setting, derivatisation, smoothing, etc.).

Prior to attempting classification, four feature vectors were obtained from the spectra (average-standard deviation (ASD), median (Med), Avg and MIQR) and the data prepared into one single file ready for statistical analysis using Matlab (The Mathworks Inc, version 7.4.0.287 (R2007a)). As with the pilot study, the classification methods of SIMCA and PCA-LDA were examined. HCA and PCA was performed using the Pirouette software and the LDA classification model with PASW Statistics 18 (SPSS, release 18.0.0). For the purposes of modelling, a 60:40 training:test data ratio was employed for each classification method.

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CHAPTER 5:

WOUND STUDY PART 2

(RESULTS AND DISCUSSION)

5.1 PILOT STUDY

5.1.1 Results

5.1.1.1 *Skin Tissue Sections - Visible Features*

To help gain an understanding of the cellular changes occurring in the wounded tissue, H&E stained sections were taken for each of the wound age groups. Figure 5-1 shows a number of examples from the various wound age groups examined in this study.

As may be observed from the examples given in Figures 5-1 to 5-4, the visible cellular changes associated with the wound healing process may be subtle over time and the wound itself more difficult to locate in well-healed tissue. This made locating a target area for infrared image capture difficult at times, although the tissue excised from each rat was cut in such a way to ensure that the wound would be located in the approximate centre. In cases where the tissue structure continuity was disrupted due to mechanical forces during cutting, or where the wound was not able to be detected under the white light of the infrared microscope's video camera, target areas for image capture had to be estimated.

5.1.1.2 *Skin Tissue Sections - Infrared Features*

Figure 5-5 provides an example of an infrared-imaged section of wounded rat skin tissue and a number of spectral profiles obtained from various regions sampled within the image. As discussed earlier, skin tissue is a complex cellular structure. One would therefore expect that each different histologic structure found in the skin will possess differing distributions of the major chemical constituents found in biological tissues: water, lipids, proteins, nucleic acids and carbohydrates.^[1]

Figure 5-1: (A) An example H&E stained section of unwounded (control) rat skin at x5 objective magnification. The epidermis (a), dermis (b), subcutaneous tissue (c) and the upper edge of the panniculus carnosus (muscle) (d) layers are all clearly visible. (B) 5 minute wound at x5 objective magnification. (C) 30 minute wound at x5 objective magnification. (D) A magnified (x20 objective) section of the 30 minute wound.

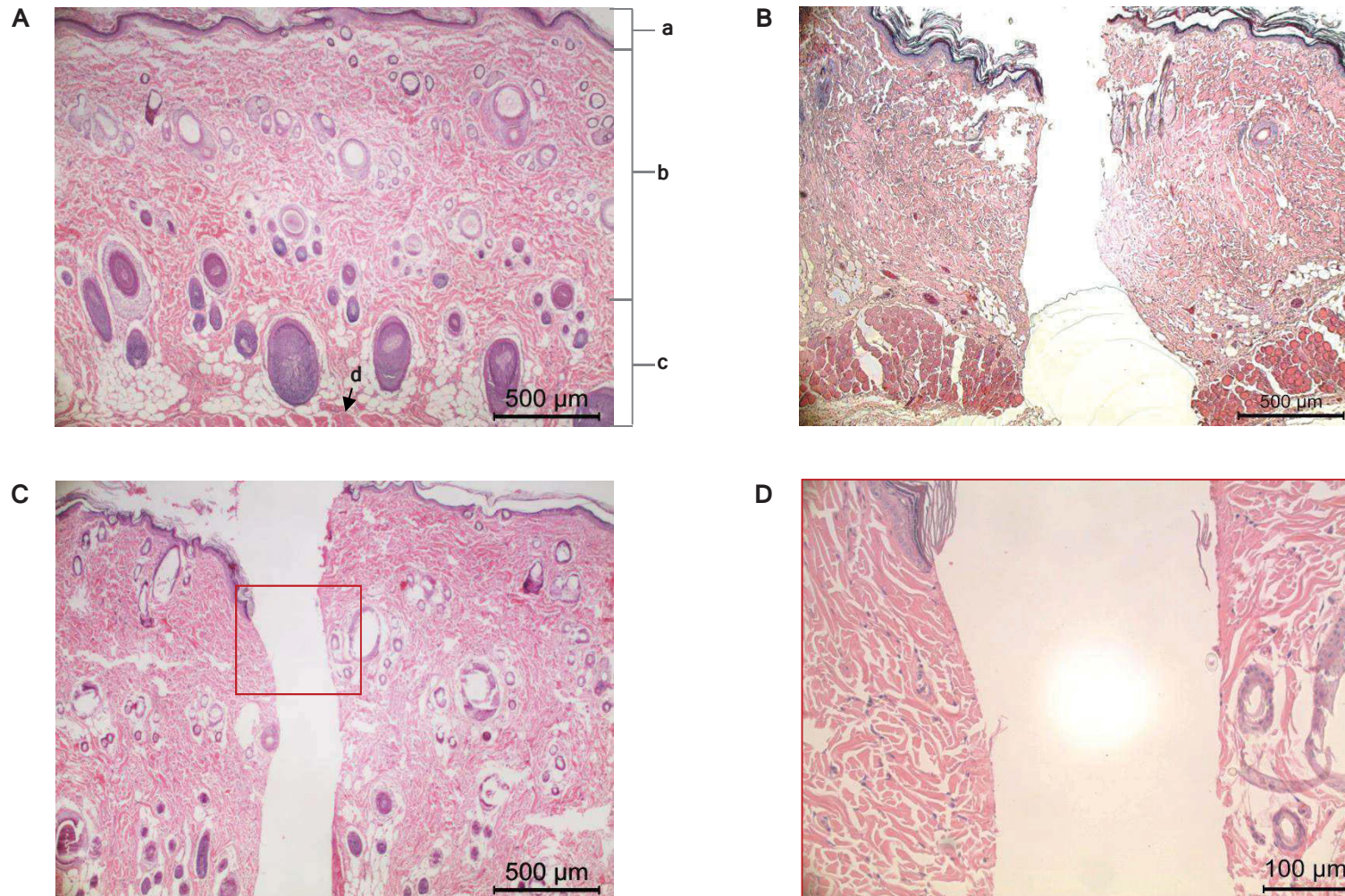
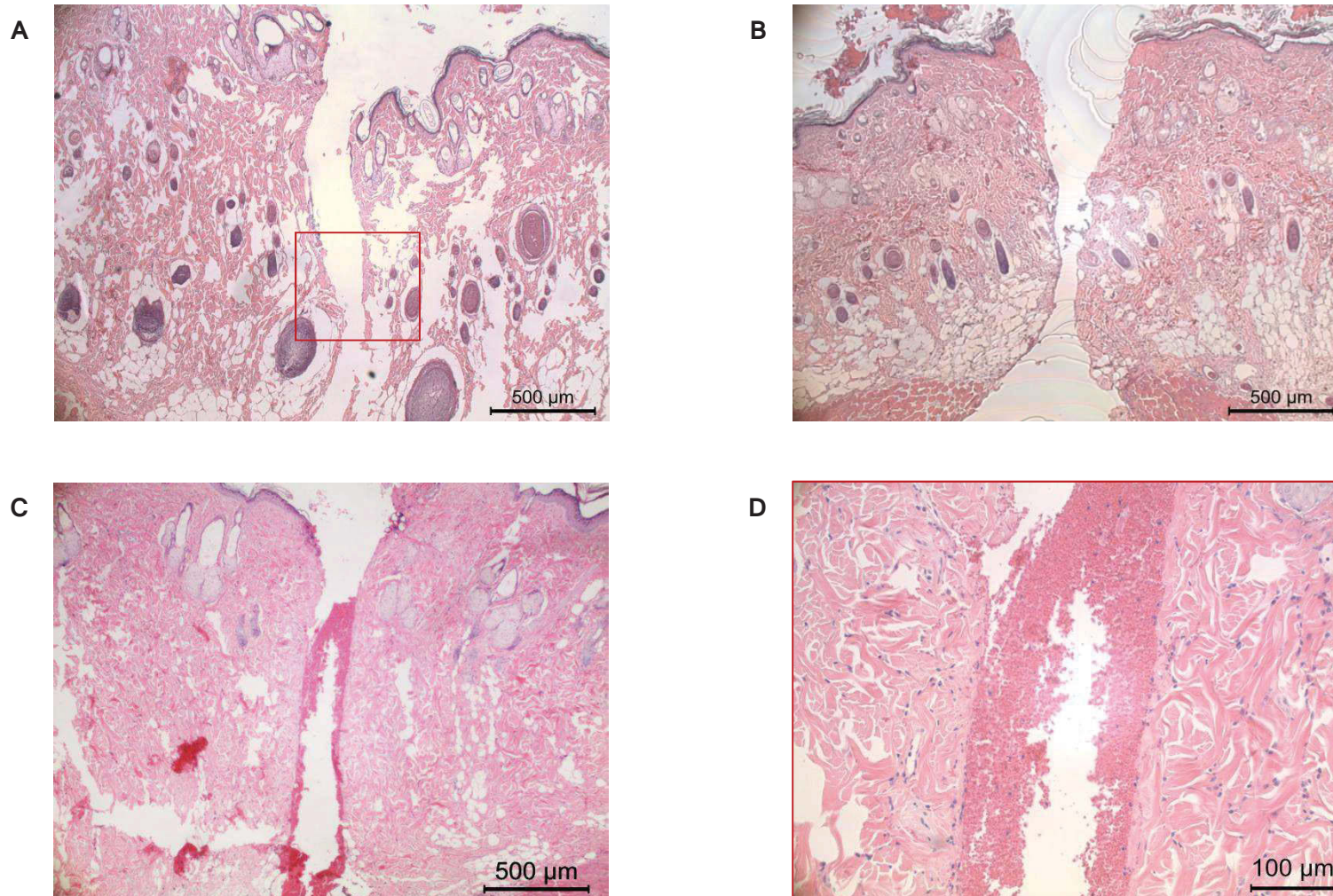


Figure 5-2: (A) 3 hour wound. (B) 6 hour wound. (C) 12 hour wound and an enlargement (D) displaying the large concentration of blood cells still present in the wound fissure.



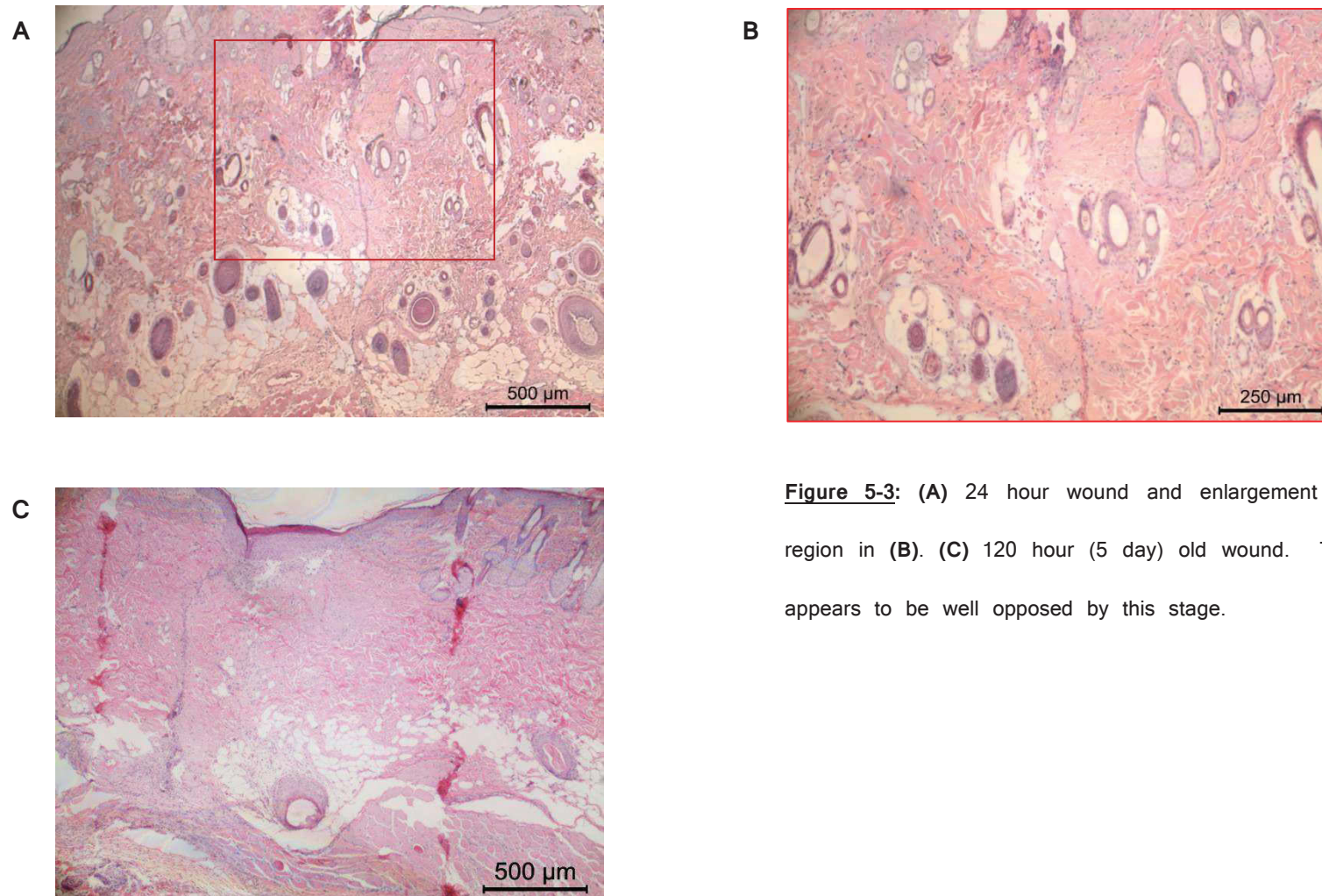


Figure 5-3: (A) 24 hour wound and enlargement of wound region in (B). (C) 120 hour (5 day) old wound. The wound appears to be well opposed by this stage.

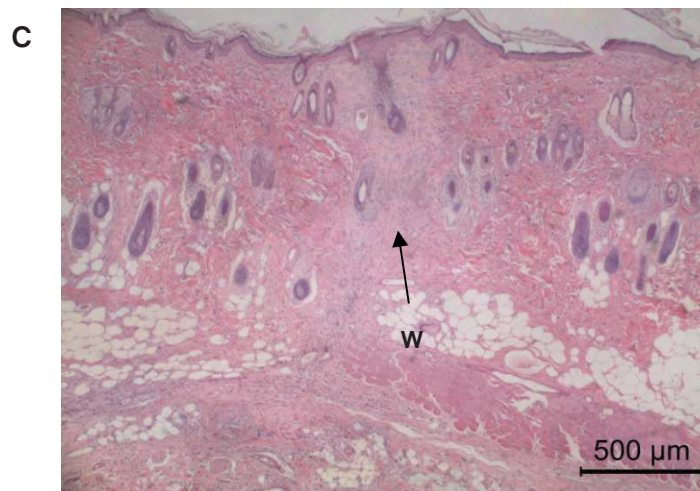
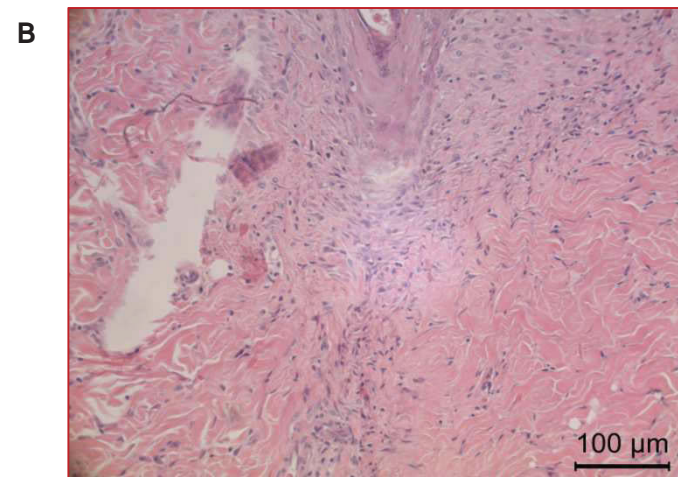
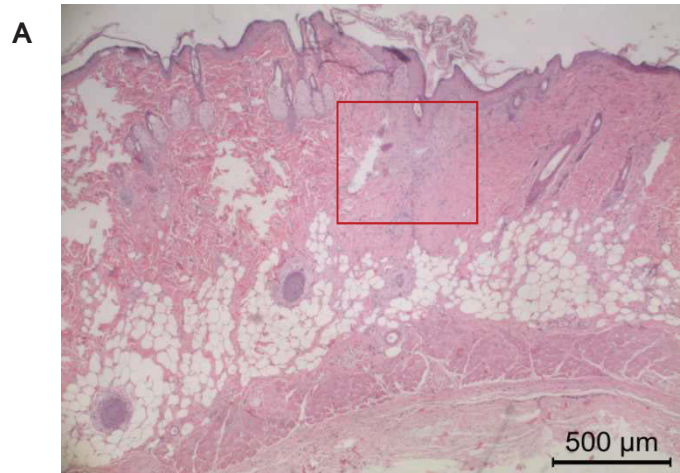


Figure 5-4: (A) 168 hour (7 day) old wound. Wound closure and re-epithelialisation has occurred. (B) A x20 objective magnification of the boxed section in B which clearly shows a portion of the thickened epidermis and the region of healed dermis below. As with the 5 day old wound, the actual wound has become difficult to detect. (C) 7 day old wound. The wound site (marked W) is difficult to detect due to the well-advanced healing that has taken place.

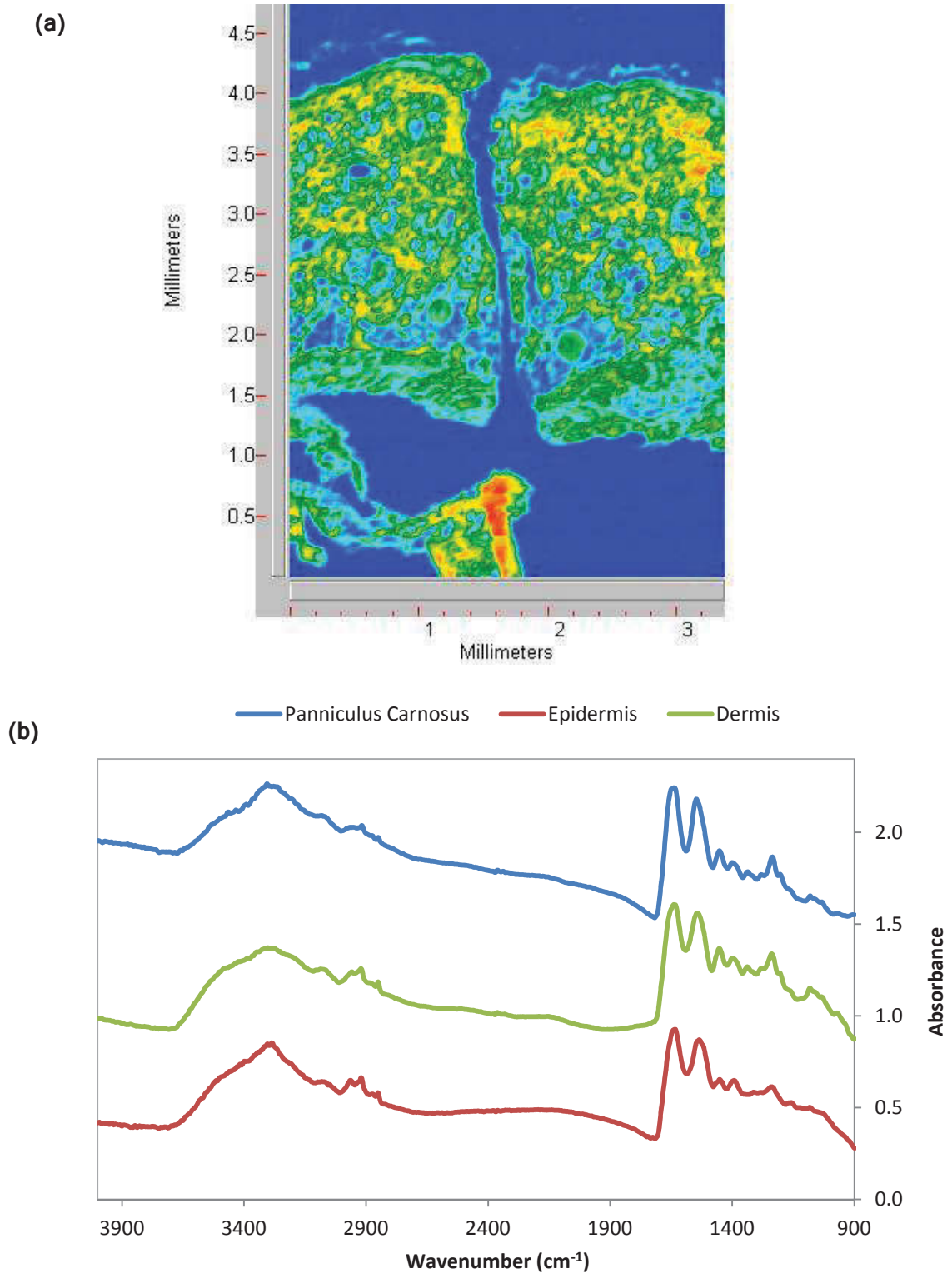


Figure 5-5: (a) Large mosaic image of a 24 hour antemortem wound tissue section as viewed using FTIR CI (8 cm^{-1} spectral resolution, displayed at 1543 cm^{-1}), and (b) example infrared spectra sampled from various regions of the image given in (a). The spectra have been off-set from each other for ease of viewing.

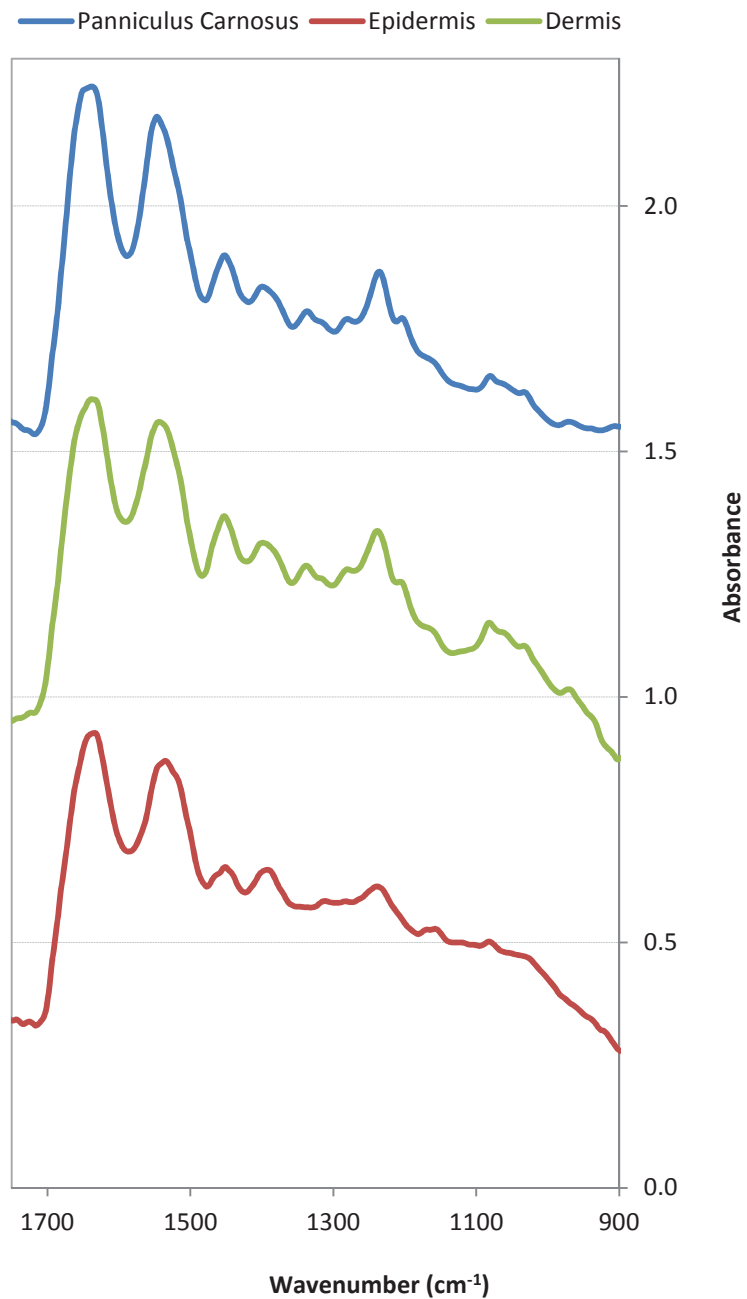


Figure 5-6: An enlarged view of the spectral region 900 - 1700 cm^{-1} for the spectra shown in Figure 5-5b. The muscle, epidermal and dermal layers all possess strong protein component peaks.

For example, hair and hair follicles possess an extremely high amount of proteinaceous material, and strong carbonyl vibration bands are considered indicative of the sebum (an oily secretion composed primarily of fatty acids and triglycerides) found in the sebaceous glands.^[2] As discussed earlier, the epidermis is composed of a number of layers, with the deeper layers being more protein-rich when compared to the outer layers such as the stratum corneum. The stratum corneum itself is composed of corneocytes, terminally differentiated keratinocyte cells, embedded in a lipid matrix. This upper keratinised layer is mainly composed of anucleitic dead cells and possesses different spectral features compared with the deeper epidermal layers.^[3]

The most intense spectral bands observed in the infrared analysis of skin are those of the amide I and amide II vibrations (located at ~ 1655 and 1545 cm^{-1} respectively). In the $1000 - 1500\text{ cm}^{-1}$ spectral region, weaker bands associated with the lipid and protein tissue components may be observed. No infrared absorption bands characteristic of skin are found in the region $1800 - 2800\text{ cm}^{-1}$. The absorptions observed in the spectral region between 2800 and 3500 cm^{-1} arise from the water stretching vibrations, the amide A and B N-H stretching vibrations (protein fraction) as well as methylene and methyl stretching. A summary of the bands attributed to the protein and lipid contributions from the various components within the stratum corneum (pig skin) is given in Table 2-7 (Chapter 2).

It is not surprising that the spectra sampled from the different skin layers, as shown in Figure 5-6, appear very similar at a cursory inspection, given that the various tissue structures found within the skin share some common biochemical components. Due to the heterogeneity of the various tissue layers found within skin, and the limited spatial resolution obtained in this infrared imaging study, it is only the averaged characteristics of the cell types within the different tissue layers that will be observed. For example, it has been shown in previous studies that the lipid and protein fractions in the stratum corneum are

not homogenously distributed.^[2] However, due to the spatial resolution employed in this study, one could not observe 'pure epidermal layers' in the recorded skin wound images.

Although wound healing involves the restitution of all tissue layers, it is in dermal layer that a large portion of the known wound healing cellular processes take place. For the purposes of this comparative study, it was decided that the dermal layer would be targeted as it represents the largest portion of the skin tissue section and thus provides the largest wound sampling region. Unfortunately, as may be seen in Figure 5-5, the bulk of the dermis is composed of loose connective tissue, which in turn produces holes or gaps through which the background slide material becomes visible and impacts on the quality of the tissue spectra being collected (e.g. dispersion artefact). To further complicate matters, the dermis is not morphologically homogenous and strewn throughout may be found widely varying tissue structures such as blood vessels, nerves, hair follicles and glands.

One advantage of employing infrared imaging for wound analysis is that it permits a larger number of representative spectra to be collected simultaneously per sample, so that the user may also selectively sub-sample regions of tissue within the image in an effort to avoid both the gaps and any tissue structures not involved in the wound healing process. Based upon a simple comparison of dermal spectra from wounds of varying ages, the only indications to possible wound age determination was the observation of strong amide I and II peaks in wounds of advanced age, which was most likely indicative of high protein concentration due to the initial stages of scar tissue formation and the associated accumulation of collagen (Figure 5-7). An alternative explanation to the observed change in spectral features in the older wound tissue might quite simply be related to spectral quality, with the more densely packed collagen fibres that accumulate in the wound region producing spectra less subject to noise (e.g. impact of the dispersion artefact is lessened).

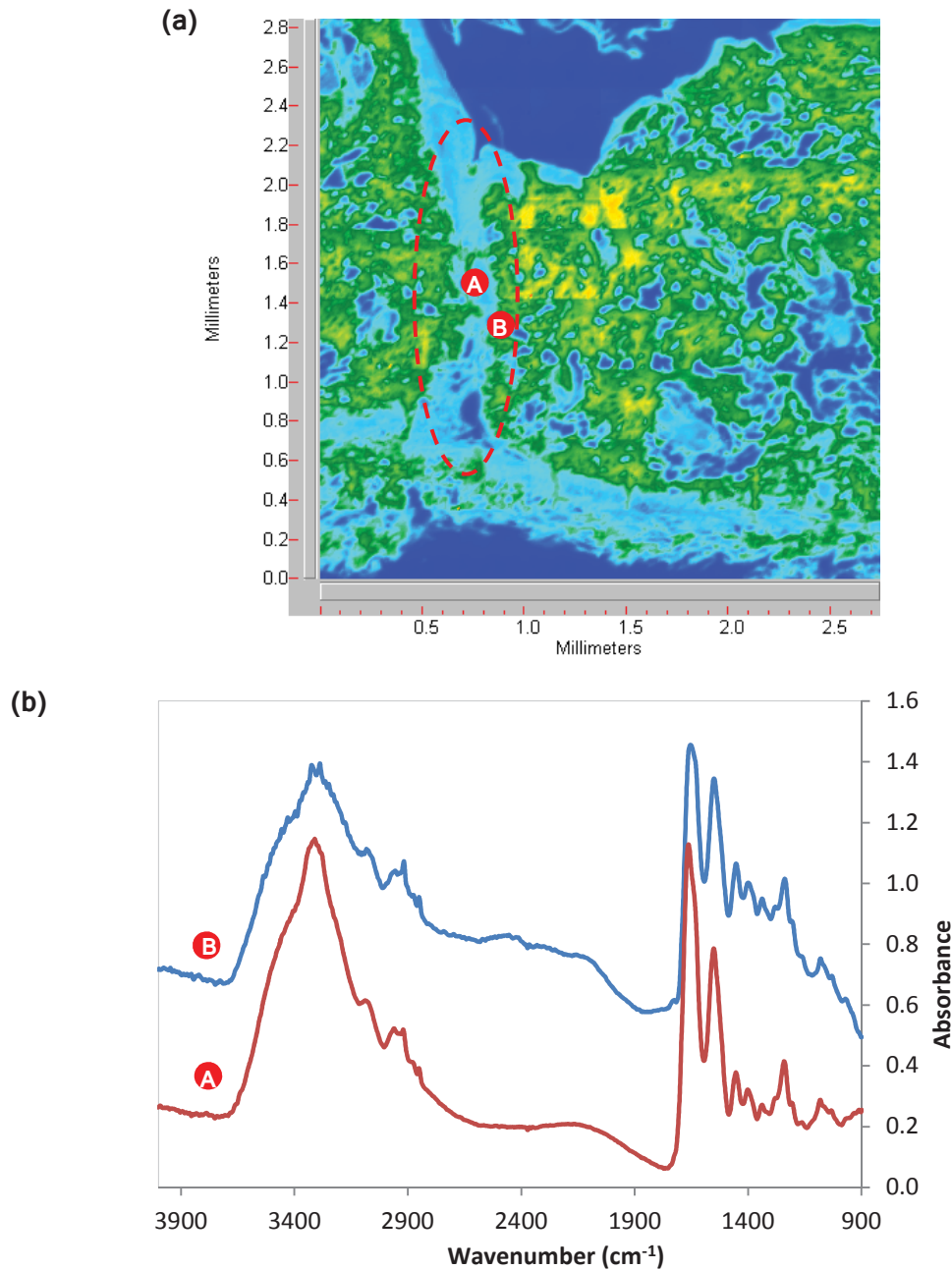


Figure 5-7: (a) A large mosaic image of a 5 day wound (8 cm^{-1} spectral resolution, image displayed at 1547 cm^{-1}). The spectra for markers A and B are given in (b). The high protein contribution and reduced noise observable in marker A's spectrum is consistent with the expected elevated concentration of protein due to the dense packing of collagen fibres formed in the wound gap following wound edge apposition. Marker B is from a region of dermal tissue in the area adjacent to the main wound site and is more typical of the spectra observed in 'normal' dermis with its 'loose' connective tissue structure.

5.1.1.3 *Data Preprocessing Approaches*

For this initial pilot study, a small subset of spectra was selected from each wound tissue image using the 'Region of Interest' selection tool in ENVI. These spectra were then exported from ENVI as an ASCII text file and imported into Microsoft Excel. The spectra were then subjected to a variety of data pre-processing steps and reduced to one representative spectrum, or feature vector, per sample.^[4] The two feature vector types selected for the Pilot Study were the average (Avg) and median-interquartile range (MIQR). A flow chart summarising the data collection and pre-processing workflow has been provided in Figure 5-8. Table 5-1 outlines the various preprocessing and feature vector calculation combinations employed in this Pilot Study.

5.1.1.4 *Principal Components Analysis / Linear Discriminant Analysis (PCA-LDA)*

This work explored the effect of the number of principal components and various data pre-processing and feature vector extraction methods on the ability to successfully group and classify the data. Due to the small number of samples examined for this pilot study, the training data was composed of five feature vectors per wound age group, with a sixth used to test the classification ability of the model developed.

Table 5-2 provides the five best classification results obtained using PCA-LDA. A scores plot providing a graphical representation of the grouping observed between the various wounds for the best classification achieved has been given in Figure 5-9. A summary of the classification capability for each pre-processing and feature vector method on the wound tissue using PCA is also provided in Table 5-3.

Figure 5-8: Imaging and data reduction process overview employed in the Pilot Study.

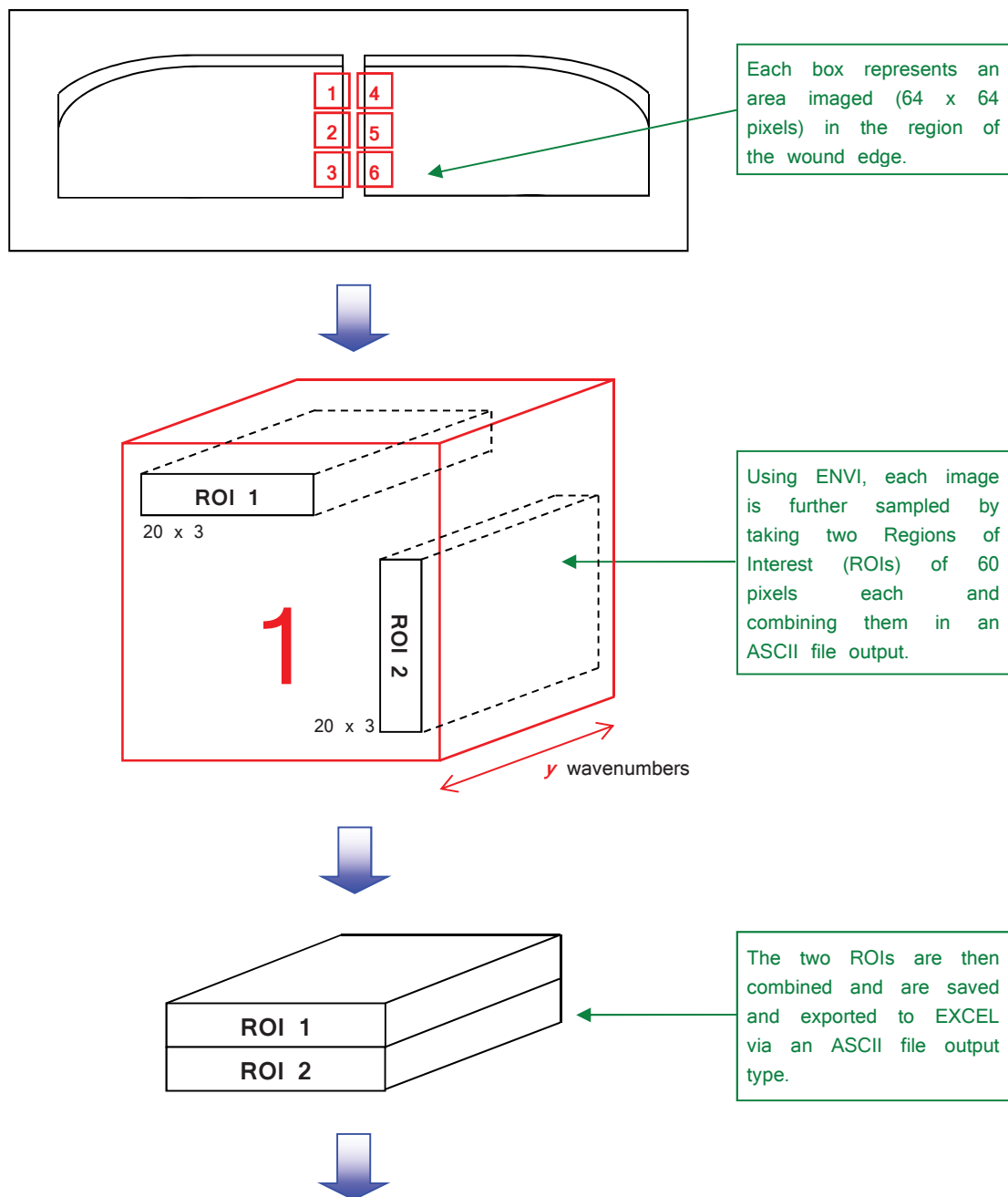


Figure 5-8 (continued): Imaging and data reduction process overview employed in the Pilot Study.

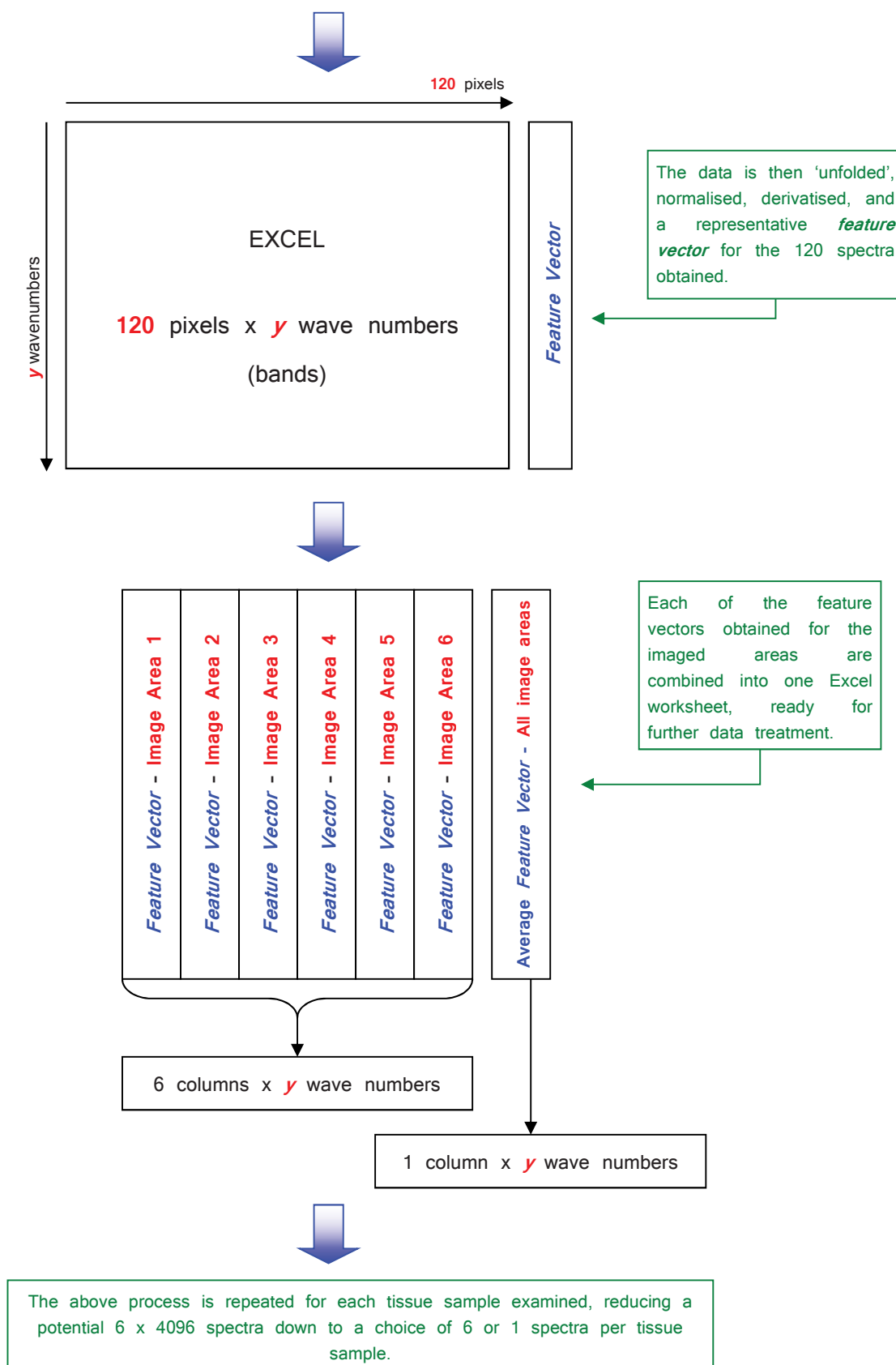


Table 5-1: Data preprocessing combinations applied to the Pilot Study wound data

Method Number	Pre-processing Technique(s) Employed	Feature Vector	Method Number	Pre-processing Technique(s) Employed	Feature Vector
1	2 nd derivative (cubic), 7 smoothing points	MIQR	11	2 nd derivative (quintic), 7 smoothing points, with vector normalisation	MIQR
2	2 nd derivative (cubic), 7 smoothing points	Avg	12	2 nd derivative (quintic), 7 smoothing points, with vector normalisation	Avg
3	2 nd derivative (cubic), 7 smoothing points, with vector normalisation	MIQR	13	2 nd derivative (quintic), 9 smoothing points	MIQR
4	2 nd derivative (cubic), 7 smoothing points, with vector normalisation	Avg	14	2 nd derivative (quintic), 9 smoothing points	Avg
5	2 nd derivative (cubic), 9 smoothing points	MIQR	15	2 nd derivative (quintic), 9 smoothing points, with vector normalisation	MIQR
6	2 nd derivative (cubic), 9 smoothing points	Avg	16	2 nd derivative (quintic), 9 smoothing points, with vector normalisation	Avg
7	2 nd derivative (cubic), 9 smoothing points, with vector normalisation	MIQR	17	Nil	MIQR
8	2 nd derivative (cubic), 9 smoothing points, with vector normalisation	Avg	18	Nil	Avg
9	2 nd derivative (quintic), 7 smoothing points	MIQR	19	Vector normalisation	Avg
10	2 nd derivative (quintic), 7 smoothing points	Avg	20	Min-max normalisation	Avg

Table 5-2: Top five PCA-LDA classification results for the Pilot Study data based on test set classification accuracy. In each case, the 3 hour wound test set sample was incorrectly classified as a 1 hour old wound.

	Data Pre-processing Method Number	Number of Principle Component Scores Used	Training Set Samples Correctly Classified (%)	Selected Cross-Validated Correctly Classified (%)	Test Set Samples Classified Correctly (%)
1	1	10	100	72	80
2	3	10	100	72	80
3	5	15	100	48	80
4	2	15	96	40	80
5	6	15	96	40	80

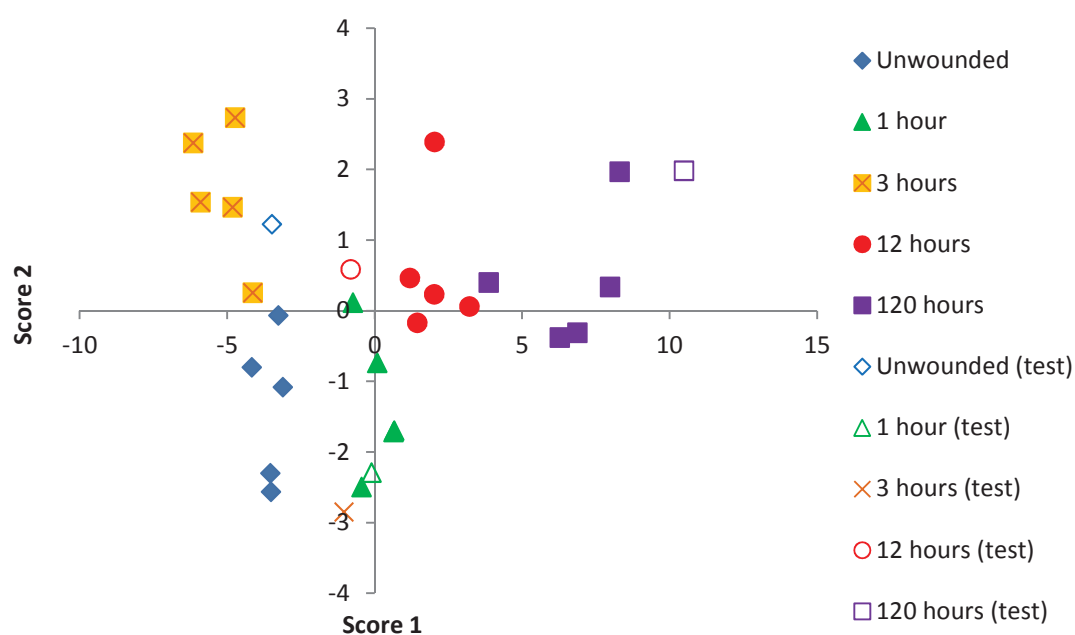


Figure 5-9: Linear discriminant analysis scores plot of the first and second score values for the wound data obtained using Method 1 (second derivative with 7 smoothing points followed by the MIQR feature vector being extracted). Ten principal component scores were input into SPSS for the subsequent LDA calculations.

Table 5-3: Best PCA-LDA classification results achieved for each data pre-processing method trialed in the pilot wound study.

Data Pre-processing Method Number	Number of Principle Component Scores Used	Training Set Samples Correctly Classified (%)	Selected Cross-Validated Correctly Classified (%)	Test Set Samples Classified Correctly (%)
1	10	100	72	80
2	15	96	40	80
3	10	100	72	80
4	5	60	36	40
5	15	100	48	80
6	15	96	40	80
7	15	100	28	60
8	15	100	28	40
9	10	84	32	40
10	15	100	28	60
11	15	100	48	40
12	15	100	20	40
13	5	56	36	60
14	15	96	32	60
15	5	60	36	60
16	15	100	28	40
17	15	96	44	40
18	15	96	52	60
19	5	56	24	40
20	15	100	44	40

5.1.1.5 *Soft Independent Modeling of Class Analogy (SIMCA)*

As with the PCA-LDA study, the effect of data pre-processing on classification was also examined when applying SIMCA to the wound tissue data using the methods listed in Table 5-1. The top five most promising SIMCA classification outcomes have been listed in Table 5-4.

5.1.2 Discussion

A number of sampling difficulties arose during both the tissue sectioning and image sampling components of this study. In regards to the tissue sectioning, the presence of sutures in a small number of the tissue blocks made microtoming more difficult and in some samples the tissue section lost its integrity. Also, skin tissue is problematic to cut at the best of times, and during the microtoming some sections were structurally altered. These unfortunate cutting artefacts produced tissue, particularly in the dermal region, which appeared 'teased' apart and possessed even more gaps than usual.

In regards to the actual collection of images and the subsequent sub-sampling of pixels, a number of problems were discovered. During the collection of infrared images, it was observed that the wound edges could be, in some cases (particularly the wounds with longer healing times), difficult to identify when using the white-light camera available in the Stingray imaging equipment. In cases where a wound location could not be clearly nominated, the central region of the tissue was imaged. Although not an ideal solution to the problem, it was the only one available given the poor visible image produced with unstained tissue sections.

Table 5-4: Top five SIMCA classification results for the Pilot Study data based on test set and training model data set classification accuracy. The incorrectly classified test data samples are shown.

	Data Pre-processing Method Number	Training Set Samples Correctly Classified (%)	Test Set Samples Classified Correctly (%)	Incorrect Classification of Test Set Samples (actual value / incorrect classification value)
1	16	84	40	Unwounded / 12 hours 12 hours / 3 hours 3 hours / 1 hour
2	12	80	40	Unwounded / 12 hours 12 hours / 1 hours 3 hours / 1 hour
3	19	76	40	Unwounded / 1 hour 1 hour / 12 hours 3 hours / 12 hours
4	13	72	40	Unwounded / 12 hours 1 hour / 3 hours 3 hours / 1 hour
5	9	72	40	Unwounded / 12 hours 1 hour / 3 hours 3 hours / 1 hour

Table 5-5: SIMCA classification results achieved for each data pre-processing method trialed in the pilot wound study.

Data Pre-processing Method Number	Training Set Samples Correctly Classified (%)	Test Set Samples Classified Correctly (%)
1	64	40
2	68	40
3	76	20
4	72	20
5	64	20
6	68	20
7	76	20
8	76	20
9	72	40
10	64	20
11	72	20
12	80	40
13	72	40
14	64	20
15	76	20
16	84	40
17	68	40
18	72	20
19	76	40
20	64	40

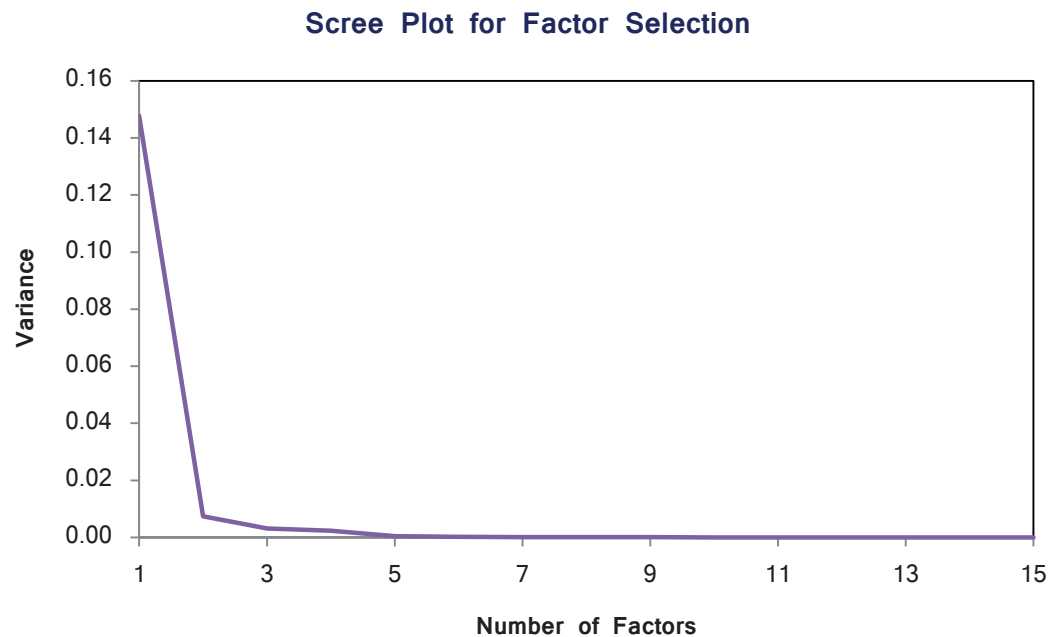
An additional problem encountered was that of the gaps present in the tissue and the very heterogeneous nature of dermal tissue. For this pilot study, regions of interest were selected by examining the spectra of the tissue in each image at various locations and selecting two areas that were the least affected by gaps or other unwanted structures (e.g. blood vessels, hair follicles, etc.). This was a manually intensive process, and it was obvious that such a method could not be employed for a larger study. An alternative pixel selection process was performed in the Main Study and will be discussed later.

As this pilot study was conducted a number of years ago, a data handling problem was also uncovered in the form of the limitations of the data dimensions imposed by Microsoft Excel 2003. At the point in time during which the pilot study was undertaken, Excel was limited to a maximum of 256 columns and 65,536 rows. This limitation dictated how much of the spectral data obtained from each image could be reasonably transformed during the various data pre-processing methods employed in the pilot study. To make data processing easier, only a small section of the infrared spectrum of each pixel was used (255 bands, roughly representing the 1200 - 1700 cm^{-1} range). The infrared spectral region selected contained a significant portion of what is referred to as the fingerprint region and this was hoped to possess sufficient characteristic information to permit classification of the wound samples. Again, this problem was easily overcome by the time the Main Study was conducted due to computer software and processor developments.

5.1.2.1 *Principal Components Analysis / Linear Discriminant Analysis*

As there is a number of possible ways to determine the most effective number of principal components (PCs) for the data analysis, it was decided that data classification would be attempted using a variety of values. After examining a number of scree plots obtained from the various data preprocessing methods utilised in the Pilot Study (an example of a scree plot obtained during the Pilot Study is given in Figure 5-10), it was determined that

Figure 5-10: An example of a Scree plot and its associated eigenvalues produced during PC analysis in Minitab for wound data pre-processed using Method 16. For this data set, it would appear that the majority of the variance within the data is explained within the first few PCs.



Factor	Eigenvalue	% Total Variance	Cumulative % Variance
1	0.14771	91.30852	91.30852
2	0.00739	4.56577	95.87429
3	0.00312	1.93086	97.80515
4	0.00236	1.45599	99.26114
5	0.00045	0.27636	99.53750
6	0.00017	0.10523	99.64272
7	0.00012	0.07242	99.71514
8	0.00009	0.05726	99.77240
9	0.00007	0.04117	99.81357
10	0.00005	0.02777	99.84133
11	0.00004	0.02515	99.86648
12	0.00004	0.02404	99.89052
13	0.00003	0.01994	99.91045
14	0.00003	0.01723	99.92769
15	0.00003	0.01572	99.94341

the LDA classifications would be performed using three score value sets comprised of the first 5, 10 and 15 PC factors.

Based on the results obtained using LDA with cross-validation, it was found that retaining a higher number of principle component scores (i.e. 10 or 15) produced consistently better classification outcomes for the Pilot Study wound data. However, retaining the first 15 factors, whilst providing high training set correct classification values, did not fare as well when compared to the classifications using 10 factors in regards to the resulting cross-validation outcomes and suggests that some over-fitting may have been occurring.

As noted in Table 5-2, for each of the top-performing preprocessing method classification outcomes, the same test data sample, the 3 hour old wound, was incorrectly classified as a 1 hour old wound. This misclassification is not surprising when one examines the scores plot given in Figure 5-9 and the nature of the data groupings along the plane of the second score value for the unwounded, 1 hour and 3 hour old wound tissue. Based on the centroids for each of the aforementioned wound age groups, one may have expected the 3 hour old wound to have misclassified as unwounded tissue instead. From a biological viewpoint, this misclassification and the 'bunching' of the earlier wound age data may indicate that there is limited variability detectable by this analytical technique for early age wounds. An alternative view may be that the data point is in fact an outlier, possibly due to biological variability (i.e. not all of the rats heal at the same rate). Unfortunately, with such a small data set examined, it is difficult to confirm such an observation based on the Pilot Study data alone.

It is clear from the classification results achieved using the PCA-LDA analysis that a measure of wound age group separation is possible, although the magnitude of variance between the age groups studied may be very small. The median-interquartile range feature vector produced the best classification results, which may be due to the fact that it contains more information from the sample spectra than the average spectrum alone.

Based on the classification results achieved, it is difficult to determine to what extent each data preprocessing technique affects the final classification achieved, as most resulted in good training set classification outcomes. The results obtained may also suggest that too many PCs were used and that overfitting was occurring. For all methods employed the cross-validation outcomes were not ideal, never achieving a value above the low seventies. It was not expected that the cross-validation value would be similar to the training set classification considering the high spectral similarity between age groups. A low cross-validation value can be determined as acceptable for purposes such as a rough screening test, but it may also indicate an inadequate analytical technique.^[5] The fact that, for some preprocessing methods, the test data set classification was relatively good indicates that the PCA-LDA classification method warrants further investigation with larger training and test data sets, with more wound age time points and sample replicates.

5.1.2.2 *Soft Independent Modeling of Class Analogy*

Although the SIMCA classification approach did not work well for the Pilot Study when compared with the results obtained using PCA-LDA, it is interesting to note that the one test data set sample that was correctly classified, regardless of the pre-processing / feature vector method used, was that of the five day (120 hours) old wound. As observed earlier, at five days the protein content observable in the wound region is increased, and it may be this component which assisted in the discrimination of this wound age group from the remaining samples. Interestingly, the next most correctly classified test data sample was that of the 12 hour wound, which was successfully identified in only four out of the twenty pre-processing / feature vector methods. The unwounded and 3 hour wound tissue samples were not once correctly classified using SIMCA in this pilot study.

Also of note is that the fact that, in over fifty per cent of the preprocessing methods employed, the 1 hour wound was misclassified as a 3 hour wound (twelve out of the twenty methods employed), and the 3 hour wound as 1 hour (eighteen instances). This

result would suggest that the two wounds, although two hours apart, are chemically too similar (based on infrared spectroscopic characteristics) to permit sufficient separation between the groups for classification purposes. What is difficult to understand is why the unwounded test data sample, in eighty per cent of the results obtained, was misclassified as a 12 hour wound, a misclassification result which was observed at times in the PCA-LDA data, but not to such a strong degree.

Based on the classification results achieved using SIMCA, it is difficult to definitively state the effect that pre-processing and feature vector extraction methods had on the classifications obtained. Whilst the quintic second derivative method was present in the top five classifications achieved, these results could not be labelled a significant improvement on those achieved using cubic derivative polynomial shape. The same also applies for the feature vector method employed, as well as whether vector normalisation had any significant impact on the classifications achieved.

5.1.2.3 Pilot Study Result Summary

The purpose of the Pilot Study was quite simply to examine the feasibility of employing infrared spectral analysis of rat skin tissue sections to enable incised wound age prediction. From a statistical viewpoint, any results obtained from the various classification methods employed are extremely limited and offer, at the most optimistic, a guide as to whether the spectra obtained possess sufficient variation between age groups for chemometric classification.

While neither of the statistical discrimination procedures produced a perfect classification of the test data, both methods were able to distinguish the older, 5 day old antemortem wound from the more closely timed remaining wounds, regardless of the data preprocessing method used. The classification of the earlier time point wounds, unlike the 5 day old wound, appeared to be more affected by the preprocessing steps used. Whilst the 1 hour

old wound test data was usually classified correctly in the majority of preprocessing methods examined, when misclassified, it was often erroneously assigned to the 3 hour old sample group (in the case of SIMCA) or the unwounded sample group (PCA-LDA).

Although various biological changes in human skin tissue have been detected as early as the first few minutes,^[6] the more common or regular detection times for these entities may be quoted at around 30 - 60 minutes. Likewise, there are parameters which are given an earliest detection time of 30 - 60 minutes, but are more regularly detected (or produce a marked reaction) an hour or more after wounding. Therefore, one would not be surprised by difficulties being encountered in the data modeling of the earlier time point wound samples, particularly after considering the dynamic nature of the wound healing process.

Although the classifications of the pilot wound data were not ideal, the results obtained indicated that a further, larger study was warranted. The goal of performing the larger, Main Study, was to evaluate the ability of PCA-LDA and SIMCA to classify wounds according to their ages, or at the very least examine the nature of the biological and biochemical variability of the wound tissue samples based on an infrared spectral analysis.

5.2 MAIN STUDY

The purpose of the main study was to focus on improving the spectral quality and increasing the model and test set sizes to provide a more statistically robust classification analysis. It was hoped that the ability to collect a larger number of sample replicates for each wound age would greatly improve confidence in the statistical models obtained, and assist in the detection of classification problems, such as overfitting.^[7]

5.2.1 Results

5.2.1.1 *Data Preprocessing Approaches*

Pixel Selection

To reduce any chances of bias regarding the selection of representative spectra from each wound image, it was decided that pixel selection would be automated using the quality test function in CytoSpec. The sample thickness test in CytoSpec calculates the integral absorption value for the spectrum over a specific range and checks this against user-determined upper and lower threshold values.^[8] Pixel integral values which fail to meet these criteria are removed from the data set. The combination of spectral range and selection criteria that gave the most consistent removal of the tissue gaps was the 3100 - 3400 cm^{-1} spectral region, with threshold values of 100:500. An example of a result from this quality testing procedure on a wound tissue image is shown in Figure 5-11.

Noise Reduction

Thin skin tissue sections, by their nature, produce infrared spectra ranging from the excellent to the extremely noisy in quality, depending on which structures or layers within the skin are being analysed. It was observed that the more closely-packed cellular structures (e.g. epidermis, blood vessels) produced the better quality spectra, with the effects of the dispersion artefact less visible also. However, it was primarily the dermal region

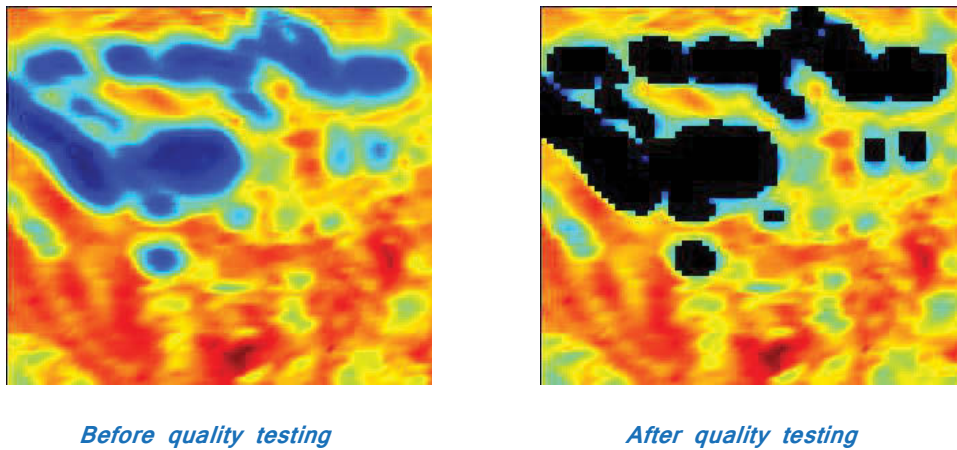


Figure 5-11: Example of the spectral quality test thickness test as implemented in CytoSpec using a 7 day old wound tissue section (16 cm^{-1} spectral resolution, imaged at $1620\text{-}1680\text{ cm}^{-1}$).

that was being sampled in this study, and with the tissue itself exposed to the atmosphere, sample spectral quality varied considerably.

Two approaches were taken in an attempt to analyse the effects of spectral noise on subsequent classification. For this main study, infrared spectral data were collected at three resolution values: 4 , 8 and 16 cm^{-1} . By collecting data at these varying resolutions, the effect of spectral resolution on the classification capability of the data was able to be observed. At 8 and 16 cm^{-1} spectral resolution, water vapour interference is less obvious, and consequently data classification may be improved by minimising the effect of the water peaks on feature extraction.

A second approach was to perform PCA-based noise reduction as a data preprocessing step in a number of methods and contrast the classification results obtained with those methods which employed no noise reduction processes. PCA-based noise reduction, performed in the CytoSpec software package, involves performing a principal component analysis of the image data. The spectra are then 're-assembled' based on the number of

principal components the user chooses.^[8] It must be noted that the choice of which PCs to include or exclude is a subjective process, and the user risks the introduction of spectral artefacts. Hence, in this study two data sets subjected to exactly the same preprocessing steps, aside from the use of PCA-based noise reduction, were collected. An example of the noise observed in the wound tissue data, and the effect of PCA-based noise reduction on the wound spectra, has been given in Figure 5-12.

Figure 5-13 outlines the data collection, preparation and pre-processing employed in the Main Study. Table 5-6 contains a comprehensive listing of the data pre-processing and feature vector reduction combination methods employed prior to performing any multivariate analysis (i.e. all of the steps leading up to the final classification routines attempted). Four feature vector extraction methods were employed in the Main Study: average (Avg), median (Med), average-standard deviation (ASD) and the median-interquartile range (MIQR).

5.2.2 Discussion

It was the goal of this main study to perform a comprehensive data analysis which would explore a wide range of data preprocessing and reduction methods in an effort to determine the optimum data preparation and analysis approach for the wound data. Ultimately, the classification will be limited by the quality of the data obtained, and its inherent ability to capture features of interest which the multivariate analysis can identify and utilise for wound age discrimination.

5.2.2.1 *Principal Components Analysis / Linear Discriminant Analysis*

Table 5-7 provides the top ten most successful PCA-LDA wound age classification results ranked according to the percentage of the training data set correctly classified. Table 5-8, in contrast to Table 5-7, ranks the various preprocessing methods according to the test data set classification accuracy. A wider classification grouping, which includes samples

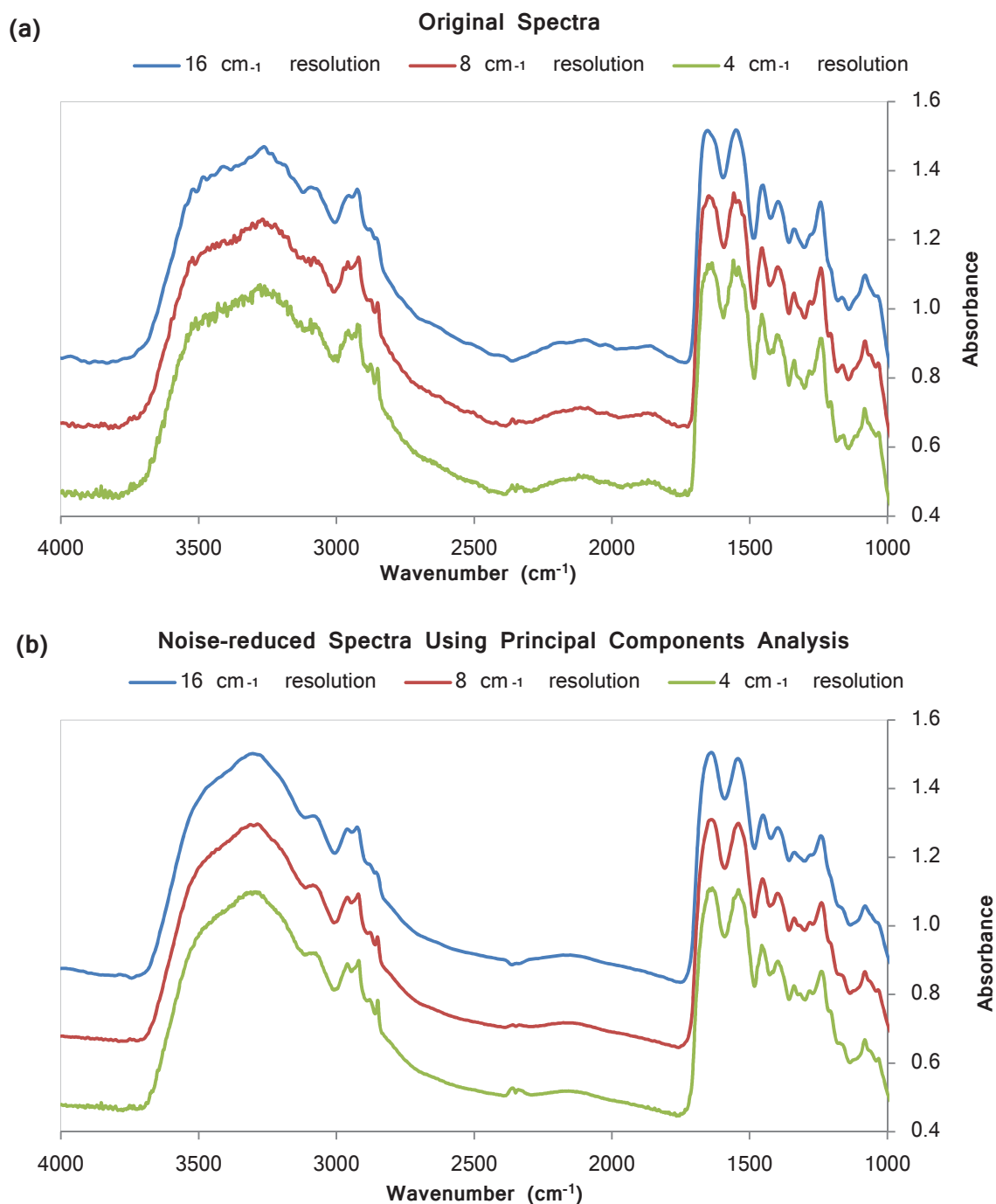


Figure 5-12: (a) Example spectra obtained from a 2 day-old wound tissue section at the same location with varying spectral resolution values. The spectra have been off-set for display purposes. (b) Example of the effect on spectral quality when PCA-based noise reduction is performed on spectra of varying resolution values from (a). The first five principal components have been retained to produce these spectra. It is clear that this noise reduction approach has the greatest impact on the 4 cm^{-1} spectral resolution data.

Figure 5-13: Imaging and data reduction process overview employed in the Main Study.

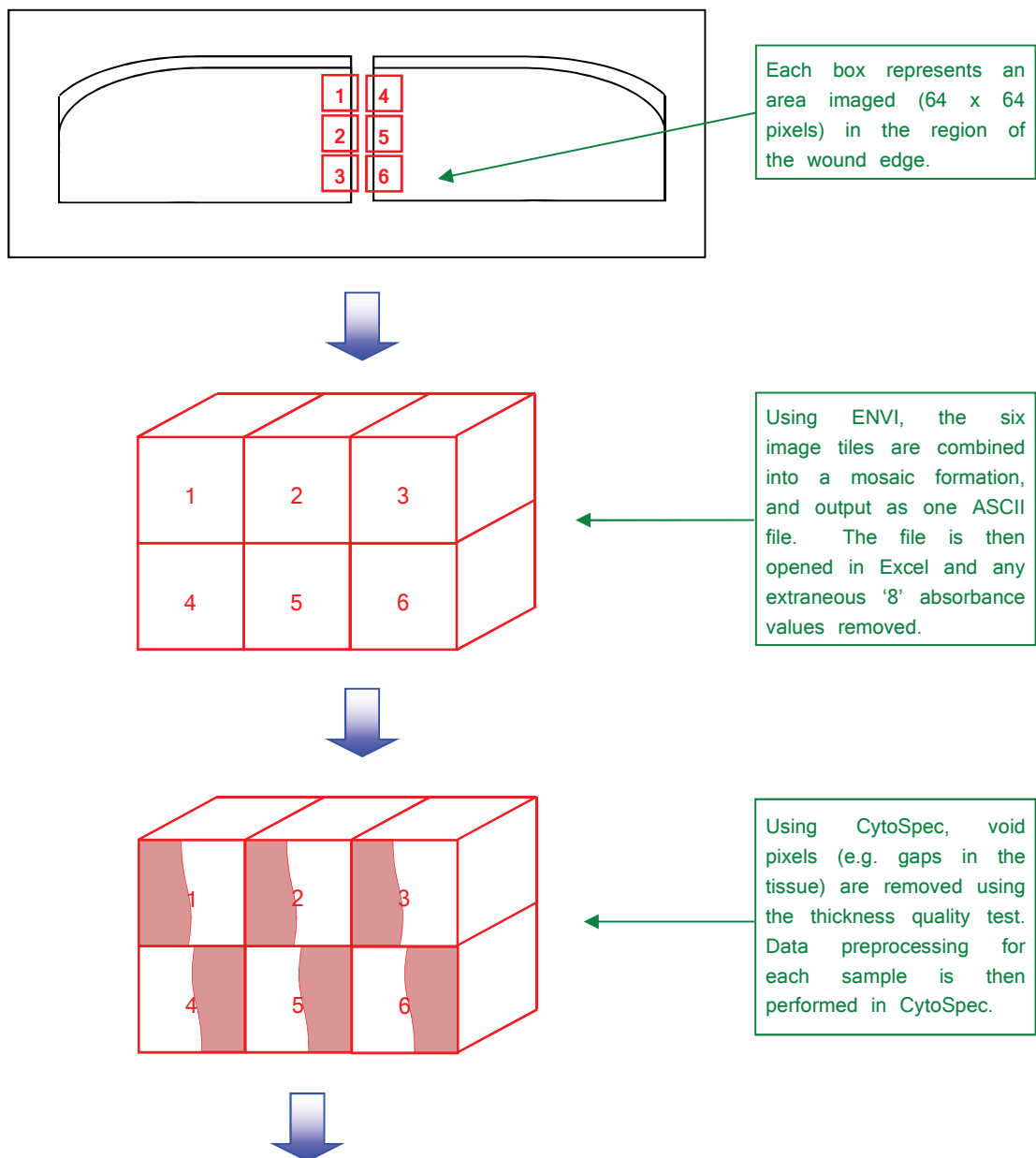


Figure 5-13 (continued): Imaging and data reduction process overview employed in the Main Study.

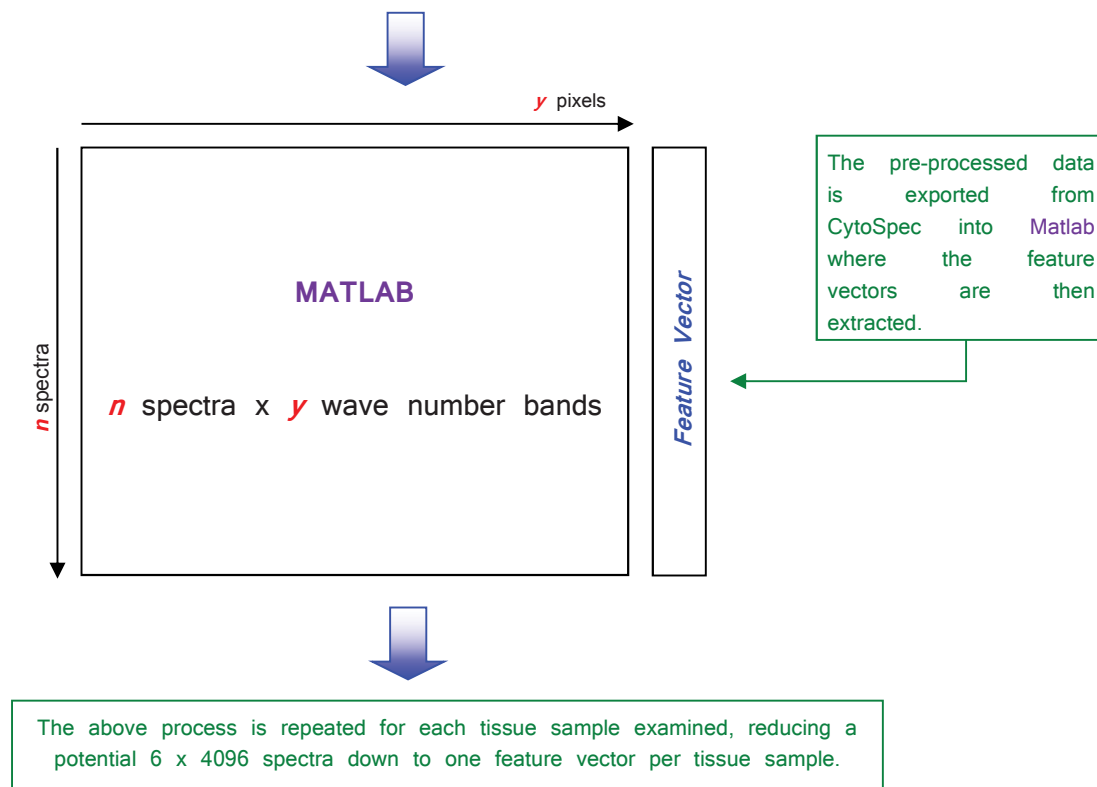


Table 5-6: Data preprocessing combinations applied to the Main Study wound data.

Method Number	PCA-based Noise Reduction	Spectral Resolution (cm ⁻¹)	Spectral Range Utilised (cm ⁻¹)	Derivative (Order / Smoothing)	Vector Normalisation	Feature Vector Calculation Method			
						MIQR	Med	Ave	ASD
21	-	4 / 8 / 16	(900 - 4000)	2 / 9	-	✓	✓	✓	✓
22	-	4 / 8 / 16	(900 - 4000) / (1200 - 1700) ⁺	2 / 9	✓	✓	✓	✓	✓
23	-	4 / 8 / 16	(900 - 4000)	1 / 9	-	✓	✓	✓	✓
24	-	4 / 8 / 16	(900 - 4000)	1 / 9	✓	✓	✓	✓	✓
25	5 / 15*	4 / 8 / 16	(900 - 4000)	2 / 9	-	✓	✓	✓	✓
26	5 / 15*	4 / 8 / 16	(900 - 4000) / (1200 - 1700) ⁺⁺	2 / 9	✓	✓	✓	✓	✓
27	5 / 15*	4 / 8 / 16	(900 - 4000)	1 / 9	-	✓	✓	✓	✓
28	5 / 15*	4 / 8 / 16	(900 - 4000)	1 / 9	✓	✓	✓	✓	✓
29	5 / 15*	4 / 8 / 16	(900 - 4000)	-	✓	✓	✓	✓	✓
30	5 / 15*	4 / 8 / 16	(900 - 4000)	2 / 7	-	✓	✓	✓	✓
31	5 / 15*	4 / 8 / 16	(900 - 4000)	2 / 7	✓	✓	✓	✓	✓
32	-	4 / 8 / 16	(900 - 4000) / (1200 - 1700)	2 / 7	-	✓	✓	✓	✓
33	-	4 / 8 / 16	(900 - 4000) / (1200 - 1700)	2 / 7	✓	✓	✓	✓	✓
34	-	4 / 8 / 16	(900 - 4000)	-	✓	✓	✓	✓	✓

* PCA-based noise reduction employing 15 principal components was performed on the 8 and 16 cm⁻¹ resolution data only.

+ The truncated spectral range was only performed using the 4 cm⁻¹ resolution data.

++ The truncated spectral range was only performed using the 8 and 16 cm⁻¹ resolution data.

Table 5-7: Top ten PCA-LDA classification results for the Main Study data based on training set classification accuracy.

	Data Pre-processing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Number of PCs Used in LDA	Training Set Samples Classified Correctly (%)	Selected Cross-Validated Correctly Classified (%)	Test Set Samples Classified Correctly (%)
1	28	8	Avg	Deriv	15	15	74.1	22.2	20.8
2	33	8	ASD	Deriv	-	15	71.6	18.5	27.1
3	31	8	Med	Deriv	15	15	70.4	13.6	25.0
4	24	8	Avg	Deriv	-	15	70.4	19.8	22.9
5	24	8	ASD	Deriv	-	15	70.4	28.4	20.8
6	32	8	ASD	Deriv	-	15	70.4	24.7	20.8
7	23	8	Avg	Deriv	-	15	69.1	22.2	31.3
8	21	8	Avg	Pre-pro	-	15	69.1	25.9	29.2
9	34	8	ASD	Pre-pro	-	15	69.1	16.0	27.1
10	25	8	Avg	Pre-pro	15	15	69.1	25.9	27.1

Pre-pro = pre-processed data block

Deriv = derivative data block

Table 5-8: Top ten PCA-LDA classification results for the Main Study data based on test set classification accuracy.

	Data Pre-processing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Number of PCs Used in LDA	Training Set Samples Classified Correctly (%)	Selected Cross-Validated Correctly Classified (%)	Test Set Samples Classified Correctly (%)
1	32	4	Avg	Deriv	-	5	40.7	17.3	35.4
2	32	4	Avg	Deriv	-	15	65.4	21.0	33.3
3	33	16	Med	Deriv	-	15	64.2	18.5	33.3
4	32	4	Med	Deriv	-	5	42.0	19.8	33.3
5	23	8	Avg	Deriv	-	15	69.1	22.2	31.3
6	27	8	Avg	Deriv	15	15	67.9	23.5	31.3
7	26	16	Med	Deriv	15	15	65.4	21.0	31.3
8	22	16	MIQ	Deriv	-	5	30.9	12.3	31.3
9	21	8	Avg	Pre-pro	-	15	69.1	25.9	29.2
10	22	16	Med	Deriv	-	15	64.2	21.0	29.2

classified in either wound age group on either side of the sample's true wound age, has also been presented in Table 5-9 and Table 5-10 to examine the extent of overlap between age groups.

To graphically represent the variance detected by the PCA-LDA approach, an example of a scores plot has been given in Figure 5-14.

5.2.2.2 *Soft Independent Modeling of Class Analogy*

Table 5-11 provides the top ten most successful SIMCA wound age classification results ranked according to the percentage of the training data set correctly classified. Table 5-12, in contrast to Table 5-11, ranks the various preprocessing methods according to the test data set classification accuracy. A wider classification grouping, which includes samples classified in either wound age group on either side of the sample's true age group, has also been presented in Table 5-13 and Table 5-14. An example of a classification result output for SIMCA has been given in Table 5-15 and Table 5-16.

5.2.2.3 *Self Organising Map (SOM) Investigation*

Due to the poor classification results being achieved using the PCA-LDA and SIMCA approaches, an examination of the data using an unsupervised classification method was investigated. A form of neural network, known as a Self Organising Map (SOM), was employed to examine the relationships between the various wound samples and hence the data structure. Unlike PCA-LDA, which is a classification technique based on factor analysis combined with a linear clustering technique, SOM is a purely non-linear clustering technique which may be used in a supervised or unsupervised manner.^[9] Because of time constraints, SOM was conducted only on a smaller subset of the preprocessing methods listed.

Table 5-9: Top ten PCA-LDA classification results for the Main Study data based on training set classification with ± 1 group accuracy.

	Data Pre-processing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Number of PCs Used in LDA	Training Set Samples Classified Correctly ± 1 group (%)	Cross-Validated Correctly Classified ± 1 group (%)	Test Set Samples Classified Correctly ± 1 group (%)
1	28	8	Avg	Deriv	15	15	86.4	59.3	50.0
2	24	8	Avg	Deriv	-	15	85.2	54.3	54.2
3	28	16	Avg	Deriv	15	15	84.0	59.3	50.0
4	22	16	Med	Deriv	-	15	84.0	50.6	47.9
5	23	8	Avg	Deriv	-	15	82.7	50.6	58.3
6	31	8	Med	Deriv	15	15	82.7	51.9	50.0
7	24	16	Avg	Deriv	-	15	82.7	59.3	50.0
8	34	8	Med	Pre-pro	-	15	82.7	53.1	50.0
9	30	8	MIQ	Deriv	15	15	82.7	60.5	50.0
10	32	8	Avg	Deriv	-	15	82.7	50.6	50.0

Table 5-10: Top ten PCA-LDA classification results for the Main Study data based on test set classification with ± 1 group accuracy.

	Data Pre-processing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Number of PCs Used in LDA	Training Set Samples Classified Correctly ± 1 group (%)	Cross-Validated Correctly Classified ± 1 group (%)	Test Set Samples Classified Correctly ± 1 group (%)
1	22	8	Med	Deriv	-	10	75.3	53.1	60.4
2	23	8	Med	Deriv	-	10	74.1	55.6	60.4
3	23	8	Avg	Deriv	-	15	82.7	50.6	58.3
4	21	16	Med	Deriv	-	10	77.8	46.9	58.3
5	27	8	Avg	Deriv	15	15	81.5	51.9	56.3
6	22	8	ASD	Deriv	-	10	80.2	58.0	56.3
7	32	4	Avg	Deriv	-	15	76.5	49.4	56.3
8	32	16	Med	Deriv	-	10	75.3	49.4	56.3
9	27	16	MIQ	Deriv	15	15	72.8	54.3	56.3
10	23	16	Avg	Deriv	-	10	70.4	50.6	56.3

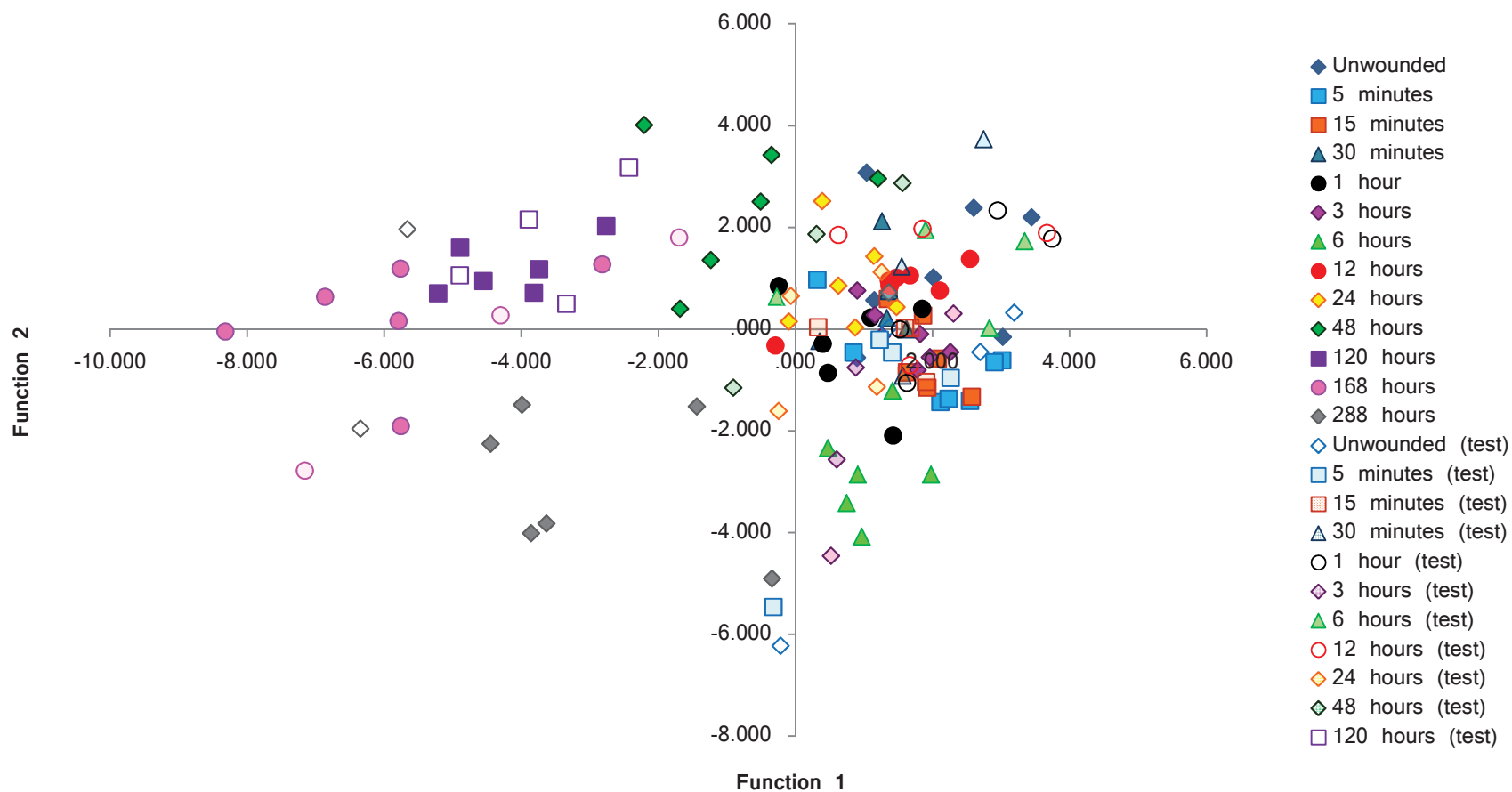


Figure 5-14: Linear discriminant analysis scores plot obtained for the 8 cm^{-1} spectral resolution ('average' feature vector) Main Study wound data which has undergone preprocessing according to Method 23. Fifteen principal component scores were provided for the subsequent LDA classification.

Table 5-11: Top ten SIMCA classification results for the Main Study data based on training model data set classification accuracy.

	Data Pre-processing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Training Set Samples Classified Correctly (%)	Test Set Samples Classified Correctly (%)
1	31	8	ASD	Deriv	15	88.9	25.0
2	26	8	Med	Deriv	15	87.7	22.9
3	31	8	ASD	Deriv	5	86.4	27.1
4	31	8	Avg	Deriv	15	86.4	25.0
5	33	8	ASD	Deriv	-	85.2	27.1
6	26	8	ASD	Deriv	15	85.2	25.0
7	26	16	Med	Deriv	15	85.2	23.4
8	31	8	Avg	Deriv	5	85.2	22.9
9	31	8	MIQ	Deriv	15	85.2	18.8
10	26	16	Avg	Deriv	15	82.7	27.7

Table 5-12: Top ten SIMCA classification results for the Main Study data based on test data set classification accuracy.

	Data Preprocessing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Training Set Samples Classified Correctly (%)	Test Set Samples Classified Correctly (%)
1	26	4	MIQ	Deriv	5	71.6	29.2
2	32	16	Avg	Deriv	-	67.9	29.2
3	26	16	Med	Deriv	15	67.9	29.2
4	30	16	Avg	Deriv	15	67.9	29.2
5	30	16	Avg	Deriv	-	66.7	29.2
6	25	16	Med	Deriv	-	65.4	29.2
7	26	16	Avg	Deriv	15	82.7	27.7
8	22	16	Avg	Deriv	-	80.2	27.7
9	31	8	ASD	Deriv	5	86.4	27.1
10	33	8	ASD	Deriv	-	85.2	27.1

Table 5-13: Top ten SIMCA classification results for the Main Study data based on training model data set classification accuracy allowing for a wider classification range.

	Data Preprocessing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Training Set Samples Classified Correctly ± 1 group (%)	Test Set Samples Classified Correctly ± 1 group (%)
1	31	8	ASD	Deriv	15	96.3	52.1
2	31	8	ASD	Deriv	5	95.1	52.1
3	26	8	ASD	Deriv	5	95.1	52.1
4	31	8	MIQ	Deriv	15	95.1	47.9
5	26	8	ASD	Deriv	15	93.8	56.3
6	26	8	Avg	Deriv	5	93.8	56.3
7	33	8	ASD	Deriv	-	93.8	47.9
8	26	8	Avg	Deriv	15	92.6	54.2
9	22	8	ASD	Deriv	-	92.6	54.2
10	33	8	MIQ	Deriv	-	92.6	45.8

Table 5-14: Top ten SIMCA classification results for the Main Study data based on test data set classification accuracy allowing for a wider classification range.

	Data Preprocessing Method Number	Spectral Resolution (cm ⁻¹)	Feature Vector	Data Block Used (Pre-processed Only / Derivative)	PCA based Noise Reduction - # of PCs Retained	Training Set Samples Classified Correctly ± 1 group (%)	Test Set Samples Classified Correctly ± 1 group (%)
1	26	4	MIQ	Deriv	5	81.5	58.3
2	26	8	ASD	Deriv	15	93.8	56.3
3	26	8	Avg	Deriv	5	93.8	56.3
4	26	8	Avg	Deriv	15	92.6	54.2
5	22	8	ASD	Deriv	-	92.6	54.2
6	31	8	Avg	Deriv	15	91.4	54.2
7	26	8	Med	Deriv	15	91.4	54.2
8	31	8	Avg	Deriv	5	91.4	54.2
9	26	8	MIQ	Deriv	15	91.4	54.2
10	33	8	Avg	Deriv	-	87.7	54.2

Table 5-15: An example of the training data SIMCA classification output obtained using the 8 cm⁻¹ spectral resolution wound data pre-processed with Method 31 (noise reduction: 15 PCs retained, ASD feature vector).

	Pred1 @2	Pred2 @2	Pred3 @2	Pred4 @1	Pred5 @1	Pred6 @2	Pred7 @1	Pred8 @2	Pred9 @2	Pred10 @1	Pred11 @1	Pred12 @2	Pred13 @2	Total Number of Samples
1. Unwounded	6	1												7
2. 5 minutes	1	6												7
3. 15 minutes		1	5											6
4. 30 minutes				5										5
5. 1 hour					4	1		1						6
6. 3 hours			1			6								7
7. 6 hours						1	5							6
8. 12 hours								7						7
9. 24 hours									6					6
10. 48 hours		1								5				6
11. 120 hours											5	1		6
12. 168 hours												6		6
13. 288 hours													6	6
Total Number of Test Set Samples Correctly Classified (%)														88.9

Table 5-16: An example of the test data SIMCA classification output obtained using the 8 cm^{-1} spectral resolution wound data pre-processed with Method 31 (noise reduction: 15 PCs retained, ASD feature vector).

	Pred1 @2	Pred2 @2	Pred3 @2	Pred4 @1	Pred5 @1	Pred6 @2	Pred7 @1	Pred8 @2	Pred9 @2	Pred10 @1	Pred11 @1	Pred12 @2	Pred13 @2	Total Number of Samples
1. Unwounded	1	1				2								4
2. 5 minutes		1					1	1					1	4
3. 15 minutes		1				1	1		1					4
4. 30 minutes		1			1			1						3
5. 1 hour					1	2			1					4
6. 3 hours	1					3								4
7. 6 hours	1		1					1	1					4
8. 12 hours		1					1	2						4
9. 24 hours			1			1		1	1					4
10. 48 hours				1					1				1	3
11. 120 hours										1	1	1	1	4
12. 168 hours											1	1	1	3
13. 288 hours	1										1		1	3
Total Number of Test Set Samples Correctly Classified (%)														25.0

To perform the SOM analysis on the wound data, the Self Organising Mapping toolbox of Matlab was employed.^[10] This permitted the feature vector data, which had been previously prepared for the Pirouette analyses, to be directly input into the neural network. In addition to using the feature vector data, the principal components extracted during the PCA study were also analysed using the SOM function. Map dimensions and training parameters were determined automatically using the software's inbuilt analysis methods.

5.2.3 Discussion

5.2.3.1 *Principal Components Analysis / Linear Discriminant Analysis*

Based on the training set classification results obtained, it would appear that the spectral resolution which produces the best results is that of the 8 cm⁻¹ data. However, if one looks at the test data classification values, there would appear to be no distinct advantage in using one particular spectral resolution value over another. If one inspects the classification accuracy when a wider classification tolerance range is employed the least desirable spectral resolution is that of the 4 cm⁻¹ data. Of the three wavenumber resolution values employed, the finer spectral resolution will be the most effective in capturing subtle peak features but is also the most subject to noise.

In regards to the value of employing PCA-based noise reduction, the classification results clearly indicate it is most beneficial for the higher resolution spectra (4 cm⁻¹), improving training correct classification percentages (CC%) by up to 17. The amount of improvement in classification accuracy was less for that of the first derivative as opposed to the second derivative data, which is to be expected considering that the second derivative will be more highly influenced by any noise present in the original data. In the cases of the 8 and 16 cm⁻¹ resolution data, any improvements in classification accuracy were generally minor and in some cases even reduced classification accuracy to a limited extent. Hence, for the lower spectral resolution data, the use of PCA based noise reduction, be it with a smaller

or larger number of PCs retained, does not demonstrate any advantage for wound age classification accuracy.

As with the Pilot Study data, it was decided that a range of principal component scores would be utilised for subsequent classification in LDA. Based on the classification results obtained for both the training and test set classifications, it is clear that the employment of fifteen principal components for the LDA classification provides the best wound age discrimination. The use of the larger number of principal component values is also supported when one observes that the majority of the poorest classification results were achieved using the smallest number of score values (i.e. five PCs).

In general, the 4 cm^{-1} data performed relatively poorly based on training set classification accuracy, particularly when no PCA-based noise reduction was employed. While the finer spectral resolution did produce some good test set classification results when compared to the other resolution data, it performed poorly when one analyses the associated training set cross validation values. In general the 8 cm^{-1} resolution spectra performed only marginally better than the 16 cm^{-1} data when one takes into account training set, test set and cross-validation classification accuracy.

Unfortunately the results obtained in this study did not clearly indicate a more suitable feature vector when compared against the others. The ASD and MIQR feature vectors provided consistently higher cross-validation correct classification values, but the average and median feature vectors produced the better test set classification values. Overall, taking the derivative of the spectra, as opposed to using the original spectra, produced better cross validation and test set classification results. However, the results did not clearly indicate any advantage in using the first or second derivative, or the optimum number of smoothing points to include.

In regards to the use of vector normalisation of the wound spectra prior to data analysis, the results produced in this PCA-LDA study did not clearly indicate any consistent benefit to classification accuracy from its use. Surprisingly, truncating the spectra to a narrow region of the fingerprint portion of the mid infrared spectrum (1200 to 1700 cm^{-1}) did not provide any improvement in classification either.

Due to the poor performance of the PCA-LDA classification approach, it is difficult to draw any strong conclusions regarding the selection of the optimum data pre-processing method for the wound data. The results achieved suggest that the PCA-LDA method is an unsuitable classification technique for the wound spectra, incapable of detecting the subtle differences which exist between wounds of different ages, or that the infrared imaging technique employed on the samples provided is unable to produce spectral data with high enough quality.

5.2.3.2 *Soft Independent Modeling of Class Analogy*

Although the classifications obtained using SIMCA were marginally better compared to those produced using PCA-LDA, the classifications obtained for both the training and test data sets were far from ideal. The optimum classification achieved using strict sample groupings produced a test set classification of just under 30%. An example of the information available to the user after employing SIMCA, such as the ability to extract information regarding the modeling power available from the training data set, has been provided in Figure 5-15, and shows the limited discrimination being achieved for the wound data.

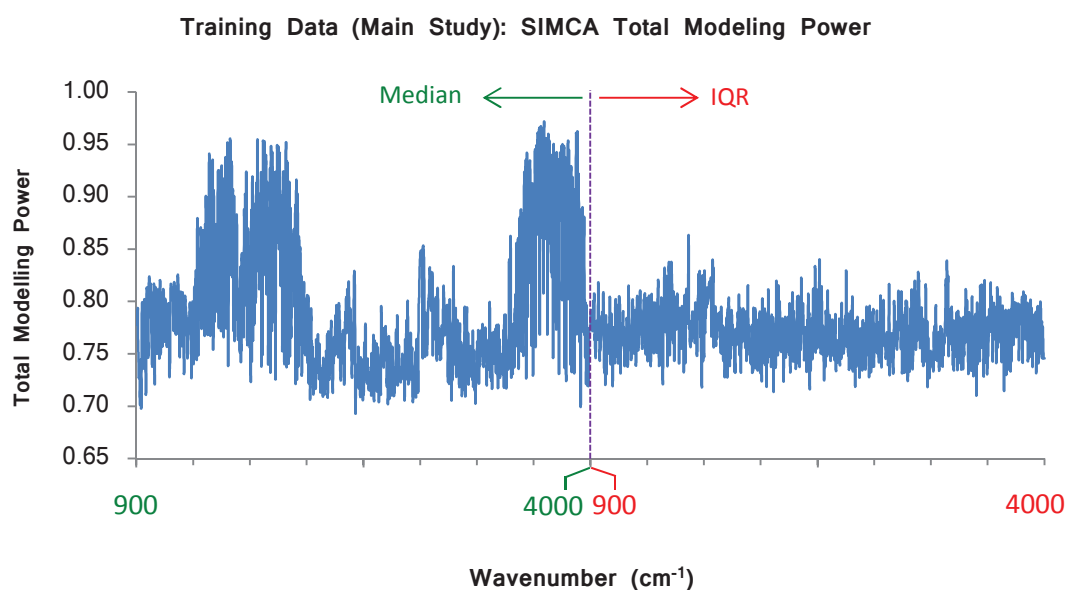


Figure 5-15: SIMCA total modeling power for data preprocessing Method 26 (4 cm^{-1} resolution, 5 PCs noise reduction) for the training data set. It would appear that the median component of the feature vector contains more discriminatory features than the interquartile range region.

Based on the data obtained in this study and that presented in Table 5-11 to Table 5-14, the higher spectral resolution of 4 cm^{-1} does not provide a consistent advantage in wound age classification over the lower resolution values, appearing only once in the top fifty highest test set classification results. While the lowest spectral resolution value of 16 cm^{-1} produced some of the highest test data classification results, the medium value of 8 cm^{-1} appears to consistently produce the best results of the three spectral resolution values examined after looking at the training and test set classification accuracy results together.

After examination of the results achieved during the Pilot Study, which by necessity had employed only a narrow region of the infrared spectrum collected ($1200 - 1700 \text{ cm}^{-1}$), it was decided that a number of the preprocessing methods utilised in the Main Study examined would also be performed using the same narrow spectral region. The region

targeted contains a large number of characteristic infrared spectral feature bands for tissue components such as collagen, nucleic acids, phospholipids, fatty acids and proteins. Spectral truncation produced a reduction in the percentage of correct classification for both the 8 and 16 cm^{-1} resolution data, with the former affected the most significantly (up to 17% and 10% drop in training and test data classification accuracy respectively). In contrast, the 4 cm^{-1} resolution spectra appeared to benefit from the narrower spectral range, with an improvement in one data set's training classification accuracy by almost 20%. This result may indicate that the lower resolution data, whilst less affected by noise, may in turn be failing to capture the more subtle spectral band features in the fingerprint region. Even with the spectral truncation, the classification accuracy of the 4 cm^{-1} data was only just meeting the classification quality of the 8 cm^{-1} data using an equivalent preprocessing method without spectral truncation.

Without question, the taking of derivatives improved spectral classification, with minimal difference observed between seven or nine smoothing points, based on the training and test set classification results achieved. In contrast, there appeared to be no strong indicators from the data obtained as to a particular feature vector producing a better result in comparison to the others.

The impact of vector normalisation on the data set classifications was most noticeable for the test set classifications when the strictest sample groupings were required: it produced a slightly lower test set classification but improved training set classification considerably. Hence, the results would indicate that while the need to use vector normalisation is not strongly supported based on the test set classifications, it does have a strong positive effect on training set classifications.

For strict sample group classifications, the use of PCA-based noise reduction did not appear to have any significantly favourable effect on the test set classifications. However, like the use of vector normalisation, the training set classifications did appear to benefit from the

use of the noise reduction algorithm. Interestingly, the noise-reduced 4 cm⁻¹ resolution data, which showed some significant improvements in both training and test classifications compared to the non-treated data, also recorded the most negative effect when the data was pre-processed using only vector normalisation. This effect was not as pronounced for the 8 and 16 cm⁻¹ resolution data.

5.2.3.3 Self Organising Map Algorithm

Figure 5-16 provides an example of a SOM output, in the form of a U-matrix, obtained from the analysis of the wound data. Although the example given does indicate a coarse form of clustering, again separating the older wounds from the remainder of the samples, this was not replicated in all of the data sets examined. In general, there were no clear signs of clustering for the majority of the data sets examined based on U-matrix inspection.

As part of the SOM Toolbox functionality, the Topographic Error (TE) and Mean Quantisation Error (MQE) for each map are calculated. High TE values were recorded for a number of the maps obtained, with values as large as 0.1, which strongly indicates that samples not necessarily represented as neighbours on the map may, nevertheless, be highly similar to each other in the input space.^[11] The MQE is a measure of map quality, and unlike the TE values varied considerably with the preprocessing method employed. For example, high MQE values were obtained for data pre-processed using the second derivative and vector normalisation, but were significantly lower for data pre-processed using the second derivative alone. Low MQE values generally indicate that the average similarity between the map and the training samples is poor.

Employing the PCs obtained from the PCA analysis, as opposed to the raw feature vector values, did little to improve the clustering results achieved, nor did the choice of feature vector used. The 4 cm⁻¹ resolution data, regardless of preprocessing method, gave consistently the highest MQE values but interestingly did not produce clear topographical maps displaying wound age clustering, in contrast to the lower resolution data.

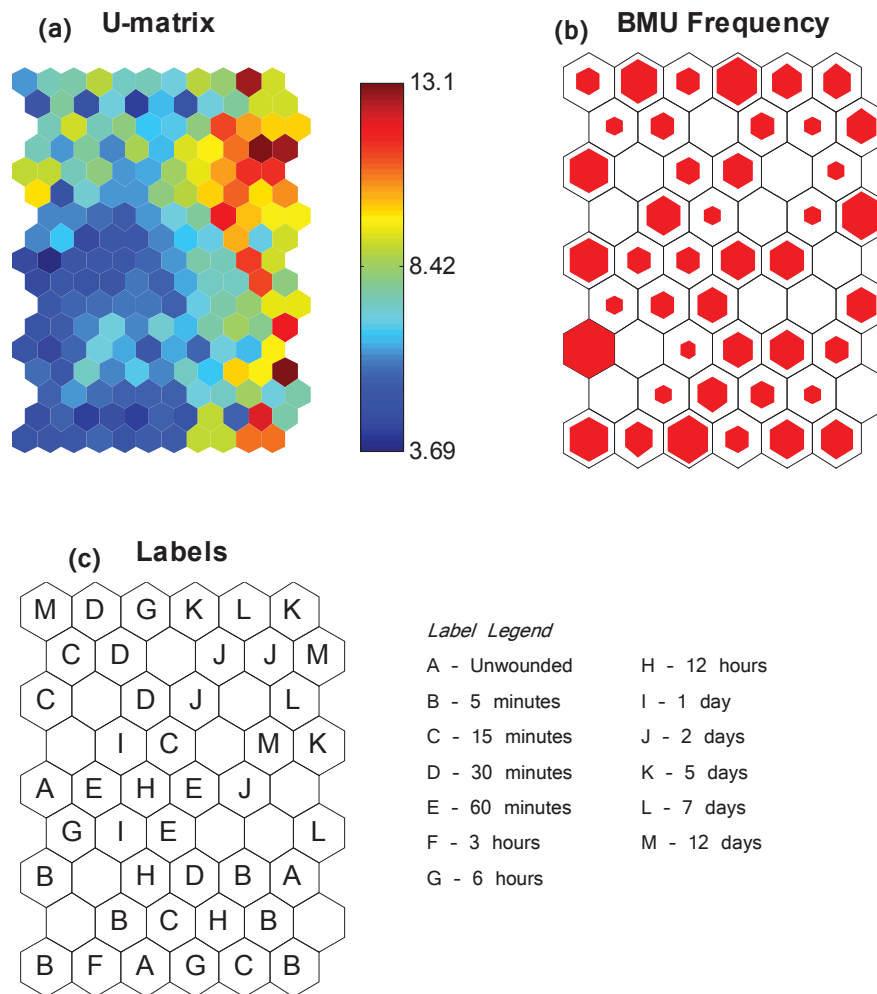


Figure 5-16: SOM results for the 16 cm^{-1} resolution data using the Method 32 pre-processing routine and the MIQR feature vector. (a) The resulting U-matrix. (b) This diagram shows the number of data points (using the red hexagon's size to indicate frequency) associated with each SOM (best-matching unit - BMU). (c) Each SOM unit's associated data label.

Although the SOM is not usually employed as a modeling tool, a form of supervised classification was attempted using the SOM to explore if any improvement might be obtained in the clustering. Rough clustering was achieved, although in some cases not all

map units for the same wound ages were located in topographically close vicinity or only a smaller number of age groups were represented (see Figure 5-17).

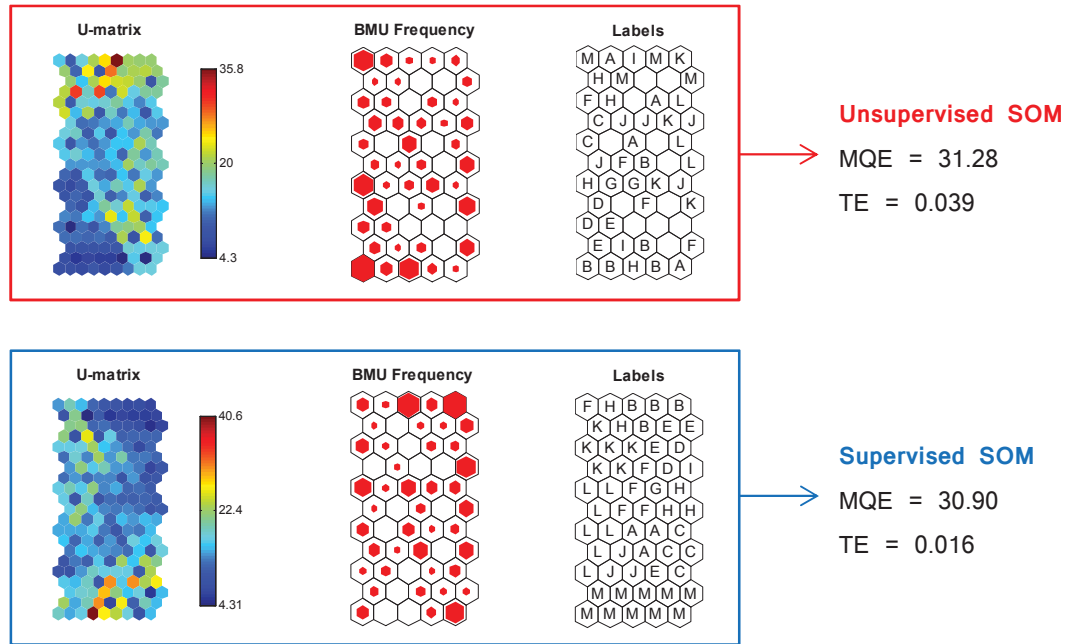


Figure 5-17: Supervised SOM results for the 4 cm⁻¹ resolution wound data pre-processed using Method 33 (MIQR feature vector). In this case the MQE value did not change dramatically as it was already quite high.

Performing supervised classification did improve the MQE and TE as one would expect (in some cases quite significantly), although as Brereton warns such results are typical when the data has been over-fitted.^[11] Ultimately, the goal of employing SOM for this component of the wound age sample analysis was purely for exploratory reasons and, compounded with a lack of available time, no further attempt to use SOM as a modeling / wound age predicting tool was made.

After considering the TE and MQE values, and examining the resulting wound sample maps, it is clear that SOM was not capable of effectively performing unsupervised clustering of the wound samples into their assigned wound age groups. This result would strongly indicate, when considered with the results obtained using PCA-LDA and SIMCA, that perhaps the data obtained using the sample preparation and analysis approach explored in this study was unsuitable for wound age classification and prediction.

5.3 MINIMUM NOISE FRACTION INVESTIGATION

Although PCA-LDA, SIMCA and SOM were all shown to produce poor classification of the wound data, it is difficult to conclusively state that the data itself was at fault and not the choice of data analysis technique. Although it was impossible to exhaust all chemometric options regarding the pre-processing and classification of the wound data, it was decided that a thorough data analysis approach should include a fourth alternative technique for the wound study. The method selected was that of pre-treating a training data set using a modified form of the maximum noise fraction transform, referred to as the Minimum Noise Fraction (MNF) transform, followed by endmember spectra extraction (implemented in this study using the software program ENVI). Classification of a test data set using these endmembers would then take place using a spectral classifier.

To perform MNF, two hyperspectral images were constructed for each wound data group analysed: one for the training data, and one for the test data sets. These images were constructed by tiling the spectra taken from each wound image into one large composite wound image. This composite image was then subjected to the MNF transform and the most coherent images identified using the in-built spatial coherence measure.^[12]

End member spectra were obtained from the wound data following MNF transformation using two methods. The first technique involved the examination of the two-dimensional scatterplots produced from the MNF bands (components) and the manual selection of

regions of interest (ROIs) which contained the pixels characteristic of spatial regions within the image (i.e. wound age). For the second approach, a semi-automated method was chosen which made use of the features in the ENVI Spectral Hourglass tool, which performs a Pixel Purity Index (PPI) calculation and combines this result with the MNF data in an interactive visualisation tool known as the *N*-dimensional visualiser.^[13,14] Classification was then attempted using these derived endmembers on both the training and test images using a variety of spectral classifying tools such as the spectral angle measure (SAM), mixture-tuned matched filtering (MTMF) and unmixed abundance images.^[12] A supervised classification approach using ROIs taken directly from the training image and then applied to the MNF-transformed test data was also attempted using SAM, MTMF and matched filtering (MF).

Although there were a variety of preprocessing combinations used to process the Pilot and Main Study data, only two data sets from each study were selected for this data analysis approach. The first data set examined had not been subjected to any preprocessing. In contrast, the second data set had been subject to various preprocessing steps such as quality testing and derivatisation. As each wound composite image was composed of the component wound age groups arranged in chronological order, it was hoped that the classified test composite images would reflect this order and produce an image possessing distinct spatial regions (assuming wound age discrimination was achieved based on the training data supplied).

An initial examination of the Pilot Study data using MNF revealed what appeared to be a form of wound age discrimination. When one examined the first three MNF bands (refer to Figure 5-18), the first band was observed to contain a feature most common in the 120 hour old wounds. Analysis of the test data sets using MNF alone also revealed similar groupings within the first five bands, with three groups again visible: unwounded tissue, 120 hour wound tissue, and a combined group for the 1, 3 and 12 hour-old wounds. Attempts

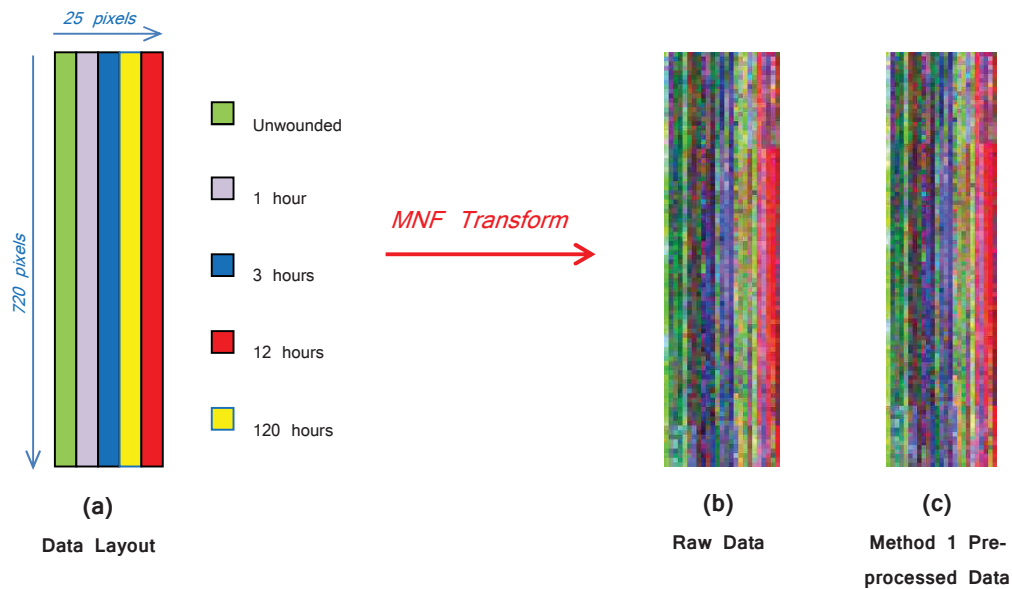


Figure 5-18: MNF comparison of the first three components for the Pilot Study training data (both original and pre-processed). (a) Depicts the layout of the data showing the five columns of pixels from each wound age group. Images (b) and (c) are the resulting RGB images constructed from the first three MNF-derived bands.

at using the two extraction methods listed above to identify the spectral endmembers characteristic of each of the wound age groups failed. A possible reason for this may be observed in the high similarity in the MNF component (band) “spectra” and the fact that the resulting scatterplots using both the MNF and PPI did not produce ‘arms’ of corner pixels consistent with good endmember estimates (refer to Figure 5-19). Varying combinations of MNF components consistently produced dense, roughly symmetrical clusters of data points with no clear corners and therefore no distinct endmember pixels.

Examination of the Main Study data using the above methods did not produce any visible wound age groupings in the training data sets and classification of the training images using endmembers selected from their own component pixels failed to produce sample

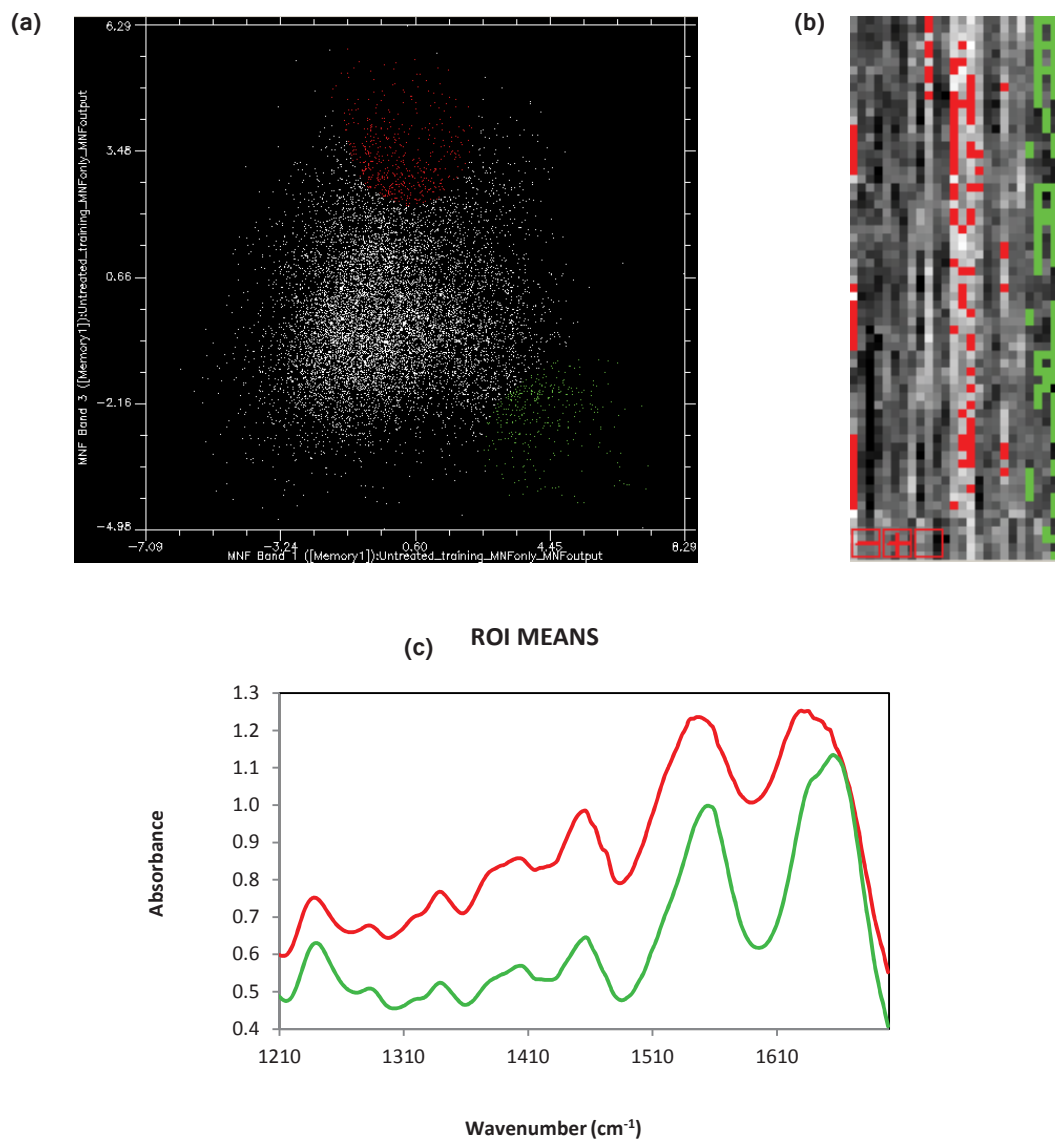


Figure 5-19: Example of the scatterplots produced using the Pilot Study data during the MNF transform in ENVI and how they may be used to select endmembers manually. In (a), two ROIs have been selected from the 'arms' of the scatterplot cloud constructed from the first and third MNF bands. The spatial locations of these pixels are highlighted in the image in (b). (c) depicts the corresponding average spectrum for each ROI.

groupings. Interestingly, an inspection of the N -dimensional visualiser scatter plots did display clusters of pixels in 'corners', but when selected these pixels did not correspond with tissue sections of a particular age group or groupings. While disappointing, this result was consistent with the lack of wound age discrimination observed for the previous data analysis approaches. Once again, it is postulated that the high spectral similarity between pixels from different wound age groups prevents good quality classifications from being achieved, and that the high number of spectra collected may have possibly swamped any pixels possessing infrared-detectable features characteristic of a particular stage in the wound healing process.

As with the previously attempted classification methods, this approach could not clearly distinguish the spectra representative of each wound age group. For the Pilot Study data, the wound age groups, albeit somewhat merged, illustrated once again that there is a limited degree of age discrimination achievable between the wound tissue samples. However, adequate endmembers representative of this grouping were not obtained and hence the predictive capability of the methods tested was poor. Based on the results achieved, precise allocation of the sample's spectra into a specific wound age has once again proven elusive using the FTIR CI data.

Whilst a quantitative approach such as counting the relative abundances of the various endmember spectra in each tissue sample image may be feasible for the analysis of the wound data, further studies would need to be performed to determine the area of tissue requiring sampling. For instance, if the characteristic features of the wound age only appear in a very small margin of the tissue, but a wide margin is imaged, these features may be swamped by the common features to all skin tissue and therefore make discrimination between wound ages very difficult. Unfortunately, due to time limitations, a quantitative approach to the wound data analysis was not attempted during this study.

5.4 GENERAL DISCUSSION

5.4.1 Comparison of Pilot Study to Main Study

Based on the classification results obtained for all data preprocessing methods, it is clear that the analytical technique employed in the Main Study, including the chemometric models applied, is not capable of providing reliable wound age discrimination and classification.

The question that arises from this observation is what might be the cause of such poor classifications, particularly in light of the promising results observed in the Pilot Study? Ultimately, no matter how well a chemometric model may be expected to perform, if the original data being utilised for classification is of insufficient quality, no amount of preprocessing or number of chemometric methods will be able to compensate for the poor original data. Based on the results observed using the PCA-LDA, SIMCA, SOM and MNF, it is clear that the various wound ages are difficult to separate into distinct age groups, particularly in the first few hours and days of the wound healing process. While, as discussed earlier, histochemical techniques have claimed some success in distinguishing the early wound age groups, the same could not be said of the analytical approach attempted in this study.

Besides increasing the number of wound age groups, there were a number of differences in procedure between the Pilot and Main Studies. In regards to the initial tissue sample collection and tissue preparation the methodology was kept the same, but the tissue deparaffinisation was conducted in an alternate laboratory with slightly different solvents. Only spectroscopic grade solvents were employed in this component of the Main Study, and the issue of contamination did not arise based on the results obtained. Tissue section thickness was also increased in the Main Study compared to the value used in the Pilot Study to improve spectral quality, but again this change would not be expected to impact negatively on the classification results obtained.

In relation to chemometric processing, the greatest change implemented between the Pilot and Main studies was the method by which spectra were selected. For the Pilot Study, a labour intensive process was employed which involved manually identifying regions of good quality spectra in the image. To improve data pre-processing times, a spectral quality test method available in CytoSpec was implemented for the Main Study, that in theory should have removed the poorer quality spectra attributable to gaps in the tissue, as well as thick tissue structures such as blood vessels and any epidermis inadvertently captured during imaging. Nonetheless, this approach was far from perfect and 'noisy' spectra were still observed in some images. It would be interesting in the future to test the quality of wound age discrimination using the manual spectra selection process employed in the Pilot Study on the Main Study wound images. Unfortunately the time required to perform such a process was unavailable to the author.

All of the results obtained in the Main Study clearly show that younger aged wounds are highly similar in spectral qualities and overlap greatly during discrimination. Rats are fast healers, and it may be that as the Pilot Study examined only a very small number of wound age groups with larger time spans between in comparison to the Main Study, the degree of overlap between wound ages would not be so clearly apparent. The Pilot Study did reveal some difficulty, using both PCA-LDA and SIMCA, in discriminating the one hour-old wound from the three hour-old wound, and may have been providing a hint of results to come.

Ultimately it is difficult to conclusively state why the Main Study performed so poorly in contrast to the Pilot Study. It may be that the Pilot Study was highly limited in its sample numbers and the number of wound age groups and did not reveal the limitations in discrimination as clearly as the Main Study. Further work is still highly recommended to gain an understanding as to why the younger wound age groups could not be separated

as well as a specialised study into the infrared features of the various cellular entities participating in the wound healing process.

5.4.2 Comparison of Infrared Imaging Study to Previous Wound Age Research

While this wound ageing approach did not produce the classification results hoped for, it is of some interest to compare the classification result trends obtained in this study with those achieved on the same samples by Tarran,^[15] from whom the samples utilised in this study were obtained.

For Tarran's study, a number of biological tissue analysis techniques were conducted on the wound tissue, commencing with an analysis of the rat skin wounds by an experienced pathologist. To perform this initial analysis, each of the rat skin wound tissue sections were stained with H&E and Perls' Prussian blue and forwarded, in the form of a blind study, to a forensic pathologist for wound age estimation using standard microscopic analysis. The number of wound age groups and replicates for each was not provided to the pathologist. When recording an estimate of the age of the wounds, the forensic pathologist employed a grading system which was based on morphological features observed in the tissue sections, such as epithelium regeneration, inflammatory cell presence and the scar cellularity.

As may be observed in Table 5-17, the forensic pathologist correctly identified all of the control wounds. However, in the early wound group (5 - 60 minutes), the correct age grading was assigned to none of the forty wounds, highlighting the difficult nature of wound age estimation in relatively young wounds using standard histological techniques. For the 2 and 5 day old wounds, samples were being identified as possessing properties more consistent with younger wounds. The pathologist fared better with the wounds aged greater than 5 days, accurately identifying 29 of the 30 samples submitted as presenting features

Table 5-17: Pathologist-assigned grading of wound age of the rat skin tissue sections reported in Tarran's study.^[15]

Pathologist-assigned Grading	Grading of Sample Replicate Wound Age									
	1	2	3	4	5	6	7	8	9	10
Control	A	A	A	A	A	A	A	A	A	A
5 minutes	B	C	C	C	B	C	C	B	E	B
15 minutes	C	C	B	B	B	C	A	C	C	B
30 minutes	C	B	C	C	B	B	C	B	C	B
60 minutes	B	C	B	C	C	B	B	C	G	B
3 hours	D	F	E	C	D	D	A	G	B	C
6 hours	D	E	F	F	F	E	H	D	E	E
12 hours	G	I	E	G	J	H	E	E	C	H
24 hours	G	D	E	J	J	E	D	H	D	E
2 days	I	H	J	E	C	J	I	D	C	J
5 days	K	K	E	H	L	J	K	N	H	M
7 days	L	M	L	K	L	K	M	L	J	K
12 days	N	L	M	M	N	O	O	N	M	M

- A** No wound evident - ? control ? postmortem / very early and tissue so well apposed no wound evident
- B** Possibly postmortem - no evidence of a vital response
- C** Looks antemortem (clot/blood plus inflammatory cells / vessel congestion), but no cellular response and therefore unable to comment on age
- D** Earliest acute inflammation
- E, F** Acute inflammation (E: Low grade, F: High grade)
- G** Chronic inflammation starting
- H, I, J** Chronic inflammatory stage (: Low, I: Medium, J: High grade)
- K, L, M** Healed epidermis, but still chronic inflammation (K, L, M in order of progressing healing)
- N** Scar
- O** Older scar

characteristic of being further advanced in the wound healing process. Tarran attributes the pathologist's ability to discriminate the older wounds more accurately to the fact that there is a larger number of features for which the pathologist may look, such as degree of epidermal regeneration, in comparison to the earlier wounds, which differ mainly based on inflammatory infiltrate features (or their absence).

The immunohistochemistry, flow cytometry and proteomic studies performed by Tarran, were again unable to distinctly group each wound age sample analysed. In the immunohistochemistry study, markers such as CD68 (macrophages), myeloperoxidase (neutrophils) and CD3 (T lymphocytes), were found to provide an indicator of a wound being above, or below, a particular age, but were not able to be used to group each wound tissue section into a particular age range.

The flow cytometry method was shown to offer no advantage over immunohistochemistry for skin wound ageing and was also destructive, consuming the tissue during processing. Using a haemoglobin protein marker, the proteomic study was able to detect varying levels of haemoglobin in the wounds, but haemoglobin is not temporally unique to wound age and may be overly abundant in early wound age samples. Ultimately, Tarran suggests that the combination of immunohistochemistry of inflammatory cells with the tissue sections treated with the routine stains of Perls' Prussian blue and H&E could provide a more precise identification of the cells involved at each wound healing stage, and that further research into additional protein markers may improve the applicability of proteomic analysis.

While this infrared and chemometric study did not produce the distinct wound age classifications hoped for, its performance in comparison to the results observed by Tarran may suggest that there are a number of factors that have impacted the classification results achieved. Firstly, it is clear from the observations made by the pathologist in Tarran's study that the inter-sample variability is difficult to control, even in the structured animal wound healing model employed. An example of this has been provided in Figure 5-20.

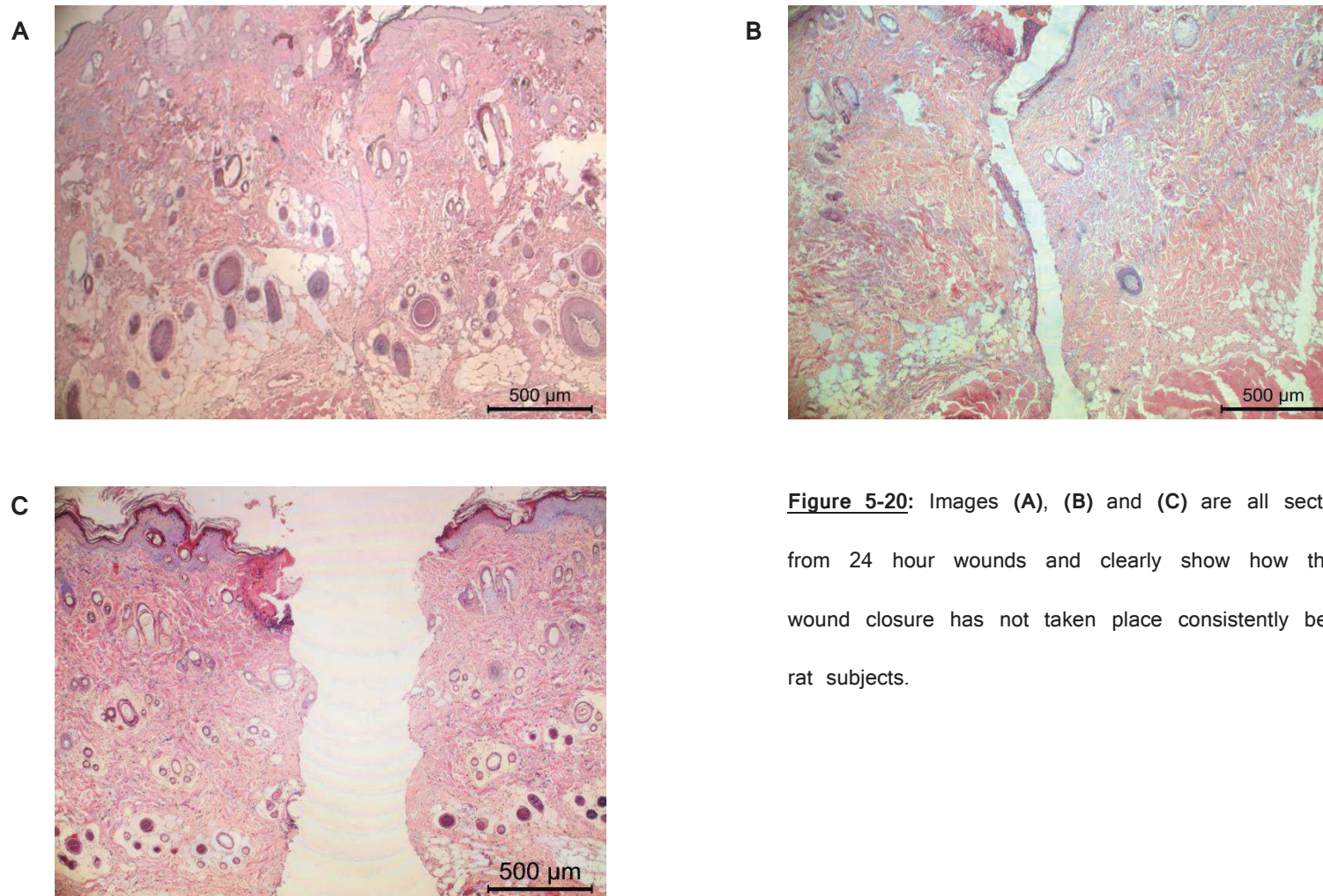


Figure 5-20: Images (A), (B) and (C) are all sections taken from 24 hour wounds and clearly show how the rate of wound closure has not taken place consistently between the rat subjects.

By performing the large number of replicates in this study for each wound age group, it was hoped that such variation may be overcome. Nevertheless, the question remains as to whether such inter-sample variability between healing wounds can adequately be overcome, particularly considering the complex biological process involved.

In relation to the large volume of wound ageing literature, it is difficult to compare the results obtained in this study with many of those published before due to the unique nature of the analytical approach taken. As stated in Chapter 4, the majority of the wound ageing techniques employed in the literature examine the wound tissue for specific markers, many of which are suited only to identify wounds exhibiting cellular entities that appear at particular wound healing stages and are hence not temporally indicative of all age groups.

5.4.3 Further Considerations

An additional issue which may have contributed to the poor wound age test set classification results is the impact that the formalin fixation and subsequent paraffin removal may have had on the various biochemical attributes of the wound tissue. A recent study by Hackett suggests that amino acids, carbohydrates, lipids, phosphates and ions are leached from biological tissues into the fixative medium - primarily from the extra-cellular and intra-cellular fluid of tissues.^[16]

While studies such as those by Tosi *et al* indicate that biological tissue classification is still possible using infrared imaging following formalin fixation and paraffin, the question remains as to whether, in the case of the wound samples, that the subtle biochemical differences between the samples of various ages was even further reduced or perhaps removed by the tissue processing technique performed in this study.^[17] The only way to definitively answer such a question would be to perform the same study using alternative tissue processing techniques such as frozen tissue sections, or perhaps leaving the paraffin in the tissue

sections, neither of which could be performed in this study due to sample accessibility and time limitations.

Another possible approach to improving the data classification on this study's data or infrared data similarly obtained would be to explore alternative feature extraction and classification algorithms. For example, Cheng *et al* successfully applied wavelet feature extraction followed by classification using a process based on statistical learning theory principles - support vector machines (SVMs) - for the detection of colon cancer in rat tissue.^[18] SVM analysis has also been employed for remote sensing and pathology tissue hyperspectral data analysis.^[19-21] Alternatively, other researchers have applied a non-linear feature extraction method based on maximum multinomial logistic regression (MRDF) and then used sparse multinomial logistic regression (SMLR), a Bayesian classification method, to hyperspectral data obtained from various biological tissues.^[22,23] A more recent approach to infrared histologic data analysis was recently proposed by Kwak *et al*, which involves a process known as frequent pattern mining.^[24] This approach effectively discretises or 'prunes' the spectral data initially into the most relevant sub groups which are then in turn subjected to a classification algorithm such as SVM. A possible area for future research would be to analyse the data obtained in this study using such techniques and possibly obtain an improved classification model.

5.5 CONCLUSIONS

Although the Pilot Study gave some positive indications as to the ability of infrared imaging combined with chemometric analysis to discriminate rat skin wound tissue of varying ages, the larger, more comprehensive Main Study was considerably less successful in achieving sample classification. Analysis of the infrared spectra using the various chemometric techniques employed did demonstrate some variation between the earlier and later wound

samples, but the predictive ability of the PCA-LDA and SIMCA models fell considerably short of producing a robust wound ageing tool for skin wound tissue of unknown age.

Although it was not possible to numerically compare the results obtained in Tarran's study to those obtained in the Main Study using FTIR CI (given Tarran's methods mainly produced an age 'range' while the FTIR CI sought to produce a precise age classification), it appears that the pathologist's analysis of the H&E/Perls' Prussian blue-stained tissues produced the most 'reliable' wound age determination for the wound samples examined. It should be noted also that the pathologist was unable to give a definitive and highly accurate wound age classification (aside from perfectly identifying the control samples) for the samples, which was the goal of this study. This suggests perhaps that the impact that biological variability of wound healing rates on wound age classification studies can be considerable in addition to the fact that the biochemical changes associated with each wound healing stage may be extremely subtle. There are many variables that may need to be considered when attempting wound age classification, such as the possible biological variation in wound healing rates of the rat subjects, the removal of key biochemical markers by tissue preparation and the introduction of artefacts and even the imaging approach taken.

Given the numerous variables identified above, it not surprising that it is difficult to conclusively identify why this analytical approach failed to produce the classification quality striven for. However, the observation of some data trends, even if only to distinguish the older from the younger wounds, does indicate that this infrared method was able to achieve a somewhat limited level of wound age discrimination.

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CHAPTER 6:

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

The aim of this thesis was to investigate the use of infrared chemical imaging for two pathology applications: the ageing of incised wound skin tissue and the detection of melanoma in both skin and lymph node tissue. Listed below is a summary of the aims and outcomes of the investigation into each application along with a brief discussion regarding the applicability of the results to actual clinical casework as well as recommendations for future work.

6.1.1 Melanoma Study

Considering that a number of research groups had previously demonstrated the ability of infrared imaging being to detect a wide variety of cancers, including melanoma, in biological tissues, the goal of this research application was to assess some alternative image classification processes to detect melanoma in human skin tissue. In contrast to previous melanoma infrared imaging studies that tend to focus on one tissue type at a time (i.e. only skin tissues, or only lymph nodes), this study investigated using one combined tissue reference library to distinguish naevus from primary melanoma, as well as healthy lymph nodes from those exhibiting metastatic melanoma, using a single classification algorithm known as the spectral angle mapper (SAM).

As part of the reference spectra library selection step in this research, a spectral unmixing approach, known as vertex component analysis (VCA), was explored as a feature extraction technique. Unfortunately this technique did not prove to be a very effective library gathering tool, but its optimal use for the tissues examined was not fully explored and remains an area of research worth exploring further in the future.

In regards to the classification of samples of 'unknown' disease state, the SAM algorithm was shown to be capable of discriminating different tissue types within samples as well as

identifying melanoma, although the overall classification methodology employed in this study (including reference spectra library collection) did produce a wide variation in classification quality. However, given the limitations mentioned above, the SAM classification algorithm was capable of producing some excellent results and warrants further study for the identification of optimal operating conditions. Unfortunately, the goal of using one spectral reference library for all tissue types was not achieved, with libraries built using spectra from tissue of the same type as the test sample (e.g. reference spectra from skin for test skin classification) outperforming a combined tissue-type library.

It was quickly evident that the time required to image each tissue section, and the file size limitations of the computer system, were significant limiting factors that in turn affected spatial resolution and data processing times. Another problem identified was the author's lack of pathology knowledge, and the impact that limited access to a trained pathologist could have on the reference library spectra gathered. Retention of paraffin in the tissue samples was also investigated, although the chemometric removal of paraffin from the spectra was not able to be performed and remains as a suggestion for future research.

Future research should focus on a number of key aspects if automation of skin and lymphoid tissue for melanoma diagnosis is to be attained. Firstly, the speed of image collection needs to be radically improved, either by optimisation of operating parameters (e.g. determining if fewer scans can be used without compromising spectral quality) and/or instrumentation development (e.g. high speed image collection as suggested by Nasse *et al.*)^[1] Further work needs also to be conducted in the area of spectral quality in relation to water vapour and the impact of paraffin embedding or removal. Unfortunately the author had insufficient time to analyse the effectiveness of noise removal tools such as the minimum noise fraction (MNF) data processing tool, or the chemometric removal of paraffin, both areas which have been shown in the literature to have a considerable potential impact on classification quality.^[2-7] Future classification efforts need to involve a trained pathologist

more during the initial training sample analysis stage to ensure that the extraction of the reference library spectra, from which all future classifications are based, does not become a major source of misclassification of the test samples.

Another area warranting further investigation is the approach suggested by Kwak *et al*, which involves employing a combination of imaging techniques (such as FTIR CI and optical microscopy - see Figure 6-1), a process known as multimodal analysis, to perform tissue classification.^[8] Given the results obtained using alternative analysis techniques as outlined in Section 2.1.12 for melanoma analysis, the possibility of combining FTIR CI with additional imaging modalities such as LA-ICP-MS, Raman or fluorescence imaging, becomes an attractive area for further investigation also.^[9-13]

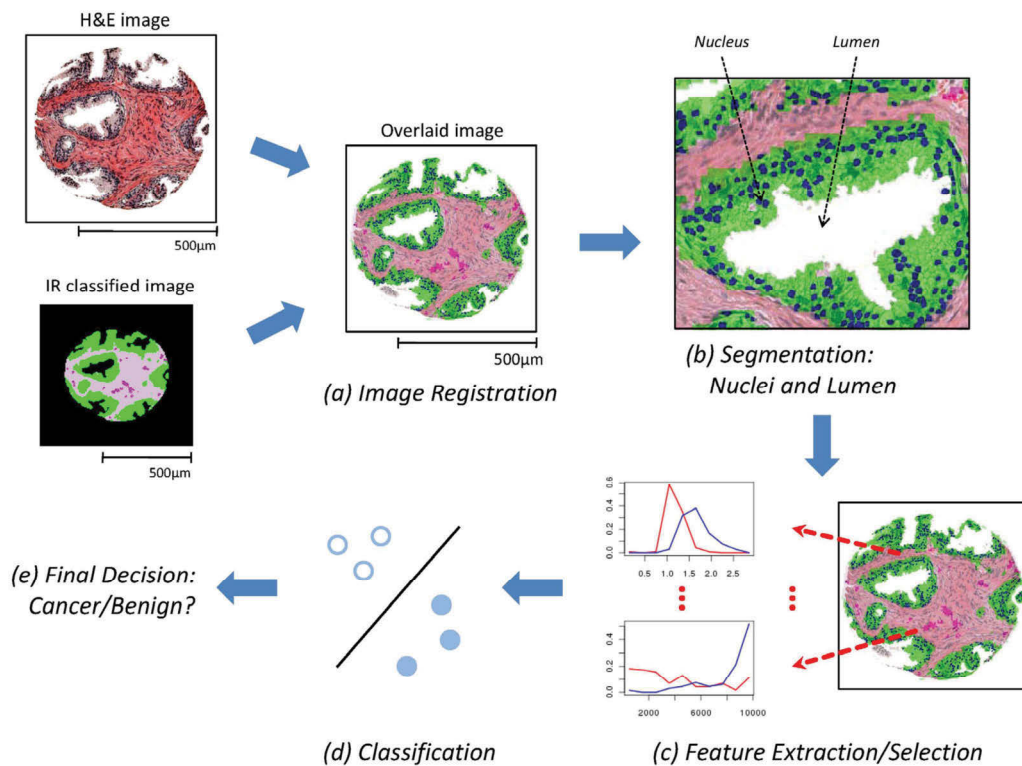


Figure 6-1: An example of an automated cancer diagnosis process using prostate tissue (from Kwak *et al*).^[8]

Unfortunately a number of different software packages were needed to provide the end classification result in this study for each sample (i.e. Varian Resolutions Pro, ENVI, Microsoft Excel, CytoSpec and Matlab), a process which could be simplified using one programming language (e.g. IDL or Matlab) to perform all of the various preprocessing and analysis tasks. Hence, a large hurdle in the development of an automated tissue classifier is gathering the knowledge necessary to write the various programs required (or at least gaining access to an individual who can).

The goal of this study and others like it was to never replace the pathologist, but to provide instead an alternative objective analysis of the supplied skin and lymphatic tissue to be used in conjunction with the pathologist's own findings. One option would be to implement such computerised techniques as FTIR CI as a preliminary screening tool to ensure the rapid identification of cancerous tissues which could then in turn be fast-tracked to the pathologist for confirmation. All computer-indicated negative results could also be later confirmed by a pathologist, and therefore any potential false-negatives identified. This would permit a form of 'triaging' to be conducted and hopefully reduce tissue analysis backlogs.

6.1.2 Wound Study

Wound age determination may be critical in criminal investigations and hence the most accurate and robust method of determination is desired by the courts. For the trained forensic pathologist, the ageing of a wound sample can be particularly problematic in the early stages of wound healing, and it was for this reason that an investigation was conducted into whether FTIR CI may be utilised to age a wound.

For this study, incised rat skin wound tissue samples of known age were obtained from Dr. Sarah Tarran, who had conducted her own wound ageing studies using proteomics and immunohistochemistry techniques, and imaged following sectioning and deparaffinisation.^[14]

Unlike the melanoma study, the 'imaging' component of the wound tissue research was primarily used to provide a large number of spectra for each sample. These large data 'cubes' were then reduced using a variety of data processing techniques and a reference library of tissue sample spectra constructed. Using a variety of chemometric approaches (PCA-LDA, SIMCA, SOM, MNF) spectra from additional tissue sections nominated as 'test' samples were classified and the accuracy of each chemometric technique assessed. These results were also compared with the classification results for the same tissue samples obtained from a pathologist cited in Tarran's own study.

It was observed during this study that the quality of the spectra obtained from the skin tissue was quite poor, and a number data treatment methods, such as principal components analysis (PCA)-based noise reduction, derivatives and normalisation, were attempted to improve spectral data quality. The impact of spectral resolution on classification was also examined by collecting the wound images at a number of resolution values (4, 8 and 16 cm^{-1}).

Based on the results obtained, each of the chemometric approaches utilised in this study experienced difficulties in accurately separating a wound tissue sample of 'unknown' age into its correct age group, particularly when the wound healing process was in its early stages. At best, the methodologies employed could only detect variation between earlier and later stage wound samples. The data collected indicated that the variation in classification quality was less affected by certain preprocessing techniques relative to others, but given the poor classification achieved overall, it was difficult to conclusively identify a most ideal data preprocessing methodology. Unfortunately the classification results for the Main Study did not replicate those of the more promising Pilot Study, an outcome that may be attributed to a variety of reasons including the method of pixel selection used in the Main Study or even the fact that the wound ages were less chronologically 'spaced' in the Pilot Study. However, the Pilot Study results were extremely encouraging and further investigation into

why the Main Study did not classify well and a more detailed study of the chemical properties of the cells involved in the wound healing process is certainly warranted.

The accuracy of the pathologist versus the FTIR CI technique was shown to be superior in determining a wound age range, although the former was only capable of 'grading' the tissue rather than assigning a precise wound age and still suffered from misclassification of samples (with the exception of the control tissue). Of particular interest was the observation by the pathologist of certain tissues of a particular wound age showing features more commonly encountered in tissues of more advanced or lesser wound healing times, indicating that there was a large amount of biological variability inherent in the samples used. The wound healing process is highly affected by a number of variables, such as the subject's overall health, and although the rats were wounded in a controlled manner, it is impossible to control all of the variables outside of the timing of the wound. This observation in turn highlights the difficulty in ever performing a human study, aside from the ethical concerns, given the variety that factors such as the subject's age, physical health (including various diseases known to affect wound healing rates), the presence of drugs, as well as environmental conditions to which the cadaver is exposed, which must be taken into consideration during any wound age determination attempt.

Any future FTIR CI wound ageing studies of a necessity must include an analysis of the impact of paraffin removal on tissue classification. Unfortunately only deparaffinised tissues were examined in this study, and it would be of great interest to assess the impact of the paraffin removal given the high spectral similarity observed between the wound tissue samples. Improving spectral quality, such as reducing the impact of water vapour (of greatest concern in the 4 cm^{-1} spectra), is also an area which requires further investigation. Following this, it is possible to consider that alternative classification techniques may yield better wound age discrimination, but their accuracy will be severely hampered if an

optimised tissue processing, image collection and data preprocessing method is not developed first.

6.1.3 Final Conclusions

In its most simple form, an automated tissue analysis model will first require the establishment of a set of reference metrics (following feature extraction from a set of known disease-state/wound-age samples) that may then be used for subsequent classification of 'unknown' cases (an example of such a process was given in Figure 6-1). Following the development and optimisation of any automated classifying process, calibration and validation data must then be obtained before any laboratory may routinely employ such a tissue analysis technique. As with any new method, the robustness of the overall process must be thoroughly investigated and any potential sources of error identified and quantified. Risk assessments must also be conducted to examine the effects of any potential errors in the system and to develop means to mitigate the impact of such incidents on both the patient and the laboratory.

Ultimately, for an automated tissue cancer diagnosis method to be attractive to a pathology service provider, the method must be shown to be more efficient in time, accuracy and cost. According to Scolyer *et al*, a pathologist takes, at a conservative estimate, one minute to analyse a lymph node tissue section.^[15] This makes the infrared imaging method employed in this study (in which large images took several hours to collect) seem far from time efficient. Development of faster imaging instrumentation may help to close this gap somewhat. Weigh also into consideration the fact that pathologists take years of training to attain their expertise, which is in turn a very costly process. In addition to this, one human cannot effectively operate twenty-four hours a day, and human decision-making capability is highly susceptible to factors such as fatigue and overall mental state. In contrast, a FTIR CI device requires very little in the way of manual intervention to run and

hence is far from being a labour-intensive process for a human technician to perform. Ultimately it will be the sensitivity of the technique which will weigh heavily into the consideration of adopting computerised classification methods, particularly if the automated method is capable of detecting the subtle changes that may be easily missed or are undetectable by the human pathologist.

In regards to the two applications and methods examined in this study, FTIR CI is a considerable distance away from routine application in actual pathology casework. The results obtained from both applications indicated that a considerable volume of work is yet to be completed, including the identification of the most ideal tissue preparation technique as well as assessing the impact of instrument performance and data preprocessing on classification accuracy. The results obtained in each study clearly demonstrate that further research into both applications is a worthwhile pursuit, particularly in the area of software development so that one program application may be used end-to-end. Given advances in technology, particularly in regards to image collection and computational power, and in the optimisation of tissue processing and data treatment processes, it is foreseeable that the analysis of tissues using FTIR CI will become far more practical and appealing for pathology laboratories processing large case volumes.

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